Reverse vaccinology based identification of anti- *Rhipicephalus microplus* vaccine candidates.

by

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Errare humanum est, sed ab erroribus discere

Devine

Scientia est lumen ad ignorantiam

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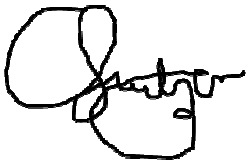

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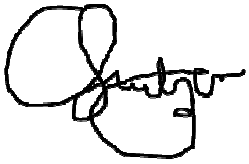

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Summary

The rapid geographic expansion of the cattle tick species, *Rhipicephalus microplus*, as well as the introduction of diseases into previously unaffected areas and the increased incidence of acaricide resistance in this species are of great concern in Africa and Southern Africa. The feasibility of developing an effective program for tick control that includes vaccination of cattle with tick protective antigens has been demonstrated sufficiently with the Bm86 vaccine. To date, a number of candidates have been identified that have been tested in cattle vaccination trials, but none could infer greater protection compared to Bm86. Genomic approaches to study *R. microplus* has only recently became possible with the availability of EST and assembled sequences databases (i.e. BmGI database with 13, 643 unique transcripts). During this study, a custom-based oligonucleotide microarray chip was designed from available genomic data allowing the simultaneous transcriptional analysis of some 13,456 *R. microplus* transcripts. This tool allowed a global view of the transcriptome of various adult female tissues and the immature life stages of *R. microplus* during feeding on South African cattle breeds. Additional clustering and annotation of transcripts resulted in the identification of biochemical processes and protein families that are unique to or conserved for the different life stages. Consequently global gene expression indicated 85 transcripts as shared between all life stages, along with a number of transcripts that were life stage specific or shared between the life stages tested. A reverse vaccinology approach was followed and the transcriptome data was subjected to bioinformatic and immuno-informatic analysis. Consequently, a set of potential antigens were identified for future evaluation as anti-tick vaccines and the applicability of reverse vaccinology in rational anti-tick vaccine discovery was assessed.

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List of Abbreviations

A	Adenosine
Å	Angstrom
AC	Adenylate cyclase
ADP	Adenosine diphosphate
Ala	Alanine (A)
AMP	Adenosine monophosphate
Arg	Arginine (R)
Asn	Asparagine (N)
Asp	Aspartic acid (D)
ATP	Adenosine triphosphate
BLAST	Basic local alignment search tool
bp	Base pair
BSA	Bovine serum albumin
C	Cytosine
^{o}C	Degrees Celcius
$CaCl_2$	Calcium chloride
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
cDNA	Complementary DNA
C-terminal	Carboxy terminal
Cy	Cyanine
Cys	Cysteiene (C)
Da	Dalton
DDT	Dichlorodiphenyltrichloroethane
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
ds	Double stranded
DTT	Dithiotritol
EC	Enzyme commission
EDTA	Ethanol diamine tetra-acetic acid
EST	Express sequence tag

G	Guanidine
GEO	Gene Expression Omnibus
Gln	Glutamine (Q)
Glu	Glutamic acid (E)
Gly	Glycine (G)
GO	Gene ontology
GPI	Glycosylphosphatidyl inositol
GSP	Gene specific primer
h	Hours
His	Histidine (H)
Ile	Isoleucine (I)
kbp	kilo base pairs
kDa	kilo Dalton
KOG	Eukaryotic gene ontology
Leu	Leucine (L)
Lys	Lysine (K)
Met	Methionine (M)
$MgCl_2$	Magnesium chloride
min	Minutes
mM	millimolar
mRNA	Messenger RNA
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MYA	Million years ago
µM	micromolar
µmol	micromole
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
NDP	Nucleotide diphosphate
ng	nanogram
NIH	National Institutes of Health
nmol	nanomole
nt	Nucleotides
N-terminal	Amino terminal

NTP	Nucleotide triphosphate
ORF	Open reading frame
PCR	Polymerase chain reaction
PDB	Protein data bank
Phe	Phenylalanine (F)
pmol	picomole
Pro	Proline (P)
RMSD	Root mean square deviation
RNA	Ribonucleic acid
RNAse	Ribonuclease
rRNA	Ribosomal RNA
RT-PCR	Reverse transcription PCR
Ser	Serine (S)
ss	Single stranded
T	Tymidine
TAE	Tris-acetate EDTA
Taq	Thermus aquaticus
TE	Tris EDTA
Thr	Threonine (T)
Tris	Tris(hydroxymethyl) aminomethane
tRNA	Transfer RNA
Trp	Tryptophan (W)
Tyr	Tyrosine (Y)
U	Units
UDP	Uracil diphosphate
UTR	Untranslated region
UV	Ultraviolet
Val	Valine
Zn	Zink

Chapter 1

Literature review

1.1. Global impact of ticks and tick control

1.1.1. Ticks: Origins to Classification

The first fossilized remains of ixodid ticks were recorded from Tertiary Oligocene amber deposits (ca. 30 MYA) collected in Wyoming (USA) (Scudder, 1885). To date, deposits have been recorded spanning both the Cretaceous (146 - 65 MYA) and Tertiary (54 -1.6 MYA) periods, with the oldest specimens dated to the middle Cretaceous around 90-100 million years of age (Klompen and Grimaldi, 2001; Grimaldi, Engel and Nascimbene, 2002). A previous hypothesis placed the origin of ticks around the Late Jurassic-Early Cretaceous periods (~140 MYA) (de la Fuente, 2003). However, following recent analysis of the 18S nuclear and 16S mitochondrial genes of the living fossil, *Nuttalliella namaqua*, it has been hypothesized that ticks originated during the Late Permian period (260-270 MYA), with the majority of their evolution and dispersal occurring throughout the Tertiary period (de la Fuente, 2003; Mans *et al*., 2011). In addition, it has also been suggested that the ancestral tick lineage may have been lymphatic feeders like its sister taxa, the Holothyrida, ultimately adapting to a blood feeding life style (Mans and Neitz, 2004).

Ticks have been well described as obligate haematophagous ectoparasites of the class Arachnida. Some of the earliest references to ticks and tick-borne diseases date as far back as ancient Egypt (ca. 1550 B.C.) and Greece (ca. 850 B.C.), from papyrus describing tick bite related “fever” and accounts from Homer’s epic poem the Odyssey (de la Fuente, 2003). The blood feeding habits of ticks have also been recorded by Aristotle (384-322 B.C.), Cato (234-149 B.C.) and Pliny (77 A.D.) stating that ticks are “a disgusting parasitic animal” infesting dogs, cattle, sheep and goats (Nuttall *et al.*, 1911). However, though ticks are obligate blood feeders, aspects of their lifecycle such as development, molting, mating and *oviposition* occurs mostly in the absence of a host animal (with the exception of one-host species). Therefore, ticks tend to be more aggressive in their feeding behavior and employ active

predation (questing behavior) or a “sit and wait“ strategy to find a host animal throughout their life cycle (Sonenshine, 1991; Lighton and Fielden, 1995).

Currently, ticks are classified into the suborder Ixodida, constituting three main families containing around nineteen genera namely, the Argasidae (193 soft tick species), Ixodidae (702 hard tick species) and the Nuttalliellidae (containing the single species *Nuttalliella namaqua*) (Barker and Murrel, 2004; Guglielmone *et* al., 2010). Though classification is based on phylogeny, morphology and biology, some basic physical characteristics can distinguish between hard and soft tick species (Sonenshine, 1991; Uilenberg *et al*., 2004). In this regard, ixodid ticks (hard ticks) bear a characteristic highly sclerotized dorsal scutal plate that acts as an attachment site for significant muscle groups, have a generally smooth integument and mouthparts (anterior) visible from the dorsal aspect (Fig. 1.1.). In contrast, Argasid ticks (soft ticks) lack a scutal plate and have a highly folded leathery cuticle that rapidly expands during feeding and the mouthparts are located ventrally not visible from the dorsal aspect (Fig. 1.1.). Ixodid ticks are generally slow-feeding organisms requiring days to feed, as the body wall needs to grow to allow expansion for the blood meal, while soft ticks feed quickly within minutes or a few hours (Sonenshine, 1991).

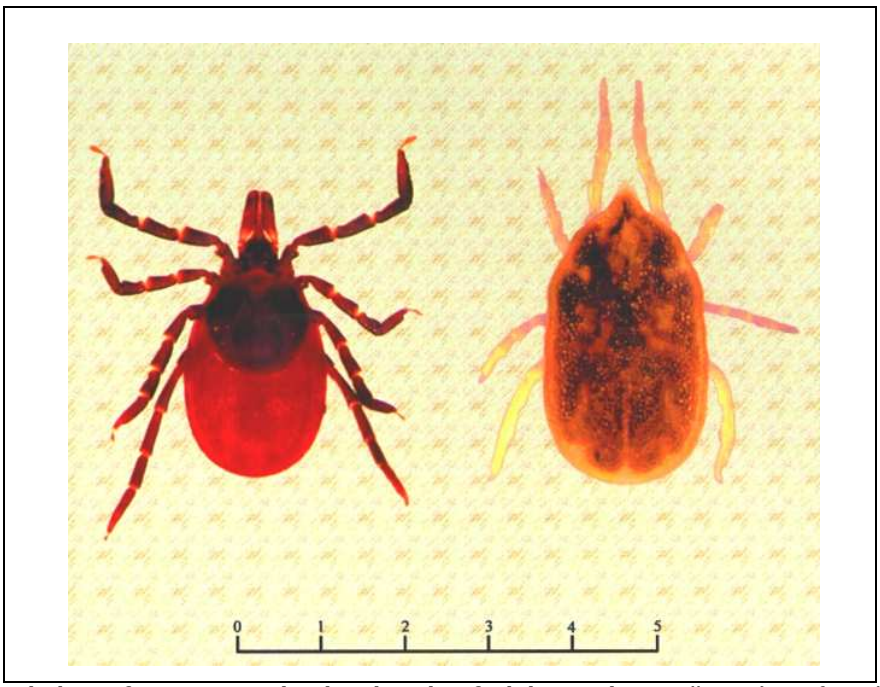

Figure 1.1. A dorsal view of representative hard and soft tick species. Indicated are females of the hard tick species *Ixodes scapularis* (left) and soft tick species *Ornithodoros hermsi* (right). Scale indicates length in millimetres (Schwan and Piesman, 2002).

1.1.2. Ticks as vectors of disease

At the turn of the 19th century, Smith and Kilborne described the transmission of Texas cattle fever by the cattle tick, *Rhipicephalus microplus* (previously *Boophilus microplus*), highlighting the economic importance of ticks as vectors in pathogen transmission (Smith and Kilborne, 1893; Murrell and Barker, 2003; de la Fuente and Kocan, 2006a). To date, about 75 species are well established as economically important vectors of protozoan-(i.e. theilerioses and babesioses), rickettsial-(i.e. anaplasmoses and heartwater or cowdriosis) and spirochete diseases (i.e. Lyme borreliosis and relapsing fever), as well as viral infections (i.e. tick-borne encephalitis and Crimean-Congo haemorrhagic fever) (Jongejan and Uilenberg, 2004). Ticks as vectors for pathogens afflicts humans, livestock and companion animals alike and ranks ticks second to mosquitoes in the transmission of a large variety of debilitating or life threatening diseases (Sonenshine, 1991). In addition, ticks can cause severe conditions such as anaemia, paralysis and toxicosis, allergic- and immune responses, irritation and secondary infection due to compromised immunity and lesion formation (Sonenshine, 1991; Jongejan and Uilenberg, 2004).

Some 240 different species of ticks have been identified in Africa, with the greatest variety of species found in the eastern and southern parts of the continent (Fig. 1.2.) (Cumming, 2000). However, the species ranges in sub-Saharan Africa are not constrained by host distribution (i.e. cattle) and expansion of suitable habitats is expected in the wake of global climate change (Fig. 1.2.) (Cumming, 1999; Leger *et al.*, 2012). About 30 species that can act as vectors, able to transmit disease causing pathogens, occur on the African continent (Table 1.1.) (Walker *et al.*, 2003). The latter also highlights the potential of ticks as vectors for the transmission of zoonotic diseases between animal reservoirs (i.e. livestock) and human hosts. In this regard, several tick-borne zoonotic diseases have been described that occur on the African continent and include: Tick-borne rickettsiosis (i.e. African tick-bite fever caused by *Rickettsia africae*), tick-borne borreliosis (i.e. tick-borne relapsing fever cased by *Borrelia spp.*), Crimean-Congo hemorrhagic fever (caused by *Nairovirus*), Human ehrlichioses (caused by species such as *Ehrlichia chaffeensis*), as well as human babesiosis (caused by *Babesia spp*.) (Cutler *et al*., 2010; Gray *et al*., 2010; Esemu *et al*., 2011; Keshtkar-Jahromi *et al*., 2011; Bitam, 2012).

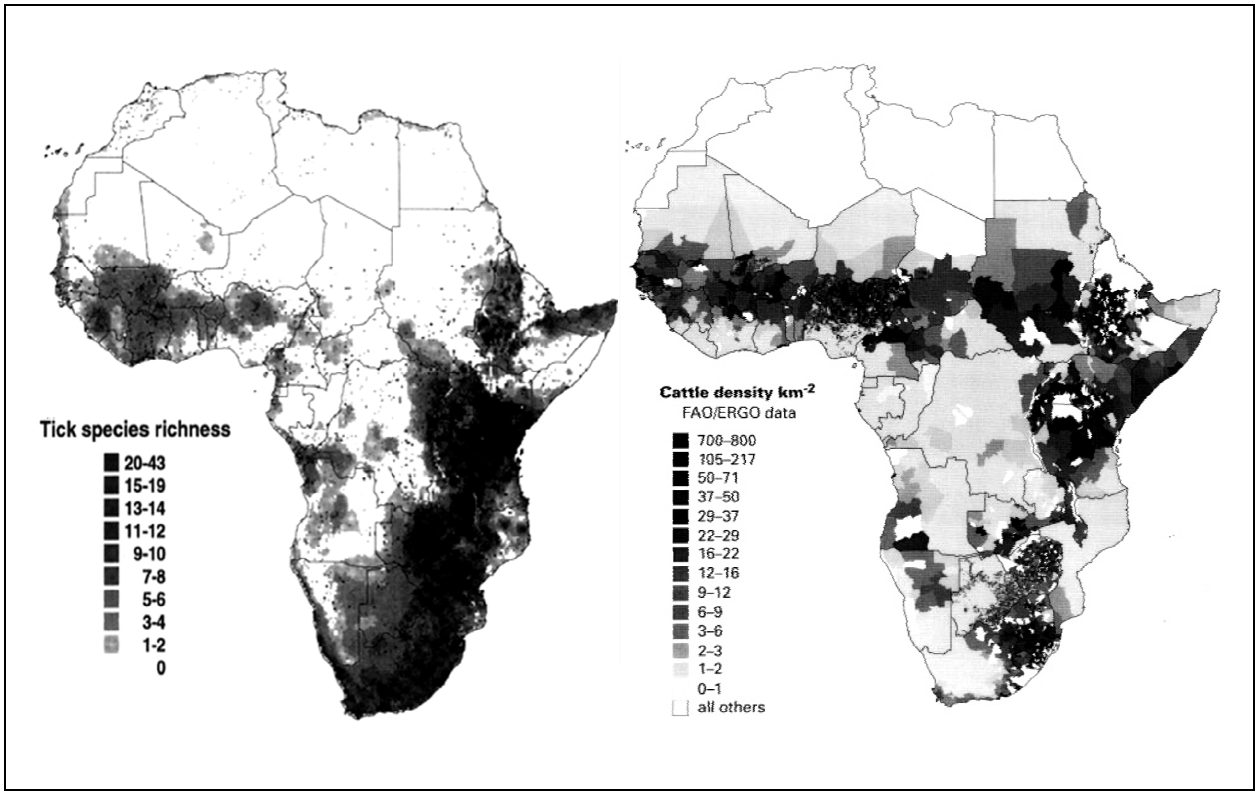

Figure 1.2. Relative distribution of tick species and cattle hosts across the African continent. Indicated are the relative distributions and species richness of ticks (left), as well as the ranges and density of cattle hosts (right), on the African continent. Figures adapted from Cummings (1999 and 2000).

Table 1.1. Pathogenic agents and vectors of tick-borne diseases affecting cattle in Africa. Indicated are some of the most prominent tick-borne diseases, the pathogens, vectors, distribution and cell tropism affecting cattle in Africa (adapted from Walker *et al.*, 2003, Jongejan and Uilenberg, 2004 and Marcelino *et al.*, 2012).

Disease	**Pathogen**	**Principal tick vector(s)**	**Main distribution**	**Cells infected (*In vivo*)**	
				Host	**Vector**
Tropical theileriosis	*Theileria annulata*	*Hyalomma anatolicum anatolicum* *Hyalomma detritum*	Northern Africa and Sudan	Macrophages	Gut cells and salivary glands
East Coast fever/ Corridor disease	*Theileria parva*	*Rhipicephalus appendiculatus*	Eastern, central and southern Africa	Lymphocytes	Gut cells and salivary glands
Tropical bovine babesiosis, Redwater	*Babesia bovis*	*Rhipicephalus microplus* *Rhipicephalus annulatus* *Rhipicephalus geigyi* *Rhipicephalus bursa*	Most tropical and subtropical regions, including Sub-Saharan Africa	Erythrocytes	Gut cells, salivary glands and ovaries
Tropical bovine babesiosis, Redwater	*Babesia bigemina*	*Rhipicephalus microplus* *Rhipicephalus decoloratus* *Rhipicephalus annulatus* *Rhipicephalus bursa* *Rhipicephalus evertsi evertsi*	Most tropical and subtropical regions, including Sub-Saharan Africa	Erythrocytes	Gut cells, salivary glands and ovaries
Malignant anaplasmosis, Gallsickness	*Anaplasma marginale*	*Rhipicephalus microplus* *Rhipicephalus decoloratus* *Rhipicephalus annulatus* *Rhipicephalus bursa* *Rhipicephalus evertsi evertsi* *Rhipicephalus simus*	Tropical and subtropical regions, including Sub-Saharan Africa and Mediterranean	Erythrocytes	Gut cells and salivary glands
Heartwater	*Ehrlichia ruminantium*	*Amblyomma astrion,* *Amblyomma gemma,* *Amblyomma hebraeum,* *Amblyomma lepidum,* *Amblyomma marmoreum,* *Amblyomma pomposum,* *Amblyomma tholloni* *Amblyomma variegatum*	Sub-Saharan Africa and Madagascar	Endothelial cells, neutrophils	Gut cells and salivary glands
Dermatophilosis or cutaneous streptothrichosis	*Dermatophilus congolensis*	*Amblyomma variegatum*	West Africa	Epidermis	Unknown

1.1.3. Livestock production in Africa and the southern cattle tick *R. microplus*

In developing nations, livestock production is a great source of revenue and cattle-farming constitutes one of the major agricultural industries, especially in the tropical and subtropical regions of the world (Perry *et al.*, 2002). Indeed the demand for livestock products (i.e. milk and meat) is rapidly rising and ticks and tick-borne diseases have a major impact on the livelihood of resource-poor farming communities in these regions (Delago *et al*., 1999; Perry *et al.*, 2002; Jongejan and Uilenberg, 2004). In Africa, cattle represents the major livestock production system in the eastern and southern parts of the continent, while smaller ruminants (i.e. sheep and goats) represent the primary livestock farmed in West Africa (Perry *et al.*, 2002; Jongejan and Uilenberg, 2004).

The southern cattle tick, *R. microplus*, is a one-host tick species and a major pest of cattle in the tropical and sub-tropical regions of the world including southern Asia, Madagascar and the southern and eastern coasts of Africa (Lynen *et al.*, 2008). This species is regarded as the most economically devastating tick species worldwide due to three main factors (Guerrero *et al.*, 2006). Firstly, *R. microplus* is a highly adaptable species that is spreading rapidly to occupy previously unaffected areas, such as the Ivory Coast in West Africa, as well as the Limpopo province in South Africa and more recently Namibia, even displacing endemic tick species (Tønnesen *et al.*, 2004; Adakal *et al*., 2013; Nyangiwe *et al*., 2013a). Secondly, *R. microplus* is a well described vector for the causative agents of Asiatic redwater (*Babesia bovis*) and bovine anaplasmosis (*Anaplasma spp.)* (de la Fuente *et al.*, 2007b; Madder *et al.*, 2007; Lynen *et al.*, 2008). Thirdly, an increase in resistance to all major classes of acaricides has been reported for this species, as well as the occurrence of a strain resistant to the current Bm86-based vaccine (de la Fuente *et al.*, 2000; Rajput *et al.*, 2006; Li *et al.*, 2007). In South Africa, *R. microplus* was first introduced from Madagascar in the early 1900s and confined to the coastal areas of the Cape and Kwazulu Natal (Howard *et al*., 1908; Howell *et al*., 1978; Walker *et al*., 2003). However, its range has since expanded to include areas of the Free State and Eastern Cape provinces and has been shown to displace endemic tick species (like *R. decoloratus*)(Fig.1.3.) (Tonetti *et al*., 2009; Nyangiwe *et al*., 2011; Nyangiwe *et al*., 2013b). It is therefore expected that the impact of this species on the cattle industry in South Africa will likely increase in the near future.

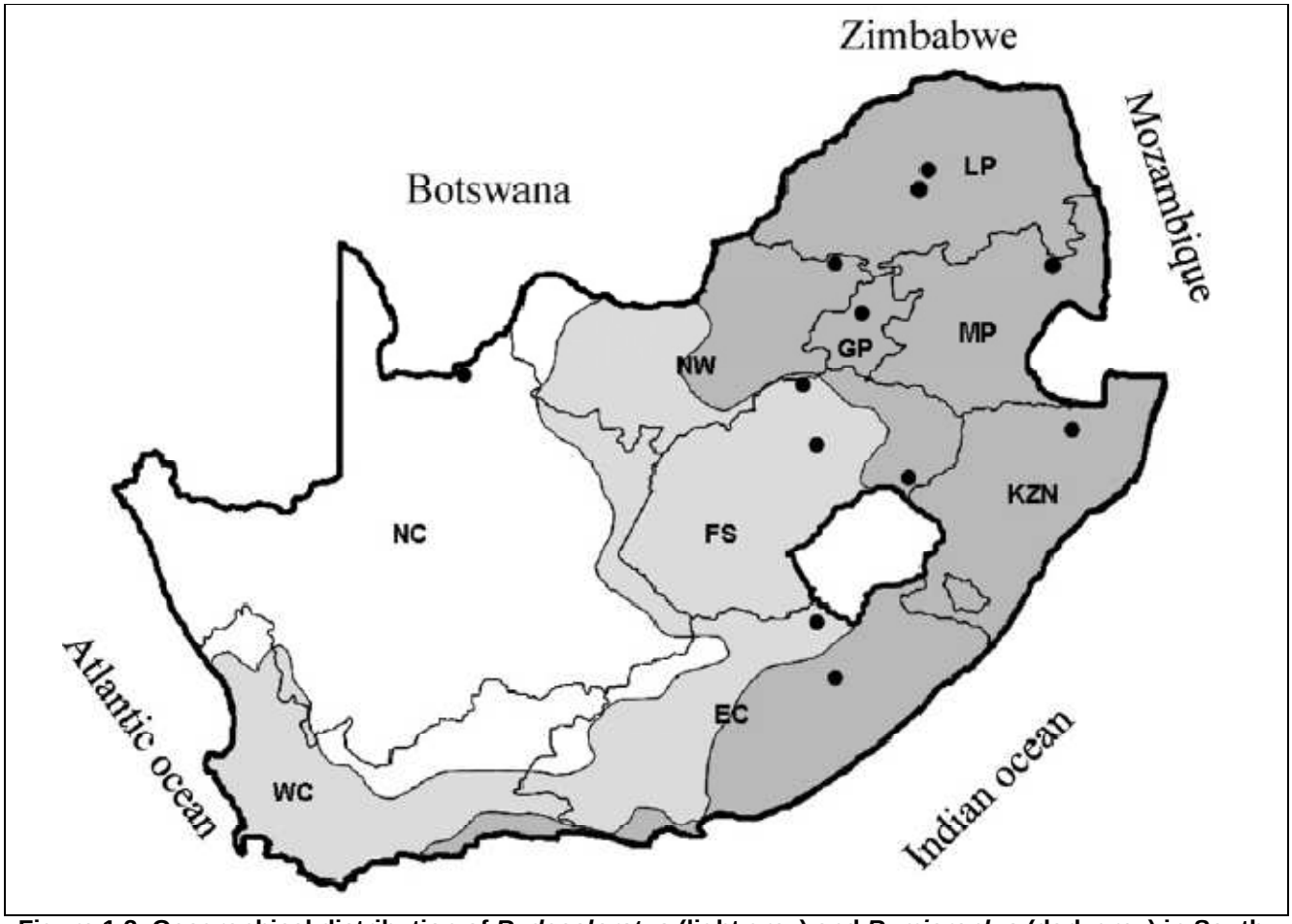

Figure 1.3. Geographical distribution of *R. decoloratus* (light grey) and *R. microplus* (dark grey) in South Africa. Distribution map reproduced from Terkawi *et al*. (2011). Black dots indicate sampling areas that were used for a serological servey of *Babesia spp.* in South African cattle.

1.1.4. Economic impact of *R. microplus* on cattle farming in South Africa

The loss of cattle condition directly due to tick infestation and cattle losses resulting from disease (i.e. redwater and gallsickness) (Table 1.1.), as well as damage to hides as a result of scarring around bite sites, poses a severe problem to the revenue stream for cattle farmers and industry (Walker *et al*., 2003; Jonsson, 2006). The cost of tick and tick-borne disease control globally has been conservatively estimated at about 7 billion US dollars (US$) per annum (McCosker, 1979). However, the latter estimation is greatly outdated and underestimated, as it was based solely on Australian figures for *R. microplus* ticks and their role in disease transmission and control. Other estimates on annual losses incurred by tick-borne diseases on livestock include theilerioses (384.3 million US$ in India) and East Coast fever (~222 million US$ for east Africa), amongst others (Jongejan and Uilenberg, 2004). During the 1980's capital losses of up to 200 million rand per year were recorded in South

Africa due to cattle losses (Bigalke, 1980). However, the latter is also an outdated estimate and based on red meat prices as a proxy from 1979/80 and 2008/09, the current costs are estimated between 1.3 and 3.7 billion rands per year due to cattle loss only (Bigalke, 1980; Directorate Agricultural Statistics, 2010; Oberholster, Unpublished data). The contribution of cattle conditioning in regards to longer production cycles, quality defects resulting from tick feeding, as well as expenses incurred from tick control (i.e. dipping) and treatment of sick animals, is not currently taken into account. Therefore, the latter estimate is still grossly underestimated.

The costs of novel drug development are estimated around the US$100 million mark and it can take a decade before a functional product appears on the market (Graf *et al.*, 2004).Thus the demand for cheap and effective tick control measures is evident, as much of the economic burden is shouldered by the poorer developing countries that lack the infrastructure and resources to combat these parasites.

1.1.5. Tick control

The encroachment of man and livestock into tick infested regions, as well as environmental changes that increases favourable habitats for infestation, will also inevitably increase the incidence of tick-borne diseases (Leger *et al.*, 2012). As most breeds of cattle introduced to the tropical and subtropical regions (including Africa) are exotic, they lack natural immunity to tick infestations, necessitating the use of acaricides (dips, sprays and injectables) for their survival (George *et al.*, 2004). Therefore, the development of effective tick control measures is vital.

1.1.5.1. Chemical control

The main method for tick and tick-borne disease control relies on the use of chemical acaricides (Guerrero *et al*., 2012a). The first arsenic based acaricides were formulated in the early 19th century and eventually led to the production of organic pesticides such as chlorodiphenyltricloroethane (DDT) in the 1940s and 1950s (Graf *et al.*, 2004). As organochlorides (i.e. Lindane and chlorophentyphos) made way for organophosphate dips (i.e. dichlorvos and toxaphene), the latter became obsolete with the introduction of amidine based chemical products, such as amitraz, that are still used today. In 2003, ectoparasiticides accounted for 22% (175 million rands) of total sales in the South African veterinary market

(Peter *et al.*, 2005). The current acaricide controls include acarine growth regulators (eg. fluazuron based on benzoylphenylurea), macrocyclic lactones (eg. ivermectin), phenylpyrazoles (eg. fipronil) and naturalytes (eg. spinosad) (George *et al.*, 2004; Graf *et al.*, 2004). The first recorded instance of acaricide resistance of *R. microplus* populations in Africa was in 1979, in South Africa and the former Transkei (Baker *et al*., 1979). Recently, populations of *R. microplus* that show resistance to several major classes of acaricides, including multi-acaricide resistant strains, have been identified in several developing countries such as Latin America and India, as well as South Africa (Andreotti *et al*., 2011b; Henrioud, 2011; Mendes *et al*., 2011; Rodriguez-Vivas *et al*., 2011; Vatsya and Yadav, 2011; Walker, 2011; Abdullah *et al*., 2012; Fernández-Salas *et al*., 2012; Lovis *et al*., 2013). Rotational programmes using multiple acaricides have proven unsuccessful to extend the long term use of an individual acaricide, as resistance traits remain relatively fixed within wild populations (Rodriguez-Vivas *et al*., 2011). Consequently, the use of more potent combinations of pesticides on livestock is necessary leading to a detrimental impact on the environment and non-target organisms (including workers), as well as increasing the potential for side effects to host animals and contamination of animal products used for human consumption (Graf *et al.*, 2004; Walker, 2011; Guerrero *et al*., 2012a).

Additional high-throughput computational biology and combinatorial chemistry approaches could be employed to create a new generation of chemical controls targeting previously unexplored metabolic and synthetic pathways (Gosh *et al*., 2007). However, replacement of current acaricides with new generation compounds will still eventually result in a step-wise gain of resistance (Rodriguez-Vivas *et al*., 2011). Therefore, monitoring and screening for markers associated with resistance will assist in the early detection of acquired resistance and will be an invaluable addition to an integrated pest management program (Gosh *et al*., 2007). Botanical extracts have also been suggested as a promising avenue for discovery of complementary or alternative chemical controls against tick infestation (Gosh *et al*., 2007). In this regard, several plant derived extracts have been tested in field trials against *R. microplus* infestation with reasonable efficiencies relative to controls (Borges *et al*., 2011). However, several factors hamper the effective use of botanical extracts as alternative acaricides in the field and include: isolation and characterization of individual active components is generally lacking, loss of efficiency between *in vitro* (laboratory) and *in vivo* (on animals) tests, low environmental persistence due to degradation (i.e., photosensitivity to sunlight, temperature, pH and microbial action), as well as variability of chemical composition of extracts in plants

obtained from different regions (Borges *et al*., 2011). Additional research is required in the formulation, delivery and production of these botanical acaricides to overcome current shortfalls in translating these compounds into a commercially viable product.

To conclude, novel acaricide discovery, development and marketing remains relatively expensive, as well as subject to consumer preference (Guerrero *et al*., 2012b). The latter are contributing factors to the development of safer environmentally-friendly, more sustainable strategies that address the current limitations of chemical tick control.

1.1.5.2. Biological control

The use of natural predators and pathogens for control of pest populations has been well documented with limited practical implementation due to the complexity of the biology and ecology of a pest species (Hogsette, 1999).

Predators

Birds represent an excellent practical example of biological tick control through predation. An example is the wild oxpecker (*Buphagus* spp.) in Africa that has been evaluated as a biological control agent of tick populations on cattle, showing a preference for *Rhipicephalus* spp. (Bezuidenhout and Stutterheim, 1980). However, the population of these birds have declined sharply in recent years due to acaricide poisoning and a decrease in game animals, requiring reintroduction into areas of Africa where they have become extinct (Samish *et al*., 2004). It has also been reported that domestic chickens (*Gallus gallus*) feed on ticks found around livestock and has been exploited by small-holder farmers as a measure contributing to tick control (Hassan *et al.*, 1992). However, consumption depends on the availability of alternative food sources and tick population density. Though predators affect natural tick populations, manipulation of their numbers to enhance their ability as a bio-control agent may also cause detrimental changes in the populations of non-targets species (Samish *et al*., 2004).

Parasitoids

The *Ixodiphagus* genus of parasitic wasps has been well documented as tick parasitoids, with *Ixodiphagus hookeri* as the most widespread and only species tested for biological control of ticks (Samish *et al.*, 2004; Lopes *et al*., 2012; Plantard *et al*., 2012). This parasite develops to

adulthood in conjunction with ingestion of blood by the host, following *oviposition* of parasitic eggs into the body cavity of nymphal ticks. Its occurrence in Africa has been reported for South Africa, Nigeria, Uganda, Kenya, Cote d'Ivoire, and Mozambique, along with a second species, *I. theilerae*, which has been described from Namibia, South Africa and Egypt (Mwangi and Kaaya, 1997). However, the efficacy of parasitoids as biocontrol agents relies heavily on inundative release of large populations of wasps for parasites to persist under natural conditions (Samish *et al.*, 2004). Furthermore, only one field trial involving *I. hookeri* as biocontrol agent of *A. variegatum* has been conducted in Kenya and further study is required to identify all existing species, determine geographical distributions (and host ranges), flight range and dispersal capacity, as well as elucidate parasitoid biology (including development within the tick host) on the African continent (Mwangi *et al*., 1997; Mwangi and Kaaya, 1997). It has also been observed that high tick densities are required for parasitoids to persist within a given tick population and that different strains have adapted to local conditions (local tick species and climate) (Samish *et al.*, 2004; Collatz *et al*., 2011). The latter explains the limited success of imported strains in tick biocontrol and therefore emphasizes the importance of identifying and enhancing the potential of native strains (Collatz *et al*., 2011). To conclude, successful cost-effective field application of parasitoids as biocontrol agents remains an impractical endeavour and requires further intensive study to fill current gaps in basic biology and phenology.

Pathogens

Microorganisms as a source for biopesticides has been explored for tick control and include entomopathogenic species of bacteria (i.e. *Bacillus thurringiensis*), fungi (i.e. *Beauveria bassiana* and *Metarhizium anisopliae*) and nematodes (Samish *et al.*, 2004).

Bacteria commonly occur on wild ticks and though not all of these bacterial species are detrimental to the tick, some species occur that are pathogenic (Samish *et al.*, 2004). The latter includes the entero-bacterium *Cedecea lapagei* that has been shown to cause 100% mortality in *R. microplus* under laboratory conditions (Brum and Texeira, 1992; Samish *et al.*, 2004). The insecticidal *B. thuringiensis* is a well documented biocontrol agent that produces lethal exo- and endotoxins that attacks the midgut of insects (Samish *et al.*, 2004). Mortality has been shown in *in vitro* conditions for several tick species, but field application is currently impractical as ingestion of the pathogen is required and ticks tend to ingest only the host's blood (Hassanain *et al.*, 1997; Zhioua *et al.*, 1999; Samish *et al.*, 2004). A recent culture-

independent screening of the *R. microplus* bacteriome using pyrosequencing technology, identified 121 bacterial genera distributed across different life stages and tissues (Andreotti *et al*., 2011b). Studies like these will expand the current repertoire of endosymbionts that occur within a given tick species and can provide the opportunity for genetic manipulation to develop novel microbial acaricides.

Entomopathogenic fungi represent an additional class of pathogens that can be considered for development of tick biocontrol agents due to their ability to penetrate the cuticle, cause mortality in several stages of the same host species and their specific virulence to a single or related host species (Samish *et al.*, 2004). The pathogenicity of entomopathogenic fungi has been well described for hard tick species such as *R. microplus* and applied in field trials with some success (Polar *et al*., 2008; Fernandes *et al.*, 2012). The potential of entomopathogenic fungi as biocontrols for the various life stages of *R. microplus* has been demonstrated *in vitro* with *B. bassiana* and *M. anisopliae* and field trials using *M. anisopliae* produced between 25% and above 70% efficacy in field and pen trials (Fernandes and Bittencourt, 2008). However, entomopathogenic fungi suffer from various disadvantages that limit their use as biocontrol agents and include: susceptibility to environmental conditions that limit persistence of fungal conidia in the field (i.e. high temperatures, strong ultraviolet irradiation from the sun and desiccation), the slow onset of mortality in host animals, high concentrations of fungal inoculum are required relative to other insects pests, multiple applications needed that would influence cost efficiency and the potential for non-target effects on other arthropods (Ginsberg *et al.*, 2002; Samish *et al.*, 2004; Polar *et al*., 2008; Fernandes *et al.*, 2012). Improvements can be made by identifying or creating new highly virulent strains, optimization of the fungal inoculum formulation and application strategies to increase persistence in the field and improve transmission efficacy, as well as seasonal timing for biocontrol application (Ostfeld *et al*., 2006; Fernandes *et al.*, 2012).

Entomopathogenic nematodes of the Heterorhabditidae and Steinernematidae families are well known parasites of insects (Samish *et al.*, 2004). The infective juvenile stage actively locates from the soil to enter the host haemocoel via natural orifices and releases symbiotic bacteria that cause mortality. For insects, the juvenile nematodes feed on the bacteria and host material to multiply and mature into adults, while in ticks they die shortly after entry into the haemocoel (Samish *et al.*, 2004). Ticks were first shown to be susceptible to nematode infection by Samish and Glazer in 1991 and since then several *Steinernema* and

Heterorhabditis species have been identified that are pathogenic to tick hosts (Samish and Glazer, 1991; Samish *et al.*, 2004). For the cattle tick, *R. microplus*, nematode species *H. bacteriophora, H. amazonensis* and *Steinernema glaseri* were found to afford around 100%, 68% and above 80% treatment efficacy, respectively in *in vitro* tests relative to controls (de Oliveira Vasconcelos *et al*., 2004; Monteiro *et al*., 2010; Monteiro *et al*., 2012). The application of entomopathogenic nematodes as a biocontrol agent may be limited to defined ecological niches, as treatment is greatly influenced by variability in pathogenicity of the different nematode strains and species, variability in susceptibility of host strains and species, as well as susceptibility of nematodes to environmental conditions (i.e. depth in the soil, moisture content of soil, composition of soil, temperature and sensitivity to UV irradiation), (Samish *et al.*, 2004).

In general, the practical use of pathogens as biocontrol agents is highly dependent on the virulence of the strain, mode of pathogenicity, effective transmission (delivery of pathogenic agent to the tick host), environmental factors (eg. humidity and temperature), potential of non-target effects, as well as safety concerns for use on animals and human contact (Samish *et al.*, 2004). Therefore, further research in this field is required for development of biocontrols based on tick pathogens.

1.1.5.3. Genetic control

Resistance to infestation is a heritable trait and cattle naturally resistant to tick infestation, such as zebu (*Bos indicus*) cattle, can be selectively bred to produce highly tick resistant breeds. An excellent example is the Belmont Adaptaur breed, developed from *Bos taurus* breeds that are highly resistant to infestation of *R. microplus* (Frisch, 1999). However, selection based on host resistance is an arduous process that can take decades for the establishment of a new breed and may negatively affect production traits such as meat and milk production.

Sterile insect techniques is another potential avenue for tick control whereby ticks can be sterilized via crosses with sterile hybrids (Hillburn *et al.*, 1991), treatment with chemicals or irradiation (Hayes and Olivier, 1981; Galun *et al.*, 1972), as well as RNA interference (RNAi) (de la Fuente *et al.*, 2006b; Merino *et al*., 2011a) and reintroduced to reduce the reproductive potential of natural tick populations (Merino *et al*., 2012). A limitation of sterile techniques is that the release of large numbers of affected individuals is necessary to instil the trait into the

local population and reduce the reproductive fitness of the wild tick population (Merino *et al*., 2011a). Additionally, the requirement of specialized skills/training and equipment to implement sterile techniques as a control measure using some of the aforementioned procedures like RNAi, is impractical and further research is required to make this a viable control method.

1.1.5.4. Immunological control: Anti-tick vaccines

A relatively new stride in control of tick infestations and tick-borne disease transmission is the development of anti-tick vaccines that offers a renewable resource with little drawback in acquired resistance and residue problems associated with acaricides (Graf *et al.*, 2004; de la Fuente *et al.*, 2007a; de la Fuente *et al*., 2007b; Guerrero *et al*., 2012). Though current vaccines lack efficacy for stand-alone use, their integration into current control strategies could give producers an edge in resistance management (Willadsen, 2006; de la Fuente *et al*., 2007b).

Immunological control of ticks is currently approached in two ways. The first is based on investigation into the physiological and immunological responses elicited by repeated attachment of ticks to their host. Antigens involved in these responses are typically components directly “exposed” to the host during feeding and involve mostly secreted anticoagulants and structural proteins from tick salivary glands or mouthparts. The second approach is based on the stimulation of an immunological response that does not occur as a result of natural parasite-host interactions. Antigens involved in these types of responses are “concealed” from the host immune system and typically include components from the midgut (Opdebeeck, 1994; Willadsen and Jongejan, 1999). Acquired immunity toward infestation for both approaches should result in a reduction of the number of engorged ticks, body weight and fecundity of ticks feeding on vaccinated animals (Willadsen, 2004; de la Fuente and Kocan, 2006a; Nuttall *et al.*, 2006). These two approaches in immunological control are expanded on in the following sections.

Exposed antigens

Acquired immunity from vaccination with exposed antigens has for the most part focused upon the tick attachment site, especially components of the cement cone used in ixodid attachment. However, the inclusion of proteins from tick salivary glands has widened the repertoire of available antigens as potential vaccine targets (Willadsen, 2004). Using truncated constructs of a putative cement protein from *Rhipicephalus appendiculatus* (64P, 15 kDa), a reduction in engorgement- and egg weight, as well as a 48% and 70% mortality rate in nymphs and adults, were observed (Trimnell *et al.*, 2002). Additional trials using trypsin inhibitors isolated from *R. microplus* larvae, also demonstrated a 72.8% efficacy in vaccinated cattle (Andreotti *et al.*, 2002; Andreotti, 2007). A 29 kDa matrix-associated protein related to vertebrate collagens, identified from *Haemaphysalis longicornis* salivary glands, provided protection during vaccination trials. Results showed a reduction in adult female engorgement and a 56% mortality rate of nymphs post-engorgement (Mulenga *et al.*, 1999). Other antigens that may be employed to obtain acquired immunity include secreted calreticulin (identified for *Amblyomma americanum; Dermacentor variabilis* and *R. microplus*), anti-complement and histamine-binding proteins (identified from *Ixodes scapularis*) (Valenzuela, 2004; Willadsen, 2004). Though, the use of exposed antigens as vaccine targets has the added benefit of enabling a direct priming of the host immune responses against tick infestation during feeding, co-evolution between the tick parasite and bovine host has resulted in ticks evolving specific molecules and systems to circumvent host immune responses to exposed tick proteins (Guerrero *et al*., 2012; Brake and Pérez de León; 2012). Furthermore, a lack of vaccine efficacy may be also be due to functional redundancy, since many of the exposed antigens identified and/or tested in vaccination trails to date are members of large gene families (i.e. histamine-binding proteins, serine proteases and trypsin inhibitors) (Table 1.2.) (Guerrero *et al*., 2012). Other draw backs to acquired resistance via exposed antigens are that few antigenic targets have been fully characterised and validated in vaccination trials, as well as determinants that could infer cross-protection are not clear and requires further in-depth investigation.

Concealed antigens

Concealed antigens represent another class that has been explored for novel vaccine development. For ticks, traditionally the gut lumen is regarded as a source for concealed antigens as it is exposed to antibodies ingested from the blood meal (Nuttall *et al*, 2006; Guerrero *et al*., 2012). However, as long as a tick protein is able to interact with host

immunoglobulins it can be considered as a concealed vaccine antigen. Furthermore, candidate antigens for evaluation as vaccines should preferably have a critical biological function that if disrupted will cause mortality in the parasite or lead to a reduction in fecundity (Guerrero *et al*., 2012). It was these characteristics that led to the most successful anti-tick vaccine to date, Bm86, a membrane-bound gut protein identified from *R. microplus*. It is the main component of commercial vaccines generally known as TickGARD plus (recently discontinued; P. Willadsen personal communication) and GAVAC (reviewed by Willadsen and Jongejan, 1999; Willadsen, 2004; Willadsen, 2006; de la Fuente *et al*., 2007c). Vaccination with this tick antigen causes destabilisation of the gut lumen, leading to leakage of gastric content into the tick body cavity during feeding resulting in so-called “red ticks”. During field trials, vaccinating cattle with native Bm86 (TickGARD plus) showed a 56% reduction in tick numbers and 72% reduction in fecundity after one generation (Jonsson *et al.*, 2000). A further 90% reduction in larvae production has also been reported for the most susceptible tick isolates and a two-third reduction in acaricide treatment required for tick control in Cuban trials (Willadsen, 2006). To date, a number of homologues have been identified in other tick species and cross-reactivity between species of ticks has been investigated (Table 1.2.) (Fragoso *et al.*, 1998; Odongo *et al.*, 2007; Canales *et al.*, 2008; Perez-Perez *et al.*, 2010; Kumar *et al.*, 2012). However, variability of the Bm86 vaccine efficacy is observed not only between different species, but also within strains. The latter variation is dependent on the characteristics of each tick population, as well as the breed of cattle that was immunized (Parizi *et al*., 2009). This inter-strain variation is likely considering the vast amount of transposons and retro-transposable elements in the genome of *R. microplus* (Guerrero *et al.*, 2010), as well as inter-strain sequence variation and differential control of gene expression (Kamau *et al.*, 2011). Another “concealed” antigen identified is the carboxydipeptidase Bm91 from *R. microplus*, which has also been shown to be effective as a vaccine candidate, as well as to increase the efficacy of Bm86 (Jarmey *et al.*, 1995; Willadsen, 2004). Though the benefit of including Bm91 in the commercial Bm86 vaccine formulation was considered negligible, this research still illustrates the feasibility of a multi-valent vaccine (Willadsen, 2008). An additional drawback of a vaccine made from concealed antigens such as Bm86 is that antibody titres are not boosted with tick infestation, but require continual booster injections (Willadsen and Jongejan, 1999; Willadsen, 2004). However, it has been suggested that a vaccine targeting both exposed and concealed antigenic target(s) would be of great benefit (Trimnell *et al.*, 2002; Guerrero et al., 2012). These dual-action vaccines combine the

advantages boosting the bovine host and keeping the immune response primed following initial vaccination (Guerrero et al., 2012).

Expression library immunisation experiments performed with salivary gland cDNA from *I. scapularis* by Almazán *et al.* (2003) in mice, identified additional concealed antigens 4D8 and 4F8 (Table 1.2). Subolesin (4D8), a tick ortholog related to insect akirins proposed to function as a transcriptional regulator involved in tick innate immunity (Naranjo *et al*., 2013), was obtained that showed a 40% reduction in *I. scapularis* infestation (44% efficiency) on mice. Consequent vaccination trials with synthetic and recombinant protein showed a 61% and 58% reduction in larval and adult infestation, respectively (71% efficacy for both) (Table 1.2) (Almazán *et al.*, 2005a; Almazán *et al.*, 2005b; Galindo *et al.*, 2009). Recent studies using a combinatorial approach involving *in vivo* subolesin gene knock-down in *R. microplus* and vaccination of cattle using recombinant protein, showed an overall vaccine efficacy against infestation of 75% and a greater than 97% decrease in the incidence of tick-borne pathogen transmission (i.e. *B. bigemina* and *A. marginale*) (Merino *et al*., 2011a; Merino *et al*., 2011b). Another antigen, 4F8, identified during these experiments showed a 50% reduction in *I. scapularis* infestation (58% efficiency) (Almazán *et al*., 2003). This putative 5'-nucleotidase (4F8), produced a 64% reduction in larval infestations (62% efficacy) during vaccination trials in mice with recombinant protein (Almazán *et al.*, 2005b). A similar antigen found in various tissues (incl. gut, ovaries and Malpighian tubules) of *R. microplus* was tested in both sheep and cattle trials, but was unable to confer protection against tick infestation in cattle though a 73% reduction in eggs was recorded for sheep trials (Liyou *et al.*, 2000; Hope *et al.*, 2010). Therefore, a deeper understanding of the factors involved in protective immunity is necessary in order to evaluate promising candidates for vaccine development. Table 1.2. summarizes some of the tick antigens evaluated in vaccine trials to date.

Table 1.2. Summary of tick antigens evaluated in vaccination trials. Indicated are various antigens with their protein identity tested for various tick species in vaccination trials. Also indicated are the source tissue(s), vaccine antigen type (native/ recombinant/synthetic), experimental animal and vaccine efficacy. Vaccine efficacies indicated in percentage were determined, otherwise individual mortality, reduction in infestation, reduction in eggs and egg viability are indicated. Denoted are no significant effect (NSE), efficacy less than 25% (*), 25-50% efficacy (**), 50-75% efficacy (***) and efficacy greater than 75% (****).

Protein identity	Antigen name	Species	Source	Protein type(s)	Experimental host(s)	Vaccine efficacy	Reference
5'-nucleotidase	5'-nucleotidase (4F8)	*Rhipicephalus microplus*	Malpighian tubules	Recombinant	Cattle	NSE	Hope *et al.*, 2010
Acid phosphatase	HL-3	*Haemaphysalis longicornis*	Various	Recombinant	Rabbits	** (28% mortality)	Zhang *et al.*, 2011
Angiotensin-converting enzyme	Bm91	*Rhipicephalus microplus*	Salivary glands	Recombinant	Cattle	* (6 and 8% reproductive efficiency and egg viability)	Lambertz *et al.*, 2012
Angiotensin-converting enzyme	Bm91	*Rhipicephalus microplus*	Salivary glands	Native	Cattle	** (~37% reduction in eggs)	Riding *et al.*, 1994
Anti-compliment protein	IRAC	*Ixodes ricinus*	Salivary glands	Recombinant	Rabbits	NSE	Gillet *et al.*, 2009
Aspartic proteinase	BYC	*Rhipicephalus microplus*	Eggs	Native	Cattle	*/** (14%and 36%)	da Silva Vaz *et al.*, 1998; Seixas *et al.*, 2012
Aspartic proteinase	BYC	*Rhipicephalus microplus*	Eggs	Recombinant	Cattle	** (25%)	Leal *et al.*, 2006; Seixas *et al.*, 2012
Calreticulin	HqCRT	*Haemaphysalis qinghaiensis*	Various	Recombinant	Sheep	* (15.6% mortality)	Gao *et al.*, 2008a
Cement protein	64TRP	*Ixodes ricinus*	Salivary glands	Recombinant	Rabbits	*** (~53% mortality)	Trimnell *et al.*, 2005
Cement protein	64TRP	*Rhipicephalus appendiculatus*	Salivary glands	Recombinant	Guinea pigs	*** (~62% mortality)	Trimnell *et al.*, 2002
Cement protein	64TRP	*Rhipicephalus sangiuneus*	Salivary glands	Recombinant	Guinea pigs	** (~47% mortality)	Trimnell *et al.*, 2005
Chitinase	CHT1	*Haemaphysalis longicornis*	Exoskeleton	Recombinant	Mice	* (~12.4% reduction in eggs)	You and Fujisaki, 2009
Component of vitellin	GP80	*Rhipicephalus microplus*	Various	Native	Sheep	*** (68%)	Tellam *et al.*, 2002
Component of vitellogenin, vitellin	VIT87	*Rhipicephalus microplus*	Eggs	Native	Sheep	*** (68%)	Tellam *et al.*, 2002
Elongation factor 1 alpha	Ef1a	*Rhipicephalus microplus*	Various	Recombinant	Cattle	** (31%)	Almazán *et al.*, 2012
Extracellular matrix protein, collagen	P29	*Haemaphysalis longicornis*	Salivary glands	Recombinant	Rabbits	**/*** (40-56% mortality)	Mulenga *et al.*, 1999
Extracellular matrix protein, Glycine-rich protein	RH50	*Rhipicephalus haemaphysaloides*	Salivary glands	Recombinant	Rabbits	** (30.5% mortality)	Zhou *et al.*, 2006
Ferritin 2	IrFER2	*Ixodes ricinus*	Midgut	Recombinant	Rabbits	**** (98%)	Hajdusek *et al.*, 2010
Ferritin 2	RaFER2	*Rhipicephalus annulatus*	Midgut	Recombinant	Cattle	*** (72%)	Hajdusek *et al.*, 2010
Ferritin 2	RmFER2	*Rhipicephalus microplus*	Midgut	Recombinant	Cattle	*** (64%)	Hajdusek *et al.*, 2010
Glutathione S-transferase	GST-Hl	*Rhipicephalus microplus*	Various	Recombinant	Cattle	** (57%)	Parizi *et al.*, 2011
Glutathione S-transferase, Vitellin-degrading enzyme and Aspartic proteinase	GST-Hl, VTDCE and BYC	*Rhipicephalus microplus*	Various	Recombinant	Cattle	**/*** (35.3 to 61.6% protection against infestation)	Parizi *et al.*, 2012

Protein identity	Antigen name	Species	Source	Protein type(s)	Experimental host(s)	Vaccine efficacy	Reference
Mating factor, voraxinα	Voraxinα	*Rhipicephalus appendiculatus*	Testis	Recombinant	Rabbits	*** (~50% reduction in eggs)	Yamada *et al.*, 2009
Mating factors, voraxinα and voraxinβ	AhEF	*Amblyomma hebraeum*		Recombinant	Rabbits	NSE (only 72% reduction in tick weight)	Weiss and Kaufman, 2004
Metalloprotease	HLMP1	*Haemaphysalis longicornis*	Salivary glands	Recombinant	Rabbits	* (15.2% and 8.8% mortality)	Imamura *et al.*, 2009
Metalloprotease	Metis 1	*Ixodes ricinus*	Salivary glands	Recombinant	Rabbits	** (~38.6% reduction in eggs)	Decrem *et al.*, 2008
Mucin	BMA7	*Rhipicephalus microplus*	Whole ticks, membrane fractions	Native	Cattle	* (~21% reduction in eggs)	McKenna *et al.*, 1998
Nucleotidase	4F8	*Amblyomma americanum*	Various	Recombinant	Mice	* (9% reduction in infestation)	Almazán *et al.*, 2005b
Nucleotidase	4F8	*Ixodes scapularis*	Various	Recombinant	Mice and Sheep	*** (62% and 33%)	Almazán *et al.*, 2005a; Almazán *et al.*, 2005b
P-selectin-binding protein	Om44	*Ornithodoros moubata*	Salivary glands	Native	Pigs	** (~50% and 43.8% reduction in feeding and fecundity)	Garcia-Varas *et al.*, 2010
Putative adhesion protein related to cement proteins	HL34	*Haemaphysalis longicornis*	Salivary glands	Recombinant	Rabbits	* (29.1% mortality, 7.9% reduction in eggs)	Tsuda *et al.*, 2001
Ribosomal protein P0	pP0-KHL	*Rhipicephalus sanguineus*	Various	Synthetic peptide	Rabbits	**** (90.25%)	Rodríguez-Mallon *et al.*, 2012
Serine protease	Longistatin	*Haemaphysalis longicornis*	Salivary glands	Recombinant	Mice	*** (72.7%)	Anisuzzaman *et al.*, 2011
Serine protease inhibitor (Serpin)	HLS1	*Haemaphysalis longicornis*	Salivary glands	Recombinant	Rabbits	* (~32.3% and ~4.4% mortality)	Sugino *et al.*, 2003
Serine protease inhibitor (Serpin)	HLS2	*Haemaphysalis longicornis*	Hemolymph	Recombinant	Rabbits	*/** (~44.6% and ~17.6% mortality)	Imamura *et al.*, 2005
Serine protease inhibitor (Serpin)	IRIS	*Ixodes ricinus*	Salivary glands	Recombinant	Rabbits	** (~33.6% mortality)	Prevot *et al.*, 2007
Serpin-3 (RAS), Serpin-4 (RAS-4) and 36kDa immuno-dominant protein (RIM36)	RAS-3, RAS-4 and RIM36	*Rhipicephalus appendiculatus*	Salivary glands	Recombinant	Cattle	** (~27% mortality)	Imamura *et al.*, 2008
Strain variant of Bm86	Bm95	*Rhipicephalus haemaphysaloides*	Midgut	Recombinant	Cattle	**** (58-89%)	Garcia-Garcia *et al.*, 2002; Sugumar *et al.*, 2011
Subolesin/Akirin	4D8	*Amblyomma americanum*	Various	Recombinant	Rabbits	* (17% reduction in infestation)	Almazán *et al.*, 2005b
Subolesin/Akirin	4D8	*Dermacentor variabilis*	Various	Recombinant	Rabbits	* (22% reduction in infestation)	Almazán *et al.*, 2005b
Subolesin/Akirin	4D8	*Ixodes scapularis*	Various	Recombinant	Mice and Sheep	*** (71%)	Almazán *et al.*, 2005a; Almazán *et al.*, 2005b
Subolesin/Akirin, unknown and nucleotidase	4D8, 4E6 and 4F8	*Amblyomma americanum*	Various	Recombinant	Rabbits	* (17% reduction in infestation)	Almazán *et al.*, 2005b
Subolesin/Akirin, unknown and nucleotidase	4D8, 4E6 and 4F8	*Dermacentor variabilis*	Various	Recombinant	Rabbits	* (22% reduction in infestation)	Almazán *et al.*, 2005b
Subolesin/Akirin, unknown and nucleotidase	4D8, 4E6 and 4F8	*Ixodes scapularis*	Various	Recombinant	Sheep	*** (58%)	Almazán *et al.*, 2005a
Subolesin/Akirin	rOeSub	*Ornithodoros erraticus*	Various	Recombinant	Rabbits	** (22% reduction in eggs)	Manzano-Román *et al.*, 2012
Subolesin/Akirin	rOeSub	*Ornithodoros moubata*	Various	Recombinant	Rabbits	NSE	Manzano-Román *et al.*, 2012

Protein identity	Antigen name	Species	Source	Protein type(s)	Experimental host(s)	Vaccine efficacy	Reference
Subolesin/Akirin	rOmSub	*Ornithodoros erraticus*	Various	Recombinant	Rabbits	** (24.3% reduction in eggs)	Manzano-Román *et al.*, 2012
Subolesin/Akirin	rOmSub	*Ornithodoros moubata*	Various	Recombinant	Rabbits	NSE	Manzano-Román *et al.*, 2012
Subolesin/Akirin	Subolesin (4D8)	*Rhipicephalus annulatus*	Various	Recombinant	Cattle	*** (60%)	Almazán *et al.*, 2010, Almazán *et al.*, 2012
Subolesin/Akirin	Subolesin (4D8)	*Rhipicephalus microplus*	Various	Recombinant	Cattle	***/**** (51% and 81% depending on construct)	Almazán *et al.*, 2010, Almazán *et al.*, 2012
Tick salivary cystatin	Sialostatin L2	*Ixodes scapularis*	Salivary glands	Recombinant	Guinea pigs	* (19% protection against attachment)	Kotsyfakis *et al.*, 2008
Troponin I	P27/30	*Haemaphysalis longicornis*	Various	Recombinant	Mice	* (~18.4% reduction in eggs)	You, 2005
Troponin T	HqTnT	*Haemaphysalis qinghaiensis*	Various	Recombinant	Sheep	NSE	Gao *et al.*, 2008b
Trypsin inhibitor	BmLTI	*Rhipicephalus microplus*	Various	Recombinant	Cattle	** (32%)	Andreotti *et al.*,, 2012
Trypsin inhibitor	BmTI	*Rhipicephalus microplus*	Salivary glands	Native	Cattle	*** (72.8%)	Andreotti *et al.*, 2002
Trypsin inhibitor	BmTI-A	*Rhipicephalus microplus*	Salivary glands	Synthetic	Cattle	* (~18.4%)	Andreotti *et al.*, 2007
Ubiquitin	UBE	*Rhipicephalus annulatus*	Various	Recombinant	Cattle	* (15% and 22% depending construct preparation)	Almazán *et al.*, 2010, Almazán *et al.*, 2012
Ubiquitin	UBE	*Rhipicephalus microplus*	Various	Recombinant	Cattle	*** (55%)	Almazán *et al.*, 2010
Unknown	4E6	*Amblyomma americanum*	Various	Recombinant	Mice	** (29% reduction in infestation)	Almazán *et al.*, 2005b
Unknown	4E6	*Dermacentor variabilis*	Various	Recombinant	Mice	* (5% reduction in infestation)	Almazán *et al.*, 2005b
Unknown	4E6	*Ixodes scapularis*	Various	Recombinant	Mice and Sheep	*** (64% and 40%)	Almazán *et al.*, 2005a; Almazán *et al.*, 2005b
Unknown	ARS antigen 1	*Rhipicephalus microplus*	Midgut	Recombinant	Cattle	**** (73-76%)	Rachinsky *et al.*, 2008; Guerrero *et al.*, 2012
Unknown	ARS antigen 1 and 2	*Rhipicephalus microplus*	Midgut	Recombinant	Cattle	*** (71%)	Rachinsky *et al.*, 2008; Guerrero *et al.*, 2012
Unknown	ARS antigen 2	*Rhipicephalus microplus*	Midgut	Recombinant	Cattle	*** (63%)	Rachinsky *et al.*, 2008; Guerrero *et al.*, 2012
Unknown	Bm86	*Amblyomma cajenensis*	Midgut	Recombinant	Cattle	* (17% reduction in infestation)	Rodríguez-Valle *et al.*, 2012
Unknown	Bm86	*Amblyomma variegatum*	Midgut	Recombinant	Cattle	NSE	de Vos *et al.*, 2001
Unknown	Bm86	*Hyalomma anatolicum anatolicum*	Midgut	Recombinant	Cattle	** (~26.8 and ~25.1%)	de Vos *et al.*, 2001, Kumar *et al.*, 2012
Unknown	Bm86	*Hyalomma dromedarii*	Midgut	Recombinant	Cattle	**** (~92% reduction in infestation)	de Vos *et al.*, 2001; Rodríguez-Valle *et al.*, 2012
Unknown	Bm86	*Hyalomma excavatum*	Midgut	Recombinant	Cattle	NSE	Galai *et al.*, 2012
Unknown	Bm86	*Hyalomma scupense*	Midgut	Recombinant	Cattle	NSE	Galai *et al.*, 2012
Unknown	Bm86	*Rhipicephalus annulatus*	Midgut	Recombinant	Cattle	**** (~100%)	Canales *et al.*, 2009; Fragoso *et al.*, 1998; Pipano *et al.*, 2003; Almazán *et al.*, 2010
Unknown	Bm86	*Rhipicephalus appendiculatus*	Midgut	Recombinant	Cattle	NSE	de Vos *et al.*, 2001, Odongo *et al.*, 2007
Unknown	Bm86	*Rhipicephalus decoloratus*	Midgut	Recombinant	Cattle	*** (70% reduction in eggs)	de Vos *et al.*, 2001, Odongo *et al.*, 2007

Protein identity	Antigen name	Species	Source	Protein type(s)	Experimental host(s)	Vaccine efficacy	Reference
Unknown	Bm86	*Rhipicephalus microplus*	Midgut	Native	Cattle	**** (92%)	Willadsen *et al.*, 1989
Unknown	Bm86	*Rhipicephalus microplus*	Midgut	Recombinant	Cattle	***/**** (51-91%, depending on tick strain)	Willadsen *et al.*, 1995; Rodriguez *et al.*, 1995; Garcia-Garcia *et al.*, 1998; Jonsson *et al.*, 2000; Garcia-Garcia *et al.*, 2000; Canales *et al.*, 2009, de la Fuente *et al.*, 2000
Unknown	Bm86	*Rhipicephalus sangiuneus*	Midgut	Recombinant	Dogs	** (~31% reduction in infestation)	Perez-Perez *et al.*, 2010
Unknown, Bm86 peptide derivative	SBm19733	*Rhipicephalus microplus*	Various	Synthetic peptide	Cattle	**** (35.87%)	Pataroyo *et al.*, 2002
Unknown, Bm86 peptide derivative	SBm4912	*Rhipicephalus microplus*	Various	Synthetic peptide	Cattle	**** (72.4%)	Pataroyo *et al.*, 2002
Unknown, Bm86 peptide derivative	SBm7462	*Rhipicephalus microplus*	Various	Synthetic peptide	Cattle	**** (81.05%)	Pataroyo *et al.*, 2002
Unknown, homologue of Bm86	Haa86	*Hyalomma anatolicum anatolicum*	Midgut	Recombinant	Cattle	*** (~68.7% and ~45.8%)	Azhahianambi *et al.*, 2009; Kumar *et al.*, 2012
Unknown, homologue of Bm86	Haa86	*Rhipicephalus microplus*	Midgut	Recombinant	Cattle	** (~36.5%)	Kumar *et al.*, 2012
Unknown, homologue of Bm86	Ba86	*Rhipicephalus annulatus*	Midgut	Recombinant	Cattle	**** (83%)	Canales *et al.*, 2009
Unknown, homologue of Bm86	Ba86	*Rhipicephalus microplus*	Midgut	Recombinant	Cattle	*** (~71.5%)	Canales *et al.*, 2009
Unknown, homologue of Bm86	Hd86	*Hyalomma excavatum*	Midgut	Recombinant	Cattle	NSE	Galai *et al.*, 2012
Unknown, homologue of Bm86	Hd86	*Hyalomma scupense*	Midgut	Recombinant	Cattle	NSE	Galai *et al.*, 2012
Unknown	Hq05	*Haemaphysalis qinghaiensis*	Salivary glands	Recombinant	Sheep	** (40% and 37% reduction in eggs and egg viability)	Gao *et al.*, 2009
Unknown	Oe45	*Ornithodoros erraticus*	Midgut	Native	Pigs	* (~23% mortality)	Manzano-Roman *et al.*, 2007
Unknown and Angiotensin-converting enzyme	Bm86 & Bm91	*Rhipicephalus microplus*	Midgut & Salivary glands	Recombinant	Cattle	**** (> 100%)	Willadsen *et al.*, 1996
Unknown and mucin	Bm86 & BMA7	*Rhipicephalus microplus*	Whole ticks, membrane fractions	Recombinant (Bm86) and Native (BMA7)	Cattle	**** (~90% reduction in eggs)	McKenna *et al.*, 1998
Unknown and nucleotidase	Bm86 & 4F8	*Rhipicephalus microplus*	Midgut	Recombinant	Sheep	**** (85%)	Hope *et al.*, 2010
Vitellin-degrading enzyme	VTDCE	*Rhipicephalus microplus*	Eggs	Native	Cattle	* (21%)	Seixas *et al.*, 2008; Seixas *et al.*, 2012

1.1.6. Factors related to immunogenicity

Past experience has indicated some essential contributing factors to protective immunity that merits consideration when selecting antigens for vaccination. These include host factors such as the presence of B- and T-cell epitopes and the type of immune response elicited as a result of vaccination and tick feeding, parasite/pathogen factors such as expression level of antigens and their sub-cellular localization, as well as chemical and physical properties of the test antigen such as its post-translational modifications and aggregation status when used for vaccination (Fig. 1.4.). These form the basis of discussion for the following sections.

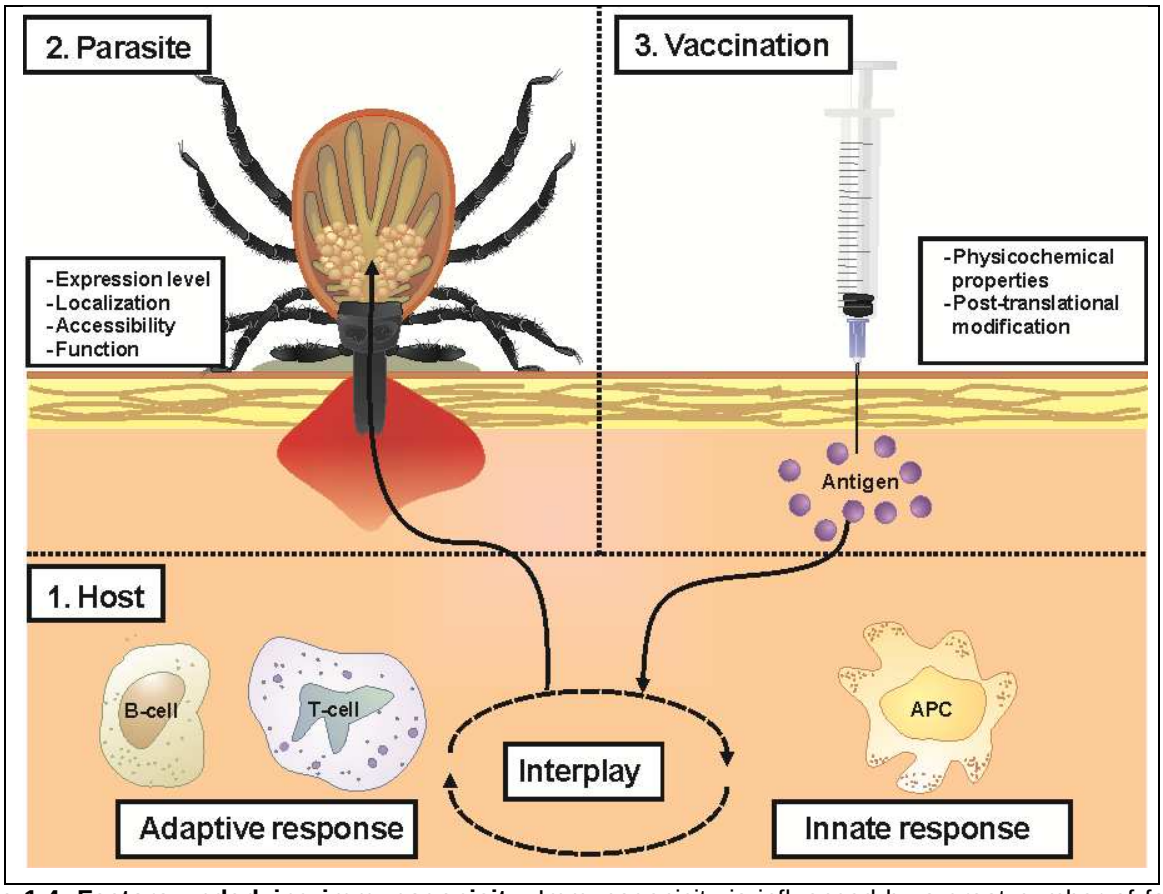

Figure 1.4. Factors underlying immunogenicity. Immunogenicity is influenced by a great number of factors, these include: (1) Factors associated with host immunity, (2) parasite proteins and (3) the vaccine antigen. Abbreviation: APC- Antigen presenting cell. Reproduced from Maritz-Olivier *et al.* (2012).

1.1.6.1. Host contributing factors

Principles underlying protective immunogenicity in humans against viral diseases, bacterial pathogens and intracellular eukaryotic parasites such as *Plasmodium* spp. are well described. However, very limited information is available for ectoparasites, such as ticks and their associated hosts. In the host, a parasite/pathogen is faced by both the innate defense mechanisms (mediated by tissue-residing macrophages and dendritic cells, as well as mobile phagocytic cells) and the adaptive immune response (mediated by large sets of molecules and cells that confer either regulatory or effector functions).

Research has revealed that innate immunity sets the scene for the adaptive response; and that innate and adaptive immunity have to interact vigorously (via antigen presenting cells) to confer protective immunity against parasite/pathogen transmission/infestation (Fig. 1.4.) (Zepp, 2010). Protective immunity against a vast amount of diseases is mediated via the adaptive immune response, meaning it is antibody -, cytokine-, B- and/or T-cell dependent (Zepp, 2010). Evidence for the possibility of induced protective immunity against ticks was provided in 1988 by Opdebeeck and colleagues, who indicated that membrane fractions from *R. microplus* midgut tissues protected cattle by 91% against tick challenge and that the levels of IgG1 and complement-fixing antibodies related to the levels of protection induced by vaccination (Opdebeeck *et al.*, 1988; Jackson and Opdebeeck, 1990). Furthermore, the anti-tick vaccine based on the Bm86 midgut protein induces a strong IgG-mediated response and it is believed that the anti-Bm86 IgG with the aid of complement is enough to damage the lumen of the tick gut, in the absence of cell-mediated responses (Kemp *et al.*, 1989; Rand *et al.*, 1989; Vargas *et al.*, 2010). In 2009, Piper *et al.* showed that both *Bos indicus* and *Bos taurus* cattle infested with *R. microplus* displayed a strong adaptive immune response. *Bos indicus* displayed a strong T-cell mediated response, while in *Bos taurus* breeds, high levels of inflammatory molecules, IgG1 and elevated MHCII gene expression was observed. It was therefore evident that an acquired T-cell response is critical to the development of tick-specific IgG and most probably to host resistance to infestation (Piper *et al.*, 2009).

1.1.6.2. Parasite/ antigen contributing factors

For proteins to be accessible to the host immune system, it is presumed that they are expressed during a reasonable period of the life cycle and that they are secreted or presented on membranes or exposed surfaces of the parasite. Currently, localization to the cell's surface

is thought to be a major determinant of immunogenicity (Flower, 2008; Vivona et al., 2008; Rappuoli and Bagnoli, 2011). As it is known that membrane fractions from *R. microplus*, and not secreted proteins, confer protection to cattle, emphasis is placed on membrane-associated proteins (Opdebeeck *et al.*, 1988; Jackson and Opdebeeck, 1990; Guerrero et al., 2012b). However, the complexity of the cell, subcellular compartmentalization and/or membrane-association, is severely under-estimated and the localization of proteins to specific organelles that may be transient, permanent or occur in multiple localizations, as well as function as multi-protein complexes, remain a difficult prospect with available tools (Flower *et al.*, 2010).

Although the function a protein performs in the parasite is irrelevant to its status as an antigen (a protein recognized and recalled by the host), targeting a protein of vital function during vaccination remains sensible. To date, proteins involved in adhesion, invasion, secretion, signaling and evading host responses, as well as lipoproteins are regarded as key players in the host-pathogen/host-parasite interface and therefore good vaccine candidates (Vivona *et al.*, 2008; Rappuoli and Bagnoli, 2011).

While biotechnology has improved significantly over the past few years, the expression of recombinant protein antigens remains a serious limitation to the production of vaccines (Gasser *et al*. 2013; Whittaker*et al*., 2013). In bacterial pathogens such as *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Porphyromonas gingivalis*, the number of vaccine candidates that can successfully be expressed, range between 30-60% of the initially identified number of candidates. Of the latter, only 1-4% of the candidates confer immunity *in vivo* (Flower *et al.*, 2010; Rappuoli and Bagnoli, 2011). This can in part be attributed to the physicochemical properties and the post-translational modifications of the antigen. Whether peptide or protein, the properties of an antigen result from the primary and secondary protein structure (as well as their associated modifications), thereby influencing its solubility, charge, aggregation status and stability. Of greater importance, is the contribution of these properties to the quality of the immune response, including binding capability of antibodies, dynamics of the peak/priming response and generation of immune memory cells (Flower, 2008; Zepp, 2010).

Glycosylation is regarded as an important factor when manufacturing vaccines, as it is known that polysaccharides can serve as a first signal for B-cell activation (de la Fuente *et al*.,

2006c; Avci *et al*., 2011). Following internalization of the glycosylated protein, the protein component is presented to T-helper cells, thereby promoting antibody switching from IgM to IgG and generation of memory B-cells (Zepp, 2010; Avci *et al*., 2011). Preliminary evidence that protective antigens in *R. microplus* midgut are either glycoproteins, or are dependent on carbohydrates for their specificity came from the study by Lee *et al.*, who showed that sodium metaperiodate (periodate) treatment of gut membrane fractions completely abolished their protective ability in cattle (Lee *et al.*, 1991). This was further supported by studies done with Bm86 expressed in *Pichia pastoris* (that allows for glycosylation), where antibodies directed against the carbohydrate determinants of Bm86 were raised. These determinants were however found not to be protective (Willadsen and McKenna, 1991; Garcia-Garcia *et al.*, 1998a; Garcia-Garcia *et al.*, 1998b). Therefore, the true role of glycosylation in tick vaccine efficacy remains to be determined.

Finally, the use of crude extracts or purified recombinant antigen, as well as the choice in adjuvant, can also contribute to the efficacy of the final vaccine formulation (Leroux-Roels 2010; Zepp 2010). Examples of the effect of these factors on immunization of cattle with *R. microplus* antigens have been published (Jackson and Opdebeeck, 1995; Almazán *et al.*, 2012).

1.2. Reverse vaccinology and vaccine discovery in the post-genomic era

The availability of completed genomes, in combination with the fast expanding amount of transcriptomic data, is enabling scientists to venture into a post-genomic era for rational vaccine design (Strausberg and Levy, 2007). By utilizing the principles of genome-based vaccine development (reverse vaccinology), it is possible to access all the proteins that are encoded by an organism using available genome or transcriptome data in combination with computational analysis, rather than starting with the organism itself (Moriel *et al.*, 2008).

In a reverse vaccinology approach, *in silico* analysis represents the central step for the identification of promising protein-based vaccines (Fig. 1.5.). Typically, the first step entails the prediction and localization of protein encoding sequences within the genome, followed by analysis of their expression profiles, sequence similarity to the host and the sub-cellular localization of the proteins within a cell (Flower, 2008; Flower *et al.*, 2010). In cases where a genome is lacking, transcriptome analyses via DNA microarrays or RNA-Seq will allow the

identification of expression patterns throughout the life cycle of the pathogen/parasite and in combination with proteomics, the associated protein expression levels (Klopfleisch and Gruber, 2012). Recognition of antigenic epitopes by T-cells, B-cells and soluble antibodies form the basis of the immune response. In this regard, various *in silico* T- and B-cell epitope predictors have been developed and used with great success for bacterial and viral pathogens (Flower, 2008; Rappuoli and Bagnoli, 2011). The use of reverse vaccinology (including immuno-informatic tools) as a process for identification and selection of potential anti-tick vaccine antigens from genomic and transcriptomic data, has not been sufficiently demonstrated to date. However, sequence data is currently available for important tick species (i.e. *I. scapularis* and *R. microplus*) that would enable rational vaccine design using a reverse vaccinology approach (Ullmann *et al.*, 2005; Guerrero *et al.*, 2006).

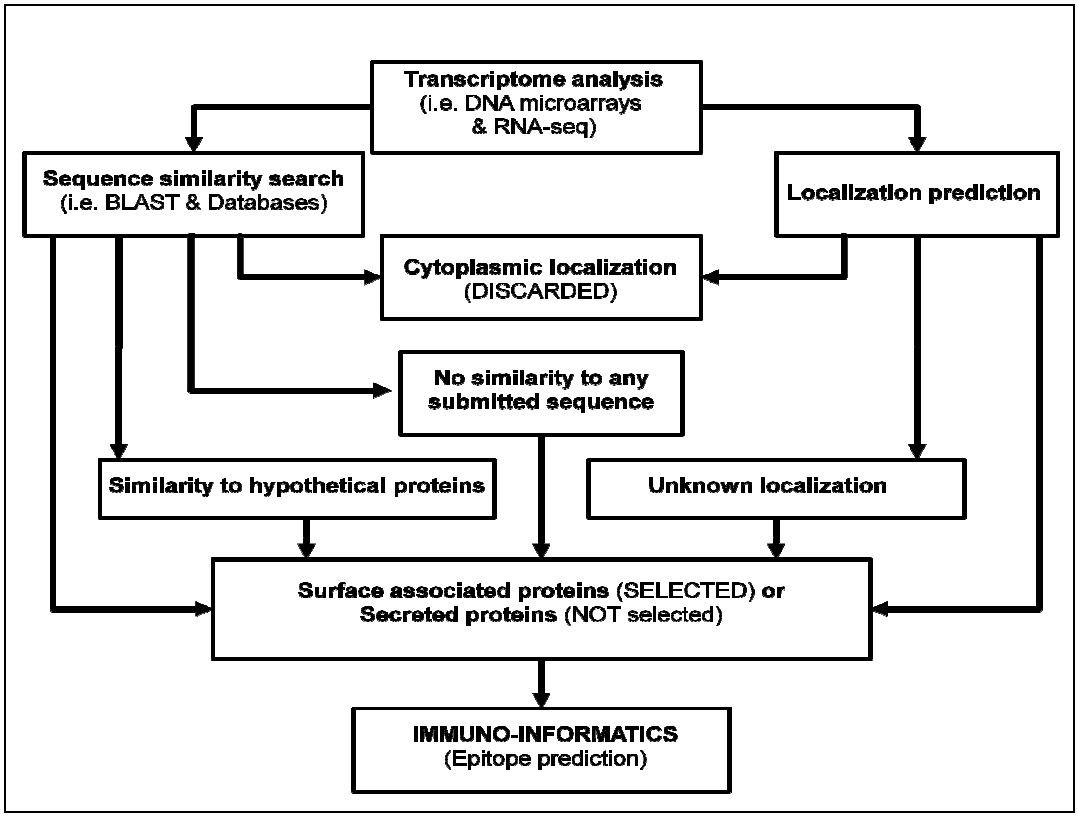

Figure 1.5. Basic strategy used for the identification of vaccine candidates using functional genomics and *in silico* reverse vaccinology approaches. Adapted from Rappuoli and Bagnoli (2011).

1.2.1. Tick genomes and nucleotide databases

In order to employ a reverse vaccinology approach to rational anti-tick vaccine design, the availability of a fully annotated genome is a considerable asset. In this regard, rationales were provided for whole genome sequencing of vector species, *I. scapularis* and *R. microplus*, due to their tremendous impact on animal and human health (Ullmann *et al.*, 2005; Guerrero *et al.*, 2006). Consequently, the *Ixodes scapularis* genome project was initiated in 2004 and currently the genome of *I. scapularis* (2.1 x10^3 mega bases) is being assembled by combining sequence data from available expressed sequence tag (EST) databases, as well as cDNA library-, bacterial artificial chromosome (BAC) library- and whole genome shotgun sequencing (https://www.vectorbase.org/organisms/ixodes-scapularis) (Hill and Wikel, 2005; Pagel Van Zee *et al.*, 2007).

In contrast, the genome size of the cattle tick, *R. microplus*, has been determined to be about 7.1 x10^3 mega bases with about 60% constituting repetitive sequences (Ullmann *et al.*, 2005) and assembly of the genome is currently in its primary stages (Moolhuijzen *et al.*, 2011; Bellgard *et al.*, 2012). Recently, a *R. microplus* expressed sequence tag database (EST) of 42,512 expressed sequence tags was assembled into a gene index (BmiGI) of 13,643 unique singleton sequences and subjected to comparative genome analysis (Wang *et al.*, 2007). These sequences were derived from various tissues, life stages and tick strains, including larvae exposed to various temperatures, host odor and acaricides. Additional EST sequence data is also currently available for *R. appendiculatus* (~18,500 ESTs) and *Amblyomma variegatum* (~3,992 ESTs) in the TIGR gene indices (Lee *et al.*, 2005).

The *R. microplus* gene index (BmiGI) has since been exploited in the first high-throughput transcriptomic analysis of acaricide-induced gene transcription in *R. microplus* larvae via DNA microarrays (Saldivar *et al.*, 2008). Though a variety of genes were identified as being significantly up- or down regulated during acaricide treatment, a great number of these genes could not be functionally assigned (Saldivar *et al.*, 2008). The latter is largely due to two factors: (1) there is considerable evolutionary distance between ticks and insects (as well as other organisms) that limits homology-based gene predictions (more than 60% of the *R. microplus* singletons remain to be annotated). (2) A lack of genomic tools and resources available for many ticks and mites (Hill and Wikel, 2005; Pagel van Zee *et al.*, 2007). Currently, information on tick protein sequences is extremely restricted and there is an urgent need for more information on the complement of proteins expressed in a variety of tick

tissues, life stages and species. In this regard, functional genomics may present a novel avenue for characterization of genes and enable identification of novel anti-tick vaccine antigens.

1.2.2. Functional genomics: Tools for anti-tick antigen discovery

Functional genomics is the research field that aims at elucidating and understanding gene and protein functions and interactions within any given organism (i.e. gene transcription and translation, protein-protein interactions and metabolomics) (Hocquette, 2005; Braga-Neto and Marques, 2006). Since a large number of sequences are currently available for ticks, such as *R. microplus*, high-throughput analyses of the available data using functional genomics tools is a promising avenue to discover novel tick antigens. Such analyses will also assist in sequence annotation of transcripts that lack corroborative functional data. A number of functional genomics techniques are currently available and include tools for transcriptional profiling (i.e. DNA microarrays and serial analysis of gene expression), protein-protein interaction or interactomics (i.e. Yeast two-hybrid and phage display) and loss-of-function or phenotype screening (i.e. RNAi and ELI) (Eisenberg *et al*., 2000; Lockhart and Winzeler, 2000; Pandey and Mann, 2000; Simson and Dobbin, 2003; Barry *et al*., 2004;, Collura and Boissy, 2007). Many of these techniques have been employed in tick research for discovery and evaluation novel tick antigens.

Expression library immunization (ELI) involves vaccination of test animals with DNA derived from a pathogen/parasite and assessing the protection conferred to the host following challenge with the test organism (Barry *et al.*, 2004). ELI has successfully been employed in tick research for high-throughput identification of novel tick protective antigens from 2705 cDNA clones in *I. scapularis* and includes the previously described protective antigen subolesin (Table 1.2.) (Almazán *et al.*, 2003). This technique however, requires the immunization and parasitic challenge of a large number of host animals (a single host for each individual candidate tested) that makes this approach painstaking, expensive and difficult to standardize (Ghosh, Azhahianambi & Yadav, 2007). In contrast, phenotypic screening via RNAi is a process of sequence-specific gene expression silencing at post-translational level (Kasner, 2008). This technique has been used extensively for rapid functional genomics analyses of a wide variety of genes in a number of tick species (including *R. microplus*), in the absence of other tools for genetic manipulation (de la Fuente *et al.*,

2007a). In a study using RNAi to screen *in vivo* for protective antigens from *I. scapularis* larval embryonic cells (IDE8), comparable results were obtained as previously obtained by expression library immunization (ELI) (de la Fuente, 2005). This research highlighted the use of RNAi as a powerful alternative tool that provides an assessable phenotype upon knockdown for identification of novel protective antigens. This technique has also been successfully applied in vaccination trials where combinatorial gene knock-down of subolesin in *R. microplus* and vaccination of cattle using recombinant protein, successfully protected against tick infestation and disease transmission (Merino *et al*., 2011a; Merino *et al*., 2011b). However, a major drawback to this methodology in screening for new vaccine candidates is that a phenotype *in vitro* does not necessarily translate to tick protective responses in cattle trials. Knock-down studies of Bm86 *in vitro* yielded a weak phenotype in spite of its efficacy as a vaccine antigen *in vivo* (Nijhof *et al*., 2010).

DNA microarrays are functional genomics research tools used for monitoring gene regulation and gene expression (transcriptome analysis) (Jares, 2006). This technique enables the identification of differentially expressed genes (under a particular set of conditions) and the elucidation of possible cellular processes in which they may participate via computational biology. DNA microarray analysis has only been explored recently as a new tool in the tick field by Saldivar *et al.* (2008), where the effects on the transcriptome of resistant *R. microplus* larvae were determined, following acaricide treatment. Results indicated that a transcript with similarity to glutathione S-transferase may play a significant role in resistance to acaricide treatment. Since then, several transcriptional profiling studies have been conducted for *R. microplus* including studies of gene regulation involved in tick feeding, pathogen infection, acaricide resistance, responses to gene knock-down and feeding on different cattle hosts (Saldivar et al., 2008; Rodriguez-Valle et al., 2010; Lew-Tabor et al., 2011; Mercado-Curiel et al., 2011; Heekin et al., 2012; McNally et al., 2012). These works will be discussed in more detail in following chapters.

To conclude, DNA microarrays are a cutting-edge technology that provides and exciting platform for the generation of large amounts of biological data and will enable the rapid expansion of tick research.

1.3. Hypothesis and aims

1.3.1. Hypothesis

By applying a reverse vaccinology approach, including large-scale functional genomics and immuno-informatic approaches, essential transcripts will be identified for evaluation as anti-tick vaccine candidates.

1.3.2. Aim of the study

A reverse vaccinology approach will be explored for identification of novel *anti- R. microplus* vaccine targets using functional genomics via DNA microarrays as the first step in a proposed multilevel strategy (Fig. 1.4.). DNA microarray analysis of cDNA prepared from selected tissues and life stages of feeding *R. microplus* ticks will enable identification of transcripts that are highly up-regulated in more than one tissue and life stage. The latter will be used to identify transcripts that are shared amongst tissues and life stages during feeding. From the latter bioinformatic and immuno-informatic analysis will be performed to identify putative antigenic transcripts that can be further evaluated for suitability as vaccine candidates in future studies.

Chapter 2 outlines the assembly of a sequence database consisting of all the available EST and gene index sequence data for *R. microplus*. This resource was utilized in the development of an oligonucleotide array that was employed in the consequent transcriptional profiling of major tissues in feeding *R. microplus* female tissues.

Chapter 3 firstly presents the transcriptional responses in the immature larvae and nymphal stages of feeding *R. microplus* ticks. This is followed by comparisons with adult tissues and a number of transcripts are identified that are shared between life stages. Lastly, the bioinformatic and immuno-informatic analysis of the global transcriptomic data is presented in order to identify potential vaccine antigens.

Chapter 4 presents a concluding discussion of the scientific contributions of this study to the tick field and outlines some of the future perspectives.

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Chapter 2

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

2.1. Abstract

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

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2.2. Introduction

2.2.1. Transcriptional profiling of *R. microplus* adult ticks

Almost three decades after its first application in expression analysis of mouse colon tumors in 1982, DNA microarrays have evolved into an essential research tool used for monitoring gene regulation and gene expression (Augenlicht and Kobrin, 1982). This high-throughput

technique has enabled researchers to investigate transcriptional regulation in various cancers, annotate transcripts, elucidate mechanisms and effects of gene silencing, alternative splicing, polymorphism and promoter mapping, as well as pathogen characterization (Kimmel and Oliver, 2006a). In terms of veterinary research, data can demonstrate how a set of specific conditions may affect regulatory networks of cellular metabolism effecting metabolic pathways in order to elucidate interactions at the vector/host/pathogen interface (reviewed by Klopfleisch and Gruber, 2012).

2.2.2. Transcriptomic methodologies, experimental design and data analysis.

The transcriptome refers to all types of complementary RNA synthesized during genome transcription and include ribosomal RNA (rRNA), transfer RNA (tRNA), as well as microRNA (miRNA) and messenger RNA (mRNA) that is currently the main RNA species analyzed in transcriptome research (Velculescu *et al.*, 1997; Klopfleisch and Gruber, 2012). Several new cutting edge methods are available for high-throughput detection and quantification of miRNA and mRNA levels and these include microarrays, deep sequencing (RNA-Seq) and quantitative real-time PCR (qPCR) arrays (Klopfleisch and Gruber, 2012). With the advent of new generation sequencing technologies that allow the sequencing of millions of base pairs of cDNA target sequences (i.e. Illumina and 454 pyrophosphate sequencing technologies), information on the full set of RNA transcribed within a given cell or tissue can be determined (Wang *et al.*, 2009). RNA-Seq is however an expensive tool for routine transcriptional analysis, but the cost per number of reads is expected to decrease in the near future as the technology develops. Currently, DNA microarrays represent the most robust and relatively cost effective approach to high-throughput transcriptional profiling.

In general, microarray screening involves the use of a solid support (glass slide or silicon chip) with known nucleotide probes cross-linked to its surface that is able to hybridize competitively to any complementary DNA (cDNA) sequence targets in a given sample. The relative abundance of bound transcripts is determined by labeling target sequences with fluorescent dyes, i.e. Cy3 (green channel) or Cy5 (red channel), and consequent measurement of their fluorescence intensities (for two-color/ channel microarrays) (Shalon *et al.*, 1996; Kimmel and Oliver, 2006a). In the case of two-color/channel microarrays the relative expression of two differentially labeled samples are determined, whereas absolute

gene expression of a single sample is determined via single-color/ channel arrays (Kimmel and Oliver, 2006a).

2.2.2.1. Microarray platforms

To date, several platforms for the production of oligonucleotide microarrays have been established (Hardiman, 2004). Several predesigned microarray slides are currently commercially available for model species, including human, mouse, rat and bovine (Affymetrix, Agilent) (Klopfleisch and Gruber, 2012). However, custom arrays can be designed for any given DNA of interest and can be tailored to each experiment. For Affymetrix GeneChip® technology, 25 mer oligonucleotides are manufactured *in situ* on quartz chips using a combination of light-directed and solid-phase DNA synthesis. In contrast, NimbleGen technology uses digital micromirrors for *in situ* synthesis of 24 to 70 mer oligonucleotides.

Agilent technology manufacture standard and custom 60 mer oligonucleotides arrays using ink-jet technology and chemical processing of each added nucleotide layer (Kimmel and Oliver, 2006a). Agilent custom microarrays also provide various slide configurations with a maximal capacity of 8 arrays of 15,000 features or 60,000 features per slide for cDNA probes. Therefore, 120,000 or 450,000 potential target sequences can be screened per slide. In a comparative study using custom arrays produced with relatively long cDNA probes (800-2,000 bp) and Agilent 60 mer oligonucleotide arrays, it was shown that Agilent slides identified a greater number of differentially expressed transcripts and therefore had a greater detection specificity and sensitivity (Hockley *et al.*, 2009). The use of ink-jet 60 mer oligonucleotide arrays, such as Agilent, has been shown to reliably detect transcript ratios at single copy level in complex biological samples (Hughes *et al.*, 2001). Therefore, Agilent custom arrays provide an excellent opportunity for transcriptional profiling of *R. microplus*.

2.2.2.2. Microarray experimental design and data analysis

A variety of experimental designs are possible in two-color arrays. The most basic consideration for selection of a particular design is based on whether the comparison is direct (within slides) or indirect (between slides) (Fig. 2.1.). For a direct comparison, differential gene expression is measured directly between two samples on the same slide and often includes dye swaps to compensate for possible dye biases (Dobbin *et al.*, 2005). An indirect comparison measures differential expression between two samples (on separate slides) by

comparison to a co-hybridized common reference that may consist of a complex mixture of RNA (universal reference) or a pool comprising of RNA from all test samples (Simon and Dobbin, 2003). It is assumed that any contribution made by dye bias will affect all arrays equally and therefore will not bias comparisons between samples. Indirect comparison via reference design is the most commonly used approach, since it is relatively simple and allows easy addition of arrays (Churchill, 2002). Moreover, inter-individual variation between samples is standardized and therefore any subset of samples can be compared to any other subset of samples, as long as they are represented in the common reference. Relative hybridization is then measured from the logarithm of the ratio of intensities of the two labels (for test sample and reference) at the same spot (Simon and Dobbin, 2003). Therefore, this approach was followed in this study.

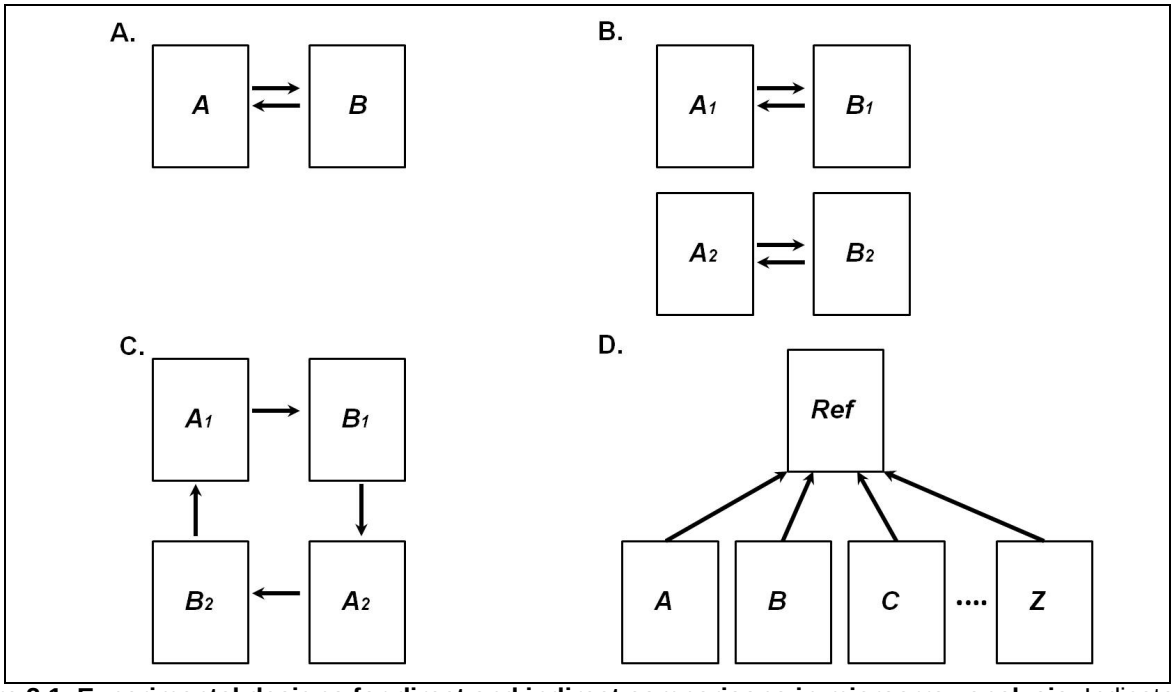

Figure 2.1. Experimental designs for direct and indirect comparisons in microarray analysis. Indicated are boxes representing mRNA samples labeled alphabetically with subscript numbers denoting independent biological replicates. An arrow indicates a single hybridization event, where the sample at its tail is labeled red (Cy5) and green (Cy3) at its head. Represented are types of direct comparisons from a dye swop comparison (A) with replication (B) to a simple loop design (C). Indirect comparison using a reference RNA sample is indicated in D. Adapted from Churchill (2002)

Following hybridization, two independent fluorescent images of co-hybridized test sample and reference (labeled with Cy5 and Cy3) are obtained at high resolution via scanning of the array slide. The relative fluorescence intensities (i.e. Cy5/Cy3) of spots are calculated and

subsequent data for each gene is reported as a $\log_2$ expression ratio known as the Minus or M-value (Eq. 2.1.).

$$\text{Gene A } \log_2\text{-ratio} = \log_2\left(\frac{\text{test sample value eg.Cy5}}{\text{reference value eg.Cy3}}\right) \qquad \textbf{Equation 2.1.}$$

In order to remove any experimental variation that is not a result of important biological variations, data normalization is used to obtain unbiased microarray variation between test samples analyzed (Quackenbush, 2001; Kimmel and Oliver, 2006b). Various statistical methods for normalization of two-color microarray data is currently available can be applied using software packages, such as the freely available R statistical environment (http://www.r-project.org).

The normalization of data can proceed in two ways, consisting of normalization of the M-value within arrays and normalization of the intensities ($\log_2$ average or A-values) between arrays to make values comparable within the dataset (Kimmel and Oliver, 2006b). Software packages like the linear model for microarray data (LIMMA) available in R statistical environment are routinely used for microarray data analysis to determine differential gene expression (Smyth *et al.*, 2008). Within-array normalization involves the calculation of a best-fit slope using linear regression analysis techniques, such as locally weighted scatterplot smoothing (LOWESS). In the case of global LOWESS, normalization of data assumes that the spotted oligos are non-differentially expressed across the array, but not that the number of genes up- and down-regulated are equal (Smyth *et al.*, 2003; Smyth *et al.*, 2008). Print-tip LOWESS is used however, when there are significant differences in the distribution of $\log_2$-ratios of spots among the print-tips (Leung and Cavalieri, 2003). As a compromise between the aforementioned, an empirical Bayes method like Robust spline normalization can be used that constructs a 5-parameter regression spline in place of curves as is the case with LOWESS regression (Smyth *et al.*, 2003; Smyth *et al.*, 2008). Between-array normalization of A-values utilize methods such as quantile scaling to correct for the individual red (R-quantile) and green (G-quantile) channels, in order to standardize the distribution of $\log_2$-ratios across all arrays (Smyth *et al.*, 2003; Smyth *et al.*, 2008). In reference design experiments, R-quantile (Cy5-labeled sample) and G-quantile (Cy3-labeled sample) normalization is extremely useful to normalize common reference values across all arrays. Following

normalization of the data set, replicate data can be consolidated and the differential transcript abundance between samples is calculated.

As microarray analysis has become a widely used tool in transcriptome analysis, standards for presenting and exchanging data has to be implemented to simplify interpretation and facilitate independent verification of data. In 2001, a proposal for the minimum information about a microarray experiment (MIAME) was published by Brazma and colleagues that outlines the necessary levels of detail required for reporting microarray experimental data, from raw image data to the quantitative output and derived measurements (Brazma *et al.*, 2001). To facilitate public access, various databases have been created where microarray data sets can be deposited. The National Centre for Biotechnology Information Gene Expression Omnibus (NCBI GEO, www.ncbi.nlm.nih.gov/geo) represents one such repository with a standard interface to review microarray data and provides an accession number for easy referencing and access to all relevant data. Furthermore, integration of data from various platforms available on repositories such as GEO is becoming a possibility as more bioinformatic tools are developed that can extract and process relevant data (Pan *et al*., 2006; Xia *et al*., 2009).

2.2.3. Transcriptome studies in hematophagous ectoparasites.

Application of microarray technology in ectoparasite research has for the most part focused mainly on pathogen biology (transmission and responses), rather than the general biology of the vector. One example is the transcriptional profiling of *Plasmodium* spp. and their vectors *Anopheles gambiae* and *Anopheles stephensi* (Dana *et al*., 2005; Xu *et al*., 2005). However, examples of transcriptional regulation of genes involved in feeding, as well as insecticide resistance have been well described for *A. gambiae* and other vector species (Sanders *et al.*, 2003; David, 2005; Marinotti *et al.*, 2005; Warr *et al*., 2007). In similar microarray studies of adult and larval stages of the obligate hematophagous horn fly, *Haematobia irritans*, candidate genes could be identified that can be further developed for control strategies of this important cattle parasite (Guerrero *et al.*, 2009).

In the case of ticks, microarrays have been done on the cattle tick, *R. microplus*, in the absence of a fully annotated genome (Moolhuijzen *et al.*, 2011; Bellgard *et al.*, 2012). In this regard, the *R. microplus* gene index (BmiGI) has been fully exploited in several high-

throughput DNA microarray analysis (using the NimbleGen microarray platform) investigating responses to acaricide-induced gene expression and *Babesia bovis* infection in larvae (Saldivar *et al.*, 2008; Heekin *et al.*, 2012), organ-specific responses to *Anaplasma marginale* infection in male adults (Mercado-Curiel *et al.*, 2011), the transcriptional effects of RNA interference-mediated gene silencing in adult females (Lew-Tabor *et al.*, 2011), as well as responses in feeding larvae and adult females on different cattle species (Rodriguez-Valle *et al.*, 2010). However, the full impact of these studies on tick biology and the identification of molecules/systems to target for vaccine development are hampered by the number of genes that are simply unannotatable. Further information on tick protein sequences is needed to elucidate of the full complement of proteins expressed throughout the life cycle of *R, microplus* and other species, using proteomic and other functional genomics techniques.

For adult tick tissues, a number of papers have been published describing the various sialomes of both hard and soft tick species (reviewed by Mans *et al.*, 2008). However, little is known about transcriptional gene regulation in the various tissues of feeding adult ticks, especially those involved in blood meal acquisition (salivary glands), digestion (midgut) and reproduction (ovaries). A study using ESTs showed that a distinct set of genes are uniquely expressed in both salivary glands (188 unique sequences) and ovaries (588 unique sequences) of female *R. microplus* during feeding. In contrast, a different expression pattern was observed in adult male salivary glands (De Miranda Santos *et al.*, 2004). More recently, DNA microarray analysis of the transcriptomes of adult male salivary glands and midgut showed a significant response in gene expression during feeding of *R. microplus* ticks (Mercado-Curiel *et al.*, 2011).

In summary, DNA microarray analysis is a valid high-throughput approach that can be used to elucidate the underlying molecular processes and biochemical pathways that are involved during tick feeding. High-throughput analysis of gene expression will therefore allow rapid expansion of our current understanding of basic tick biology, as well as facilitate the identification of pathways shared among tissues and life stages, offering novel candidates for tick control.

2.3. Materials and methods

2.3.1. Tick rearing and sample collection

Freshly hatched *Rhipicephalus microplus* larvae (Mozambique strain, provided by ClinVet Pty. Ltd, South Africa) were allowed to feed on Holstein-Friesian cattle under controlled conditions at the University of Pretoria Biomedical Research Centre (UPBRC), South Africa. Ethical and relevant Section 20 clearances were obtained from the South African Department of Agriculture, Forestry and Fisheries, as well as the University of Pretoria Animal Use and Care Committee (ethical clearance number: EC022-10). Larvae were allowed to advance through their life stages until mature females dropped off the host animal after 24 days. Ticks were sampled on days 0 (unfed larvae), 4 (feeding larvae), 5 ("replete" larvae), 7 (feeding nymphs), 13 (attached adults), 15 (feeding adults) and 20 (close to replete adult females). Collected ticks were assessed under light microscope and whole ticks, as well as dissected adult female salivary glands, midgut and ovary tissues, were placed in cryovials containing TRI REAGENT® (Molecular Research Center, Inc., USA) and snap-frozen in liquid nitrogen for subsequent storage at -70^{o}C. Adult tissues were collected and processed according to the method of Nijhof *et al.* (2010).

2.3.2. Microarray probe design from *R. microplus* sequences

Using available EST data from GenBank (http://www.ncbi.nlm.nih.gov/nucest/) and the gene index of *R. microplus* (BmiGI release 2.1) from the Harvard gene index project (http://compbio.dfci.harvard.edu/tgi/tgipage.html), a sequence data set was assembled from some 60,000 ESTs and 13,643 unique sequences to a final sequence database consisting of 13,456 contiguous sequences, using the online bioinformatic tools cd-hit-est (http://www.bioinformatics.org/cd-hit/) and CAP3 (http://pbil.univ-lyon1.fr/cap3.php) using default settings. Detection and removal of vector sequences from EST data were performed with the VecScreen tool (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen_docs.html), using the UniVec database available on the NCBI website (http://www.ncbi.nlm.nih.gov/VecScreen/UniVec.html). The final sequence dataset was then submitted online for array design using the Agilent 8x15k microarray and eArray microarray design platforms (https://earray.chem.agilent.com/earray/). Using the standard base composition methodology, a set of 60 mer probes was designed for complete representation

of all assembled transcripts, incorporating a 3'-bias for possible incomplete cDNA synthesis. Probe quality was assessed from base composition scores and unique probes were randomly distributed across the array, including additional quality control probes and a set of housekeeping controls. The latter comprised of elongation factor 1-alpha (GenBank accession no. EW679365), H3 Histone family 3A (GenBank accession no. CV442167), Ribosomal protein L4 (GenBank accession no. CV447629), TATA box binding protein (GenBank accession no. CV453818) and subolesin (GenBank accession no. EU301808) (Nijhof *et al*., 2009). The custom array was synthesized by Agilent technologies (USA).

2.3.3. Isolation of total RNA from tick tissues

Total RNA was isolated according to the manufacturer's guidelines for TRI REAGENT® and purified with the RNeasy mini kit (QIAGEN, USA) (Chomczynski, 1993). Briefly, sample material was removed from storage at -70°C and allowed to thaw. Homogenization of sample material was achieved by mechanical shearing using a whirring blender and then followed by a series of syringes equipped with 19, 21 and 23 gauge needles. RNAse activity is inhibited to certain extent by TRI REAGENT® as it contains phenol/guanidine that denatures proteins (Chomczynski, 1993). Chloroform was added to each vial (100 µl per 500 µl TRI REAGENT®), vortexed for 15 seconds and incubated at room temperature for 15 minutes. Chloroform addition cause homogenates to separate into an aqueous upper phase and an organic lower phase containing proteins, with a genomic DNA containing interphase. Reaction mixtures were centrifuged at 12,000xg for 15 minutes (4°C) and the aqueous phases were transferred to new eppendorf tubes containing 500 µl iso-propanol (100%) to precipitate total RNA. The eppendorf tubes were gently inverted several times and incubated at room temperature for 15 minutes. Following centrifugation at 12,000xg for 15 minutes (4°C), the supernatants were removed and pellets washed with a 1,000 µl of 70% (v/v) ethanol. These reactions were then centrifuged again at 7,500xg for 5 minutes (4°C) and the ethanol was removed. RNA pellets were air dried for 15 minutes then resuspended in 25 µl of DEPC-treated water. Sample tubes were then incubated at 55°C for 15 minutes, vortexed briefly and centrifuged at 12,000xg for 30 seconds. Sample volumes were adjusted to 100 µl with RNAse free water and combined with 350 µl RLT lysis buffer (proprietary, QIAGEN, USA).

The resuspended RNA was loaded onto an RNeasy spin column following addition of 250 µl absolute ethanol and centrifuged at 8,000xg for 15 seconds. The resultant flow through was

reloaded and centrifuged again to allow maximal binding of RNA material. The bound RNA was washed with 350 µl of Wash buffer RW1 (proprietary, QIAGEN, USA), centrifuged 8,000xg for 15 seconds and subjected to on-column DNase I digestion with 70 µl RDD buffer (proprietary, QIAGEN, USA) containing 10 µl DNAse I stock solution (27 Kunitz units). Columns were incubated at room temperature for 15 minutes then the membranes washed with 350 µl buffer RW1. Following centrifugation at 8,000xg for 15 seconds the flow through was discarded and the columns washed twice with 500 µl RPE buffer and finally centrifuged at 8,000xg for 2 minutes to remove residual ethanol. The RNeasy spin columns were then placed into new 2 ml collection tubes and centrifuged at full speed (~15,700xg) to ensure membranes are completely dried. The spin columns were then transferred to 1.5 ml collection tubes and RNA eluted from the membranes in 50 µl of RNAse free water following centrifugation at 8,000xg for 1 minute. The eluent was reloaded onto the column and the centrifugation step repeated. RNA concentration and quality was assessed using the Nanodrop-1000 system (Thermo Fisher Scientific Inc., USA) and samples stored at -70^{o}C.

The integrity and purity of RNA samples was assessed with the Bioanalyzer 2100 micro-fluidics system (Agilent technologies, USA). RNA was prepared and run as per manufacturer's guidelines on a fully automated chip-based capillary electrophoresis system that uses fluorescence-based sample detection (Denisov *et al*., 2008; Delibato *et al.*, 2009). The RNA integrity is expressed as an RNA Quality Indicator (RQI) value, which ranges from 1.0 (fully degraded) to 10.0 (intact RNA).

2.3.4. cDNA synthesis, labeling and microarray hybridization and scanning

A reference RNA pool consisting of equivalent amounts (µg) of RNA from each life stage was prepared including RNA from each test group, in order to allow the independent analysis of both immature and mature life stages. Test groups for the current study consisted of tissues (salivary gland, midgut and ovary) collected from 15 partially fed females (day 20), from two biological replicates.

For first strand synthesis, 3 µg of high quality total RNA (reference or test sample) was incubated with 250 pmol poly-dT primer (5'-$(T)_{25}$VN-3'; N=ATGC; V=AGC) and 750 pmol random nanomers at 70^{o}C for 10 minutes, followed by 4^{o}C 10 minutes. A reaction mixture containing 6 µl of 5x First strand buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$, pH

8.3), 10 mM DTT, 40 units RNAse inhibitor (Promega, USA) and 1.7 µl of a 10x aminoallyl-dUTP stock (5 mM of each dATP, dGTP, dTTP, dCTP, aminoallyl-dUTP) was added to the reaction, mixed and incubated at 42^{o}C for 2 minutes, before adding 340 units of Superscript^{TM} III reverse transcriptase (Invitrogen^{TM} life technologies, USA). To ensure sufficient full-length cDNA synthesis, the reaction was incubated at 42^{o}C for 17-20 hours over night, before heat-inactivating the enzyme at 70^{o}C for 10 minutes. The cDNA template was incubated with 1 M NaOH in the presence of 0.5 M EDTA (pH 8) for 15 minutes at 65^{o}C to remove contaminating RNA template.

The cDNA was purified using the PCR clean-up kit from Macherey-Nagel (Germany) as per manufacturer's indications. Briefly, the reaction mixture is loaded onto a silica membrane under high chaotropic salt concentrations with about ten volumes of binding buffer NT (2.8 M potassium acetate, pH 5.1) and incubated at room temperature for 4 minutes. The NucleoSpin® Extract spin column was centrifuged at 13,000xg for 1 minute and the flow through discarded. The column was then washed three times with 500 µl NT3 washing buffer (100 mM Tris-H_3PO_4, 15% ethanol, 1.15 M KCl, pH 6.3) with a 1 minute centrifugation at 13,000xg and followed by final drying step for 2 minutes at 13,000xg. The cDNA was finally eluted in 30 µl RNAse-free water (37^{o}C) following a 4 minute incubation step (37^{o}C) via centrifugation at 13,000xg for 90 seconds. The concentration and purity of the cDNA was determined using the Nanodrop-1000 system (Thermo Fisher Scientific Inc., USA).

2.3.5. cDNA labeling, microarray hybridization and scanning

A 1.5 µg amount of cDNA template for each sample was dried *in vacuo* and resuspended in 2.5 µl RNAse-free water, to which 5 µl 0.2 M Na_2CO_3 (pH 9) and 2.5 µl of Cyanine 3-dCTP (reference pool) or Cyanine 5-dCTP (test sample) was added for dye coupling via incorporated aminoallyl dUTPs. A common reference design was followed for microarray analysis incorporating both biological and technical repeats (Fig. 2.2.)

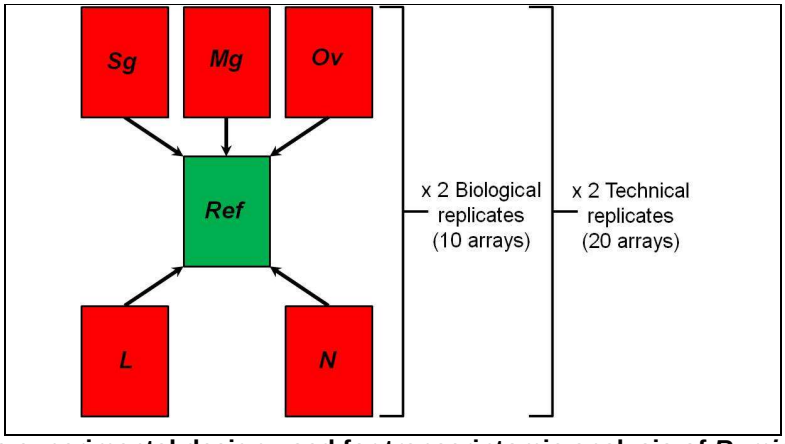

Figure 2.2. Reference experimental design used for transcriptomic analysis of *R. microplus* immature life stages and adult female tissues. Indicated are the test samples collected from larvae (L), nymph (N), salivary glands (Sg), midgut (Mg) and ovary (Ov) that were labeled with Cy5 (Red), as well as the representative reference RNA (Ref) that was labeled with Cy3 (Green). A total of 20 hybridizations were performed, consisting of two biological repeats and two technical repeats.

The sensitive labeling reaction was performed in a desiccator in the absence of light for 2 hours at room temperature and excess dye was removed using the QIAquick PCR purification kit (QIAGEN, USA). Briefly, ten volumes of PBI loading buffer was added to each labeling reaction and mixed. Following an incubation step of 4 minutes samples were loaded onto the QIAquick spin column and centrifuged at 13,000xg for 1 minute. The resultant pellet was washed three times with 500 µl of PE washing buffer and centrifugation at 13,000xg for 1 minute. The membrane was then dried with centrifugation at 13,000xg for 2 minutes and the column transferred to a new 1.5 ml microfuge tube. Labeled material was eluted from the membrane with 30 µl of pre-warmed RNAse-free water (37^{o}C) with centrifugation at 13,000xg for 90 seconds, following a 4 minute incubation step. The dye concentration and incorporation was determined using the Nanodrop-1000 system and the coupling efficiency calculated (Eqn. 2.2.). Assuming that the average cDNA length is 1,000 bases, the number of moles of dye per 1,000 bases of cDNA was calculated and a minimum of 10/1,000 labeled nucleotides was considered acceptable.

$$\text{Efficiency} = \frac{\text{pmol dye x 324.5 pg/mol dNTP}}{\text{ng DNA}} \qquad \textbf{Equation 2.2.}$$

Since template was labeled with Cy3 (reference pool) or Cy5 (test sample), equivalent picomoles of Cy3-labeled cDNA from the common reference pool were mixed with Cy5-labeled individual test cDNA (20 pmol each, 40 pmol total) in a 0.2 µl tube along with 5 µl of

10X blocking buffer (Proprietary, Agilent technologies, USA), 1 µl of 25X fragmentation buffer (Proprietary, Agilent technologies, USA) and RNase-free water in a final reaction volume of 25 µl. Following incubation at 60^{o}C for 30 minutes to fragment any remaining RNA, 25 µl of 2x GE hybridization buffer (Proprietary, Agilent technologies, USA) was added to the tube and mixed via careful pipetting (as not to introduce bubbles) and put on ice. Each 40 pmol hybridization sample was loaded onto a gasket slide placed in an assembly chamber base. The printed array slide is finally mounted on top of the gasket slide along with a chamber cover and the assembled hybridization chamber secured tightly with a clamp assembly. Hybridization of pooled reference and test samples to the custom microarray was performed in a hybridization oven at 65^{o}C for 17 hours with a rotation speed of 10. Prior to scanning, each slide was washed in wash buffer 1 (Proprietary, Agilent technologies, USA), rinsed two times for 1 minute in pre-heated (37^{o}C) wash buffer 2 (Proprietary, Agilent technologies, USA). Following a drying step by centrifugation for 1 minute, slides were scanned with the GenePixTM 4000B microarray laser scanner (Molecular Devices Inc., USA).

2.3.6. Microarray data analysis and functional annotation

Fluorescence intensities of Cy3 and Cy5 of microarray spots were extracted using default parameters in the GenepixPro feature extraction software (v6.0, Axon Molecular Devices, USA). Following manual inspection to evaluate spot quality, flagged features were given a zero weight and not considered for further analysis. Normalization within slides was performed using the LIMMA package in R (hhtp://cran.r-project.org/), employing the locally weighted scatterplot smoothing (LOWESS) technique, followed by Gquantile normalization between slides. From the normalized data, the log average (A) and $\log_2$-ratio (M) values were determined, where the A value indicates the brightness of a feature and the M value is an indication of the differences in expression between two groups. A and M values were calculated as follows: A = (logCy5 + logCy3)/2 and M = logCy5- logCy3. Therefore, the intensity value of each spot in each Cy5-labeled test group for ovaries, midgut and salivary glands were calculated. A standard pair-wise Pearson correlation (r) was performed in Microsoft Excel, using the calculated M-values from normalized data to determine the overall concordance between test samples (biological and technical replicates).

The LIMMA package was subsequently used to calculate the $\log_2$ fold change between each group pair-wise comparison, using an empirical Bayes ANOVA to identify significantly

differentially expressed transcripts with a *P* value adjusted for multiple comparison false discovery rates. Comparisons with *P* values ≥ 0.001 were ignored. In order to identify transcripts that are organ-specific, only genes with M values > 0, a Cy5 intensity > 1,000 and a $\log_2$ fold expression of one or greater in a single organ relative to all other tissues examined, were considered. All transcripts with a $\log_2$ fold expression higher than 2 were considered as highly up-regulated under the current chosen conditions. Transcripts with M values > 0 and Cy5 expression intensity > 1,000, in all of the tissues tested, were considered shared (non-differentially expressed) under the current chosen conditions. In this regard, all transcripts with expression intensities higher than 2,000 were considered strongly shared.

To functionally annotate transcripts, the desktop cDNA Annotation System (dCAS) software (v.1.4.3) was used (Guo *et al.*, 2009). This system provides a user friendly GUI interface and allows BLAST searches to be performed on large data sets where each transcript is analyzed against the following databases: gene ontology protein sequence database (GO), eukaryotic orthologous groups database (KOG), mitochondrial and plastid database (Mit-Pla), non-redundant (NR), protein family sequence database (Pfam), rRNA subset (RRNA) and simple modular architecture research tool domain family database (SMART) (http://exon.niaid.nih.gov). Though manual inspection and assignment of transcript identity is still a prerequisite, this platform has been used extensively in the annotation of large sets of sialomic data (Mans *et al.*, 2008). All BLAST searches were performed using default settings and results were stored in a tab-delimited file by dCAS system. All transcriptionally relevant sequences were manually inspected and final annotation was based on a consensus between two or more databases, using an expected value (E value) cut-off of less than 1×10^{-6}. All transcript descriptions were based on consensus with reviewed database entries from Uniprot (http://www.uniprot.org/uniprot/) and BRENDA (http://www.brenda-enzymes.org/), in the case of enzymes. Transcripts were finally classified into functional groups based on the eukaryotic orthologous group terms for gene ontology (Tatusov *et al.*, 2003). Additional alignments were performed with the Clustal W program (http//:www.ebi.ac.uk/clustalw/) and amino acid sequences/ alignments were edited with the Genedoc multiple sequence alignment editor and shading utility (http//:www.psc.edu/biomed/genedoc/, ver. 2.6.002). Subsequent phylogenetic analysis was performed with the Molecular Evolution Genetics Analysis program (MEGA, ver. 3.0) using neighbour-joining analysis (1,000 bootstraps, p-distance model) (Kumar, Tamura and Nei, 2004).

2.3.7. Microarray results validation using qRT-PCR

For the verification of gene expression results obtained for female tissues, transcripts encoded by Contig1, Contig5396, Contig5672 and Contig8723 were selected and primer sets were designed and used in relative qRT-PCR analysis (Supplementary Table S2.1, Ms. S. Richards, Unpublished data). Briefly, using the same RNA isolated for microarray hybridizations, cDNA was synthesized using the iScript™ cDNA synthesis kit (BioRad, Hercules, USA). The resultant cDNA template was utilized for relative qRT-PCR using the KAPA SYBR®-FAST qPCR kit (KAPA Biosystems, USA) in a 10 µl final reaction volume containing 2.5 pmoles of oligonucleotide primer pairs corresponding to selected sequences that were differentially expressed. The mRNA levels were normalized against Contig8723, as well as elongation factor 1 alpha that was previously validated as a reference gene with stable expression (Nijhof *et al.*, 2009). Relative semi-quantitative PCR was performed in triplicate on a LC480® light cycler (Roche Applied Science, USA) using cycling parameters consisting of an activation step at 95°C for 5 minutes, followed by 45 cycles of 95°C for 3 seconds, 55°C for 7 seconds and 72°C for 1 second. A melt analysis was performed consisting of 95°C hold for 30 seconds, followed by incubation at 55°C for 30 seconds and a slow increase to 95°C for melting curve analysis. All assays were first optimized on a control cDNA pool that was also used for between- and in-plate calibration reactions, using elongation factor 1 alpha primers. The relative transcript levels for selected genes were evaluated using the extracted Cq-values and expressed as a fold change (on a $\log_2$ scale) relative to the reference genes selected using the qBase software (Hellemans *et al.*, 2007). These normalized values were used to calculate the fold change for selected transcripts between microarray contrasts. Linear regression analysis was performed to correlate fold changes calculated by qRT-PCR analysis with fold changes determined from microarray data.

2.4. Results and Discussion

2.4.1. Isolation of total RNA from tick tissues

Due to the random nature of infestation, there is no standard method to synchronize the life cycle of ticks during infestation and therefore not all individuals that are placed on a host develop at the same rate (attachment, feeding and molting). The time points sampled were based on attachment and feeding status and assessed under light microscope to ensure that the correct life stage was collected. The groups selected comprised a mixture of individuals in various stages of attachment and feeding and therefore are a reasonable representation of the life stage tested. The latter is also why a reference design (with an expanded reference pool) was followed. The latter standardizes the inter-individual variation within samples (and allows comparison between different sub-sets of samples), as all test samples are compared to the same reference pool (Simon and Dobbin, 2003). Finally, the test groups for microarray analysis consisted of feeding larvae (day 4), nymphs (day 7) and adult female salivary gland-, ovary- and midgut (day 20) tissues collected post infestation. While additional unfed larvae and feeding adults (day 13 and day 15) were collected for addition to the RNA reference pool to be more representative of the life cycle (Chapter 3). The results for isolation and hybridization of larvae and nymph test groups will be discussed along with the adult female tissues in the following sections. Expression analysis results for the immature stages will be discussed in Chapter 3.

RNA quality was measured and expressed as a RNA integrity number (RIN) or RNA quality indicator (RQI) that can be used to assess each sample. This value is based on mapping the electropherogram profile of a test sample and expressing the RNA quality as a value between 1 (fully degraded) and 10 (intact) (Denisov *et al.*, 2008). RNA with RQI values between 7 and 10 is considered as good quality. All samples tested had RQI values of around 8 or better, indicating that the RNAs were of high quality (Fig. 2.3.).

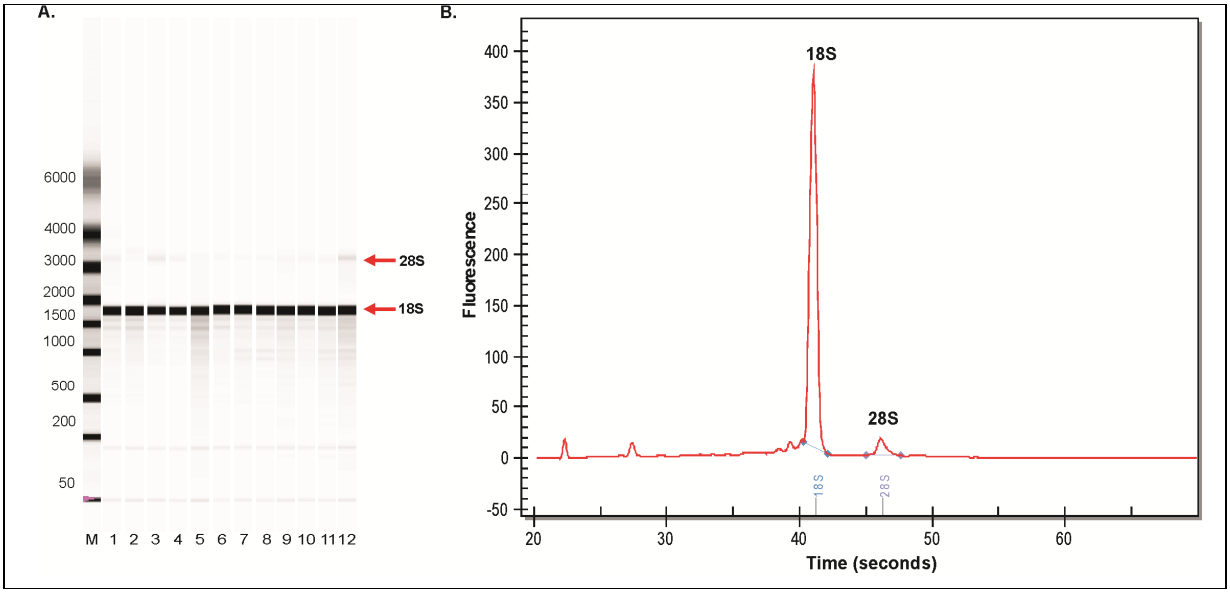

Figure 2.3. Purity and quality assessment of *R. microplus* RNA to be used for microarray analysis. [A] Indicated is a virtual gel image of representative RNA samples isolated from unfed larvae (1), larvae collected on day 4 (2), nymphs collected on day 7 (3-4), adults collected on day 13 (5), adults collected on day 15 (6), salivary glands (7-8), ovaries (9-10) and midguts (11-12). [B] A representative electropherogram indicating the position of 18S and 28S ribosomal subunits (also indicated by red arrows).

Though the electropherograms clearly indicate distinguishable bands representing intact 18S rRNA, little to no bands are visible for the 28S rRNA and 5.8S rRNA (Fig. 2.3.A.). It has been shown previously for insects that the 28S rRNA consists of two separate smaller strands of RNA associated to each other via hydrogen bonding (Winnebeck *et al.,* 2010). This association is extremely sensitive to heat denaturation, causing the 28S rRNA to dissociate and resolve with the 18S rRNA. Similar resolution of electropherograms for heat treated *Apis meliffera* RNA was achieved with no marked decline in RNA quality (Fig. 2.4.).

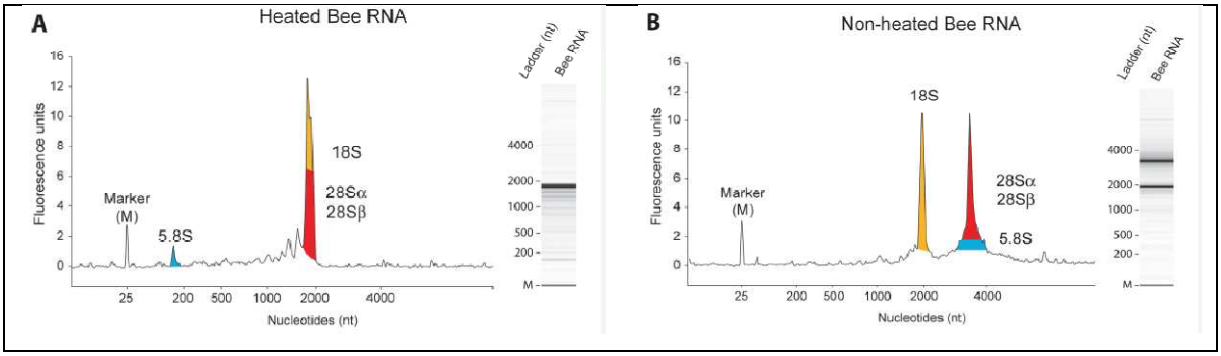

Figure 2.4. Electropherograms and virtual gel images of RNA isolated from the honey bee, *Apis meliffera*. RNA profiles following heat denaturation (A) and without heat treatment (B). rRNA species are indicated 5.8S (blue), 18S (yellow) and 28S components (red). Reproduced from Winnebeck *et al.* (2010).

Since ticks are part of the arthropod order they may share a similar rRNA structure to insects (Gillespie *et al*., 2006). It is therefore hypothesized that similar RNA denaturation occurred for the RNA material isolated in this study, as it was incubated at 55℃ to dissolve the RNA pellet (Section 2.2.3.). Furthermore, as no smearing or peak shifts in the electropherograms are visible, indicating degradation of rRNA, samples were considered acceptable for use in microarray analysis. High RNA yields were obtained ranging from ~25 µg for larvae to ~120 µg from midgut tissues.

2.4.2. Preparation of microarray platform for transcriptome analysis of *R. microplus*

From the *R. microplus* gene index (BmGI) and Genbank nucleotide sequence database, some 60,000 ESTs and 13,643 singleton *R. microplus* sequences were assembled using the CAP3 clustering program (Huang and Madan, 1999). CAP3 clustering program has been used extensively in genome and EST data assembly as it incorporates automated 5' and 3' clipping of low quality sequence data, overlap constraints that allows more stringent assembly of low quality sequence data (Huang and Madan, 1999; Scheibye-Alsing *et al.*, 2009). This program is also currently being used to assemble genomic data for the construction of the *R. microplus*, genome (Bellgard *et al.*, 2012).

Using a sequence identity threshold of 100% and default settings, 13,456 contiguous sequences were assembled then stripped of any contaminating vector sequences via similarity searches to the univec (vector) database (Vecscreen). The final assembled sequence database was used for microarray chip design. These assembled sequences had average sequence lengths ranging from 100 to 4,000 nucleotides, with the largest subset of sequences ranging from 600 to 1,500 base pairs (Fig. 2.5.). The latter equates to putative proteins ranging from 22 kDa to 55 kDa in size, if an average molecular weight of 110 Da is accepted per amino acid.

Of the 20,929 assembled tentative consensus (TC) sequences available for *I. scapularis* on the Harvard gene index site (http://compbio.dfci.harvard.edu/), 9,327 (~44.6%) are smaller than a 1,000 base pairs and 10,909 (~51%) occur between 1 kb and 2 kb pairs. In contrast, the 13,456 transcript database assembled for *R. microplus* in this study shows that 9,073 (67.4%) transcripts are less than 1,000 base pairs and 4,071 (30.3%) transcripts occur between 1 kb and 2 kb pairs (Table 2.1., Fig.2.5.). It is interesting to note that comparable

results were obtained for the BmGI tentative consensus sequence database (9,551 transcripts) that was used for the assembly of the database in this study (Table 2.1.). This database consisted of 5,651 (~59%) sequences smaller than 1,000 base pairs and 3,981 (~41%) sequences occurring between 1 and 2 kb pairs.

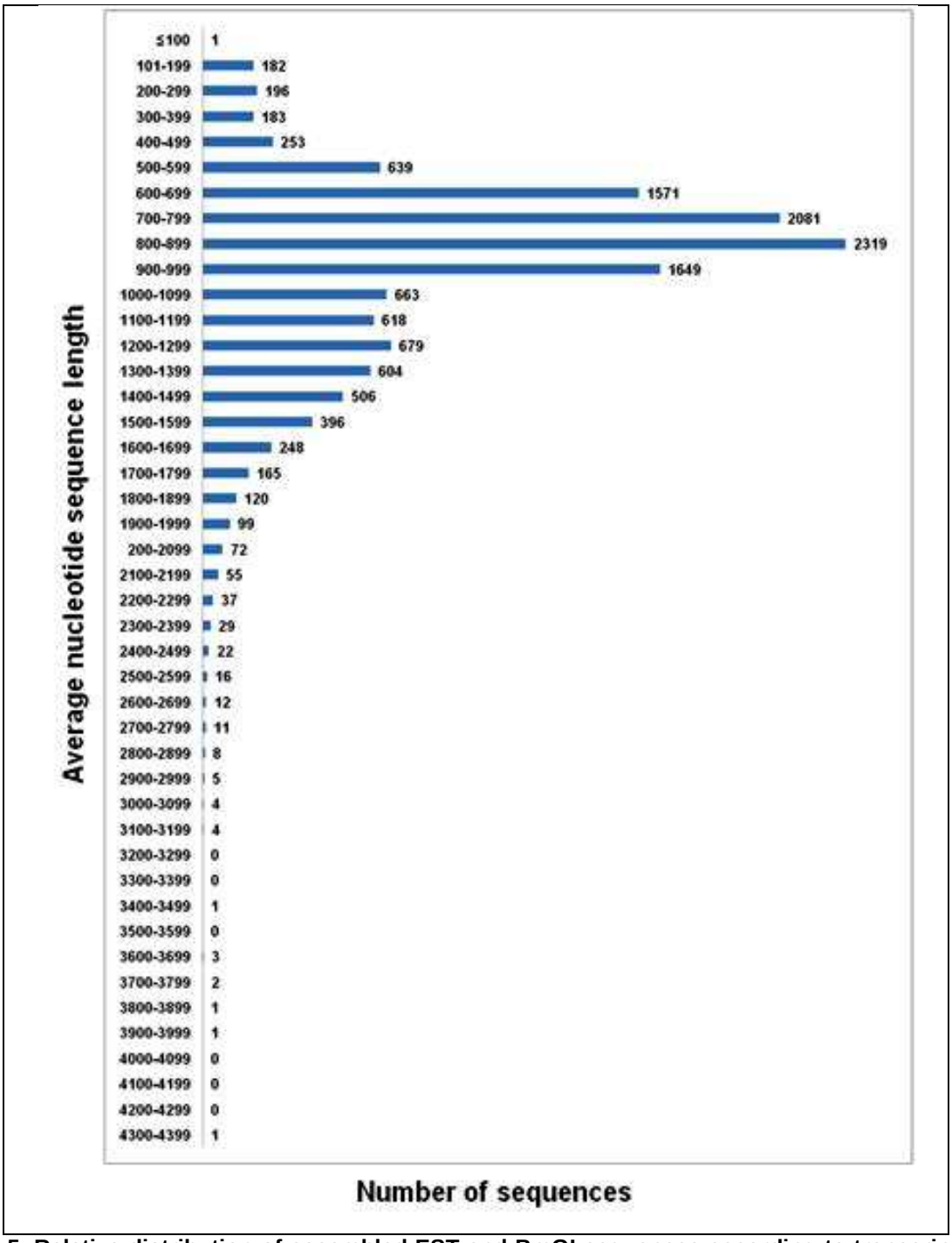

Figure 2.5. Relative distribution of assembled EST and BmGI sequences according to transcript length. Indicated are the numbers of assembled sequences (left) that fall into a particular sequence length (in base pairs) category.

Table 2.1. Comparison of tentative consensus (TC) sequences available on the Harvard gene index (http://compbio.dfci.harvard.edu/). Indicated are the number of unique sequences and their size ranges assembled for *I. scapularis* and *R. microplus* from EST and genomic sequence data, as well as the database assembled.

	I. scapularis	***R. microplus* (BmGI)**	***R. microplus* (Assembled)**
< 1 kb	9,327	5,651	9,073
1 kb < <2 kb	10,909	3,981	4,071
2 kb < <3 kb	661	206	267
3 kb < <4 kb	32	11	16
4 kb<	3	2	1
Total number of TCs	20,929	9,551	13,456

The assembled sequences were then submitted for probe design on the eArray server for the *in silico* design of the microarray slides. Using standard settings, 13,464 probes (60 mer each) were designed that were randomly distributed across the array slide design. The probe quality was assessed by blast analysis of the probes against the constructed database. A base composition score (BC) is calculated and a value of 1 to 4 is given, with 1 being the best. Only 22 probes scored lower (BC = 4), while 18 had better scores (BC = 3) and 13,416 were of the best quality (BC = 1 or 2). A control grid consisting of a set of control probes is automatically added to the array design to assist in image orientation for data extraction, also enabling a measure of labeling efficiency and background (Giles *et al.*, 2010). These probe sets consist of endogenous sequences that produce a high signal following hybridization (bright corners) and hairpin structures unable to hybridize cDNA efficiently (dark corners).

The predicted probes (including controls) were used in an 8 x 15k slide configuration, chosen to maximize the experimental design. It was determined that 3 µg starting material for each test sample was sufficient to perform 20 hybridizations (biological and technical repeats) (Fig. 2.2.). An example of a typical scanned microarray slide is presented to illustrate some technical aspects of the hybridization experiment (Fig.2.6.).

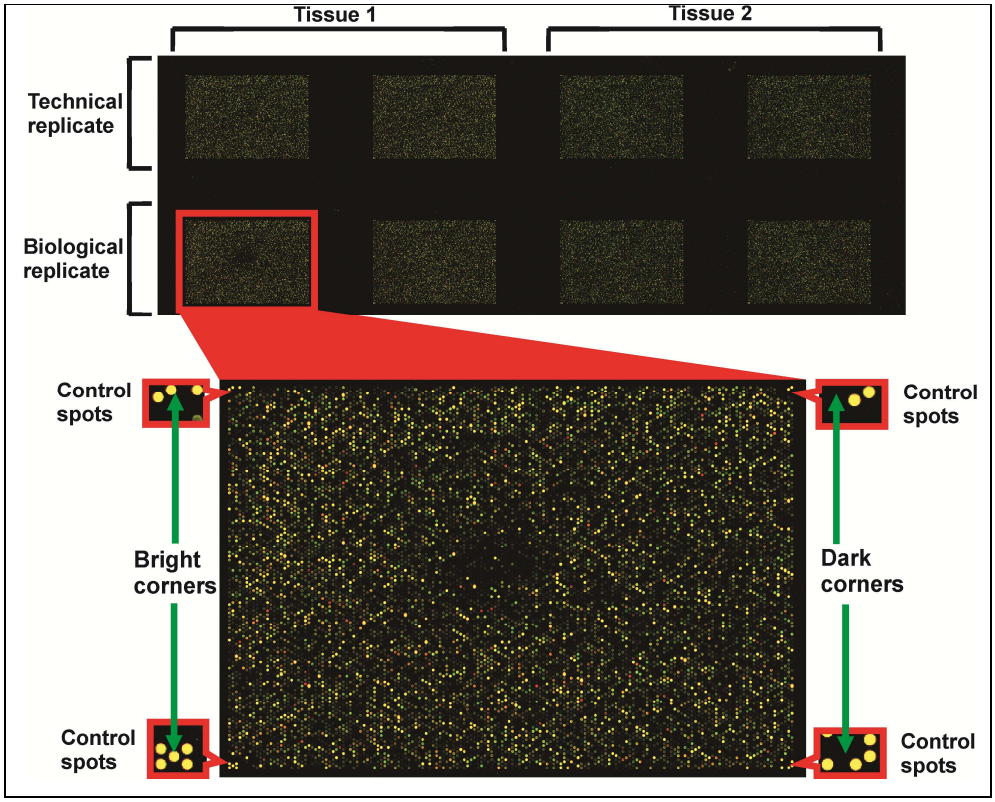

Figure 2.6. Agilent microarray slide used for expression profiling. Presented is a scanned image of a typical microarray slide, consisting of 8 arrays (15,000 spots), following hybridization with Cy3- (reference pool) and Cy5- (test sample) labeled cDNA. The experimental design consisting of biological and technical replicates for each test sample (i.e. Tissue 1 and Tissue 2) is indicated. A single array is highlighted to illustrate control spots consisting of dark and bright corners that are used during data extraction.

2.4.3. Normalization of microarray data

To compensate for experimental variation, normalization of data was approached in two ways, consisting of background correction of the M-values within each array to compensate for any possible dye biases and then normalization of the intensities (A-values) between arrays to make values comparable across datasets (Kimmel and Oliver, 2006b; Chiogna *et al.*, 2009). For within-array normalization, global LOWESS linear regression analysis was applied, assuming that the spotted oligos are non-differentially expressed across the array, but not that the number of genes up- and down-regulated are equal (Smyth *et al.*, 2003; Smyth *et al.*, 2008). Results indicated that interquantile variation was improved for all $\log_2$-ratios with a better mean distribution around 0 (Fig. 2.7.B.). Individual sample sets generally clustered well together with similar mean $\log_2$-ratios. However, insufficient normalization was observed for $\log_2$-ratios (M-values) for certain replicates as outliers were observed for arrays hybridized with salivary glands and ovaries after normalization (Fig. 2.7.B.). Intensity values

were however successfully transformed and evenly distributed with improvement of interquantile variation (Fig. 2.7.D.).

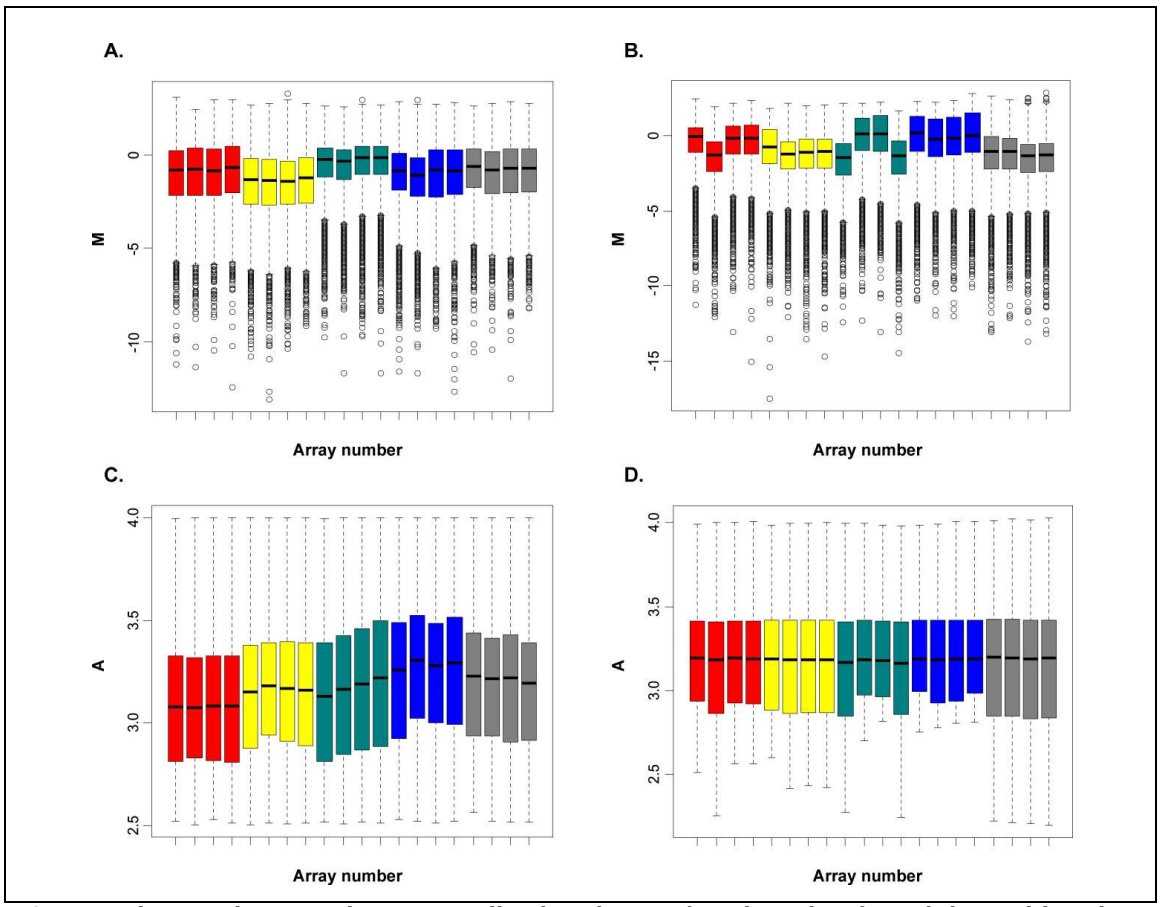

Figure 2.7. Background correction, normalization log_2-ratios (M-values) and intensities (A-values). Indicated are the boxplots of the log_2-ratios (M-values) before (A) and after (B) normalization, as well as the intensities (A-values) before (C) and after (D) normalization. Colors indicate arrays hybridized with the same class of test sample midgut (red), salivary glands (yellow), ovaries (green), larvae (blue) and nymphs (grey).

To assess microarray quality, MA scatterplots before and after normalization were constructed for each array and evaluated. For MA plots the M-values should ideally be distributed about zero and with a ratio independent of intensity (A-value). However, due to inherent variances between M and A, M will never be zero and therefore, a MA plot will never give a straight line. Following normalization of the data all arrays show marked improvement in distribution about the median axis (Fig. 2.8.A. and B.).

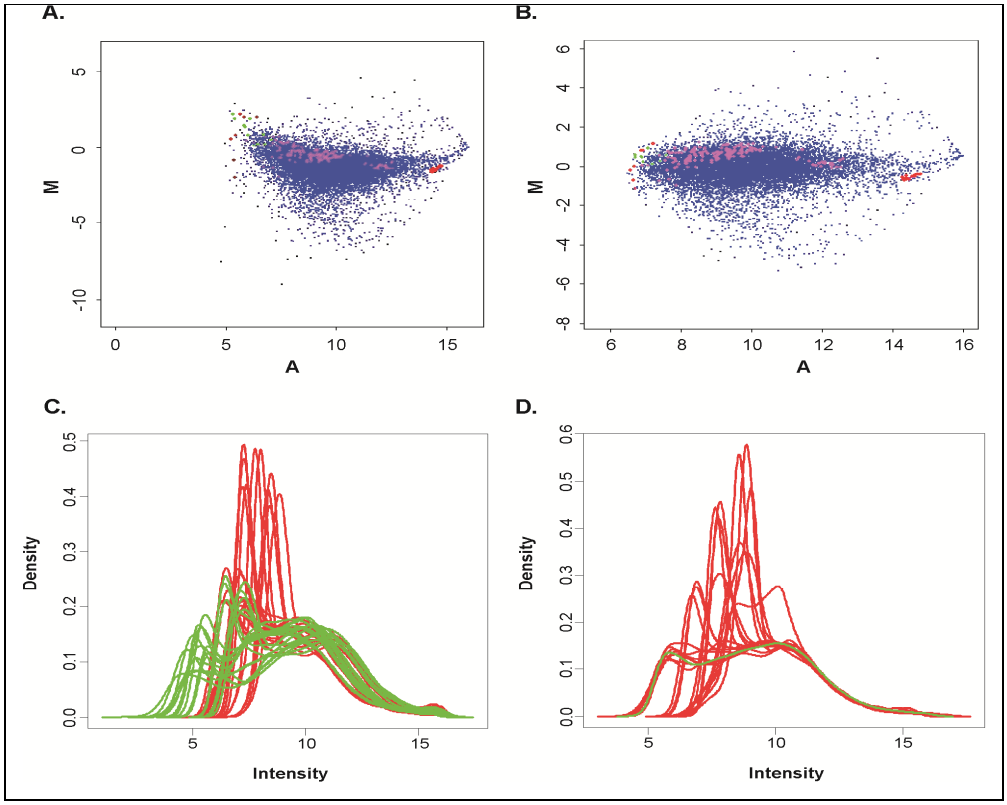

Figure 2.8. MA and RG-density plots obtained during pre- normalization and post-normalization. Indicated are representative MA plots before (A) and after (B) data normalization. Density plots for Cy5 (red) and Cy3 (green) are also indicated before (C) and after (D) data normalization. The reference values (green) are reduced to a single value (D) for all consequent sample comparison analysis.

In a reference design as employed in this study, quantile normalization is extremely useful to normalize a common reference value across all arrays. As seen from the red/green density plots there are great differences in test sample intensities (i.e. dye biases), as each test group resolves differently to each other (Fig. 2.8.A. and C.). Between-array normalization of A-values (intensities) was performed using G-quantile scaling to correct for the green (reference) channel, in order to standardize the distribution of $\log_2$-ratios across all arrays (Smyth *et al.*, 2003; Smyth *et al.*, 2008). All reference samples were consequently normalized to a single representative curve, as all reference samples are essentially the same (Fig. 2.8.C. and D.). The arrays were subsequently normalized according to the green channel reference enabling the comparison of $\log_2$-ratios (i.e. Cy5/Cy3) (Fig. 2.7.D.). Differential transcript abundance between tissue and life stage comparisons was finally calculated using LIMMA.

To determine the concordance between sample data sets, standard Pearson correlation coefficients were calculated. This approach has been used extensively to determine the reproducibility and therefore the reliability of microarray data sets (Jenssen *et al.*, 2002; Wilkes *et al.*, 2007). Positive concordance between biological replicates and their respective technical replicates were obtained above 0.66 (Supplementary Table S2.2.) The average Pearson correlations between biological and technical replicates for each life stage and tissue were calculated for midgut (0.886), salivary glands (0.830), ovaries (0.846), nymphs (0.718) and larvae (0.898) (Supplementary Table S2.2.). These results indicate an overall high level of concordance of the sample data and therefore good data reproducibility.

For all of the following adult tissue comparisons, transcripts with *P* values ≥ 0.001 were not considered to provide the best level of confidence for the sequence data analyzed. Only intensity and $\log_2$ expression values applicable to adult female tissue comparisons were used to determine transcripts that are organ-specific or shared for the adult life stage. The expression profiles related to the immature stages will be discussed in Chapter 3.

2.4.4. Microarray data validation by qRT-PCR

To verify microarray results, relative qRT-PCR was performed on 3 selected transcripts from the tissues tested (Table 2.2).

Table 2.2. Verification of differential gene expression of selected transcripts by qRT-PCR.

Contig[a]	Description [b]	Comparison [c]	Microarray fold change [d]	qRT-PCR fold change [e]	Direction of change
Contig8418	Elongation factor 1 alpha	MG/SG	1	0.94	Unaffected
Contig8723	Putative 60S ribosomal protein L9, Ribosomal protein L6P family	MG/SG	0.93 [f]	1.06 [f]	Unaffected
Contig1	Putative angiotensin-converting enzyme, Peptidase M2 family	MG/SG	0.12 [f]	6.43E-03 [f]	Down
Contig5396	Putative mucin	MG/SG	19.61 [f]	17.66 [f]	Up
Contig5672	Unknown protein	MG/SG	2.81 [f]	7.05 [f]	Up

[a] Assigned contiguous sequence identification for transcripts following assembly of all available expressed sequence tags and the *R. microplus* Gene Index version 2.1.

[b] The functional annotation of genes based on comparison of BLAST outputs from seven databases outlined in materials and methods. All transcript descriptions are based on consensus with database entries from Uniprot (http://www.uniprot.org/uniprot/) and BRENDA (http://www.brenda-enzymes.org/), in the case of enzymes.

[c] Tissue comparison indicated represents the product of calculated fold change for midgut (MG) and salivary gland (SG) comparisons

[d] Fold change (absolute) for microarray calculated from the $\log_2$ expression level for the gene (*P* value ≤ 0.001).

[e] Fold change data identified from qRT-PCR. The data was normalized against Contig8418 and Contig8723 and fold changes are expressed according to the current tissue comparison (i.e. midgut to salivary gland).

[f] These values demonstrated a high correlation at 0.96 with the microarray and qRT-PCR data, calculated by using Pearson linear regression (R squared value of 0.93).

These transcripts were: a putative angiotensin-converting enzyme (Contig1), a putative mucin (Contig5396) and an unknown secreted protein (Contig5672). An additional stably expressed 60S ribosomal protein (Contig8723) was identified (Table 2.2.). For microarray analysis validation by qPCR genes are selected that are not differentially expressed in order to use these genes as “house keeping” genes for normalization (Abruzzo *et al.*, 2005) The latter transcript was consequently used as an additional reference gene for data normalization. When individual fold change values determined from qPCR analysis were expressed relative to each other, a high degree of correlation (0.96) to microarray data was determined using Pearson regression for values pertaining to adult tissues (Table 2.2.). An overall concordance was observed in the direction and magnitude of fold change values obtained. Analysis of ovary was not included in this analysis as it was not considered a priority for vaccine development at this time. However, when considering the $\log_2$ fold transcription of the full

complement of comparisons between the adult tissues (salivary glands and midgut) and immature life stages (larvae and nymphs) tested (Supplementary Table S2.3), the values demonstrated a high correlation at 0.91 between microarray and qRT-PCR data, calculated by using Pearson linear regression (R squared value of 0.83) when only two outliers are removed from the analysis (otherwise 0.64).

It has been stated previously that quantitative gene expression analyses requires a large set of reference genes to obtain a stable fold change (Rodriguez-Valle *et al.*, 2010). As only a few reference genes have been evaluated that could correlate with gene expression data (Nijhof *et al.*, 2009), there is a great need for the identification of new reference genes of different abundance classes (low, medium and high) for use in expression analyses. Several novel transcripts have been identified during this study that may be validated for different life stages and more tissues (manuscript in preparation).

2.4.5. Gene expression in feeding *R. microplus* adult female tissues

Expression analysis identified 3,991 transcripts differentially expressed among all tissues above threshold, representing 28% of the total number of contigs (Table 2.3.). However, it was found that less than 13% (1,707 transcripts) of the full complement of genes tested was significantly regulated in any given tissue or comparison (Table 2.3., Fig.2.9.A.). Previous studies on tissues isolated from male *R. microplus* showed similar trends in expression patterns for salivary gland and midgut tissues in response to feeding (Mercado-Curiel *et al.*, 2011). A set of 588 transcripts was found to be expressed in all three tissues (Supplementary Table 2.7.). Of these, 135 transcripts were identified with more than double the intensity threshold for expression. In addition, several tissue-specific transcripts were identified that were uniquely up-regulated in salivary glands (171 transcripts), midgut (310 transcripts) and ovaries (417 transcripts) (Fig.2.9.A., Table 2.3., Supplementary Tables S2.4. to S2.7.). Therefore, a total of 1,005 transcripts were expressed in ovaries, followed by 898 transcripts in midgut and 758 transcripts in salivary glands when shared transcripts are included (Table 2.4. and Supplementary Table S2.8.).

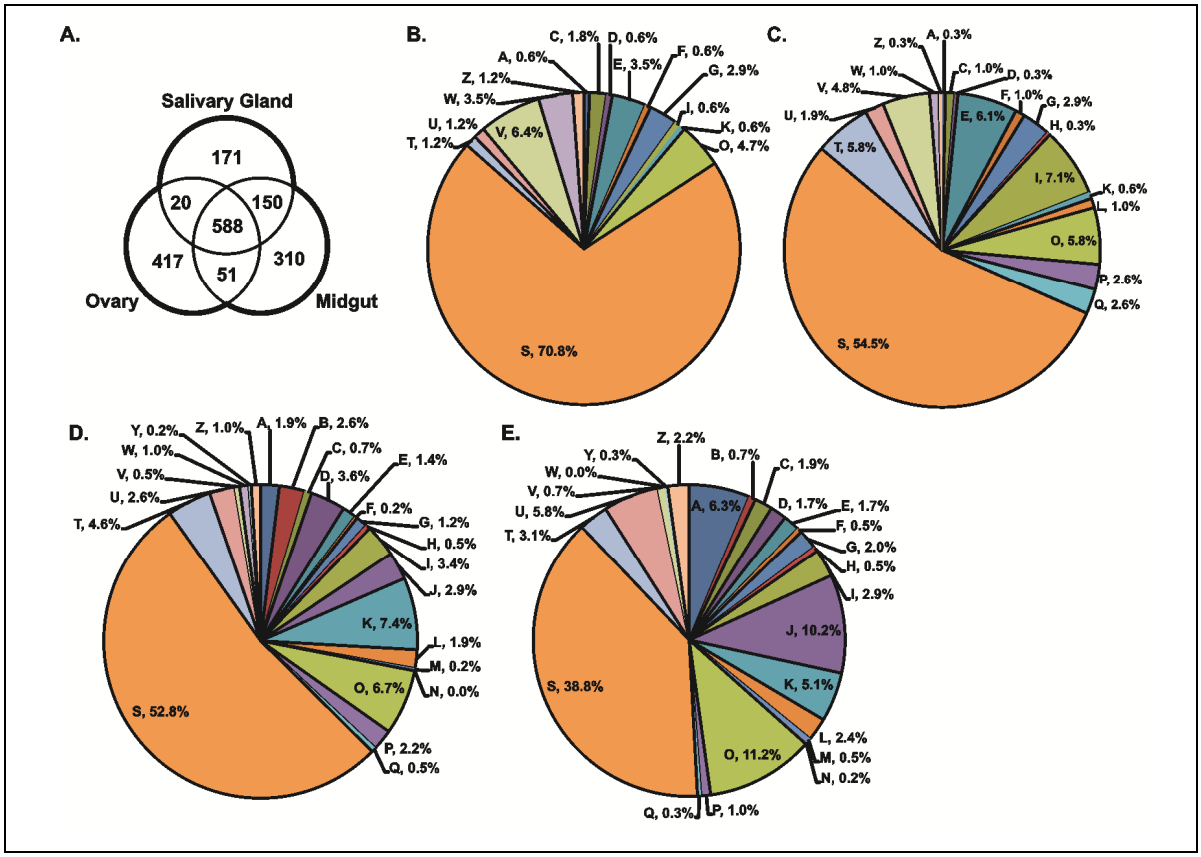

Figure 2.9. Distribution and classification of genes regulated between tissues of feeding female *Rhipicephalus microplus* ticks. Indicated are the number of unique genes shared between midgut, salivary glands and ovaries with M-values > 0, an intensity threshold above 1000 and *P* values ≤ 0.001. (A) Venn diagram indicating the number of genes that are unique to or shared between the different tissues. Regulated transcripts in all tissues are classified according to their eukaryotic orthologous functional groups (KOGs). The percentages of unique transcripts regulated in (B) salivary glands, (C) midgut, (D) ovaries and (E) shared between all tissues of female *Rhipicephalus microplus* ticks during feeding are indicated. The functional classifications (bottom right) that are represented include: A- RNA processing and modification; B- Chromatin structure and dynamics; C- Energy production and conversion; D- Cell cycle control, cell division, chromosome partitioning; E- Amino acid transport and metabolism; F- Nucleotide transport and metabolism; G- Carbohydrate transport and metabolism; H- Coenzyme transport and metabolism; I- Lipid transport and metabolism; J- Translation, ribosomal structure and biogenesis; K- Transcription; L- Replication, recombination and repair; M- Cell wall/membrane/envelope biogenesis; N- Cell motility; O- Posttranslational modification, protein turnover, chaperones; P- Inorganic ion transport and metabolism; Q- Secondary metabolites biosynthesis, transport and catabolism; S- Function unknown (also includes transcripts with only general functional predictions); T- Signal transduction mechanisms; U- Intracellular trafficking, secretion, and vesicular transport; V- Defense mechanisms; W- Extracellular structures; Y- Nuclear structure; Z- Cytoskeleton.

Table 2.3. Summary of unique transcripts expressed in tissues of feeding *R. microplus* female ticks (*P* ≤ 0.001). Number of transcripts determined using an intensity threshold of a 1,000 and an average absolute two-fold change relative to other tissues.

	Regulated	Fold change [b] / Intensity	Salivary gland	Midgut	Ovary	All tissues
Regulation of significant genes [a]	Up	>3	14	130	8	
		>2	62	210	102	
		>1	171	310	417	
	No change	>5,000*	-	-	-	74
		>2,000*				135
		>1,000*				588
Total regulated genes above intensity threshold [c]	3,991		520	552	994	588
Total regulated genes with *P* value ≤ 0.001 [d]	1,707		171	310	417	588*
% Regulated genes [e]	12.7		1.3	2.3	3.1	4.4

[a.] Data corresponds to fold change values using an empirical Bayes method to identify differentially expressed transcripts with a *P* value ≤ 0.001 adjusted for multiple comparisons false discovery rates.

[b.] Fold change corresponding to the $\log_2$ expression ratio of genes with at least a greater than 2 fold absolute expression for any given tissue comparison.

[c.] Total number of genes regulated above an intensity threshold of 1,000 and an M-value (Cy5/Cy3) > 0.

[d.] Total number of genes regulated with a *P* value ≤ 0.001 adjusted for multiple comparisons false discovery rates.

[e.] Percentage of genes regulated from 13,456 total transcripts used on the microarray.

[*.] Number of non-differentially expressed genes for all tissue comparisons based on M-value (Cy5/Cy3) > 0 and a minimum threshold intensity of 1,000.

2.4.6. Tissue-specific gene expression during feeding

The adaptation to a blood feeding lifestyle has endowed ticks with a complex repertoire of components to evade host defense mechanisms and hemostasis, to digest and metabolize the blood meal in order to acquire the metabolites and energy necessary for reproduction. Furthermore, to understand the complexity of the biological processes underpinning this adaptation and to target them for vaccine development, evaluation of the full complement of genes that are uniquely expressed in a particular tissue is necessary. The following section will deal with some of the various classes of transcripts identified that were uniquely associated with the salivary glands, midguts and ovaries.

2.4.6.1. Unique transcripts highly regulated in adult female salivary glands

In order to obtain a blood meal successfully, evasion of host defenses against tick attachment and prolonged feeding time is of primary importance. In this study, a 171 transcripts were identified that were salivary gland-specific (above threshold and *P* value ≤ 0.001), of which 70% could not be functionally annotated or classified into a specific ontology (Fig. 2.9.B, Table 2.3. and Supplementary Tables S2.4. and S2.8.). Using a minimum two-fold absolute expression, relative to other tissues, these tissue-specific transcripts represent 1.3% of the total complement of transcripts analyzed (Supplementary Table S2.8.). The major functional classes according to KOG that were represented related to defense mechanisms (6.4%), protein turnover (4.7%), amino acid transport and metabolism (3.5%), as well as extracellular structures (3.5%) (Fig.2.9.B.).

2.4.6.1.1. Proteins for defense and blood meal acquisition

Numerous proteins involved in blood meal acquisition and specifically defense mechanisms were identified that were highly expressed in salivary glands during feeding. Defense proteins represent the major functional class identified for salivary glands comprising 6.4% (11 transcripts) of the total complement of tissue specific transcripts (Fig. 2.8.B., Supplementary Tables S2.4. and S2.8.).

These include putative protease inhibitors such as serine protease inhibitors or serpins (Contig1520, Contig6586, CK178656 and Contig688) that are similar to proteins described in other tick species (Anderson *et al.*, 2008; Francischetti *et al.*, 2010; Chalaire *et al.*, 2011). Secreted serpins such as Iris from the castor tick, *Ixodes ricinus*, can also act as anti-inflammatory factors and have been found to be expressed in salivary glands, midgut and ovaries (Prevot *et al.*, 2006; Maritz-Olivier *et al.*, 2007). Recombinant Iris caused a ~28% mortality rate of feeding nymphs following small scale vaccination trials in Rabbits (Table 1.2) (Prevot *et al*., 2007). Similarly, two serpin proteins (HLS1 and HLS2) isolated from *H. longicornis* salivary glands and hemolymph, respectively, caused mortality rates of around 40% for nymphs and 11% for adults, following vaccination trials in rabbits (Table 1.2.) (Sugino *et al*., 2003; Imamura *et al*., 2005). Vaccination in cattle using a cocktail containing two serpin homologs (RAS-3 and RAS-4), caused a ~27% mortality among feeding adult *R. appendiculatus* female ticks (Table 1.2.) (Imamura *et al*., 2008). These rather disappointing results illustrate problems of using antigens (i.e. serpins) that are part of large functionally redundant protein families, as well as the differences between tick species and life stages that causes variability in protection against tick infestations (Guerrero *et al*., 2012).

An additional, trypsin inhibitor-like cysteine rich domain-containing (TIL) protein (Contig8207) with putative anti-elastase, as well as antimicrobial activities, was also up-regulated (Fogaça *et al.*, 2006; Sasaki *et al.*, 2008). Trypsin-type inhibitor proteins, such as tick Kunitz serine proteinase inhibitors, are well established effectors of anti-coagulation in the intrinsic (via Thrombin and fXa) and tissue factor assisted extrinsic pathways. Other protease inhibitors related to the thyroglobulin type-1 repeat containing proteins or thyropins (Contig300) and the alpha-2-macroglobulin family (Contig2131 and Contig1745) were identified (Supplementary Table S2.4.). Thyropins have been proposed to act as inhibitors of cysteine proteases, as well as binding partners for heparin (Anatriello *et al.*, 2010; Francischetti *et al.*, 2010). Alpha-2-macroglobulins are universal protease inhibitors involved in clearance of exogenous proteases and innate immunity. These proteins have been identified in both hard and soft tick species (Saravanan *et al.*, 2003; Buresova *et al.*, 2009).

Additional well described defense transcripts that were highly up-regulated in salivary gland tissues included putative lipocalins or tick histamine binding proteins (Contig2493 and Contig2529) (Mans *et al.*, 2008). Lipocalins or histamine binding proteins are a ubiquitous class of proteins associated with tick feeding that can perform a host of molecular functions,

including immunomodulation and clearance of endogenous and exogenous compounds (Mans *et al.*, 2008; Maritz-Olivier *et al.*, 2007).

Antimicrobial defense proteins specific to salivary glands were identified that are orthologous to *R. microplus* cysteine-rich microplusin and defensin peptide proteins (CK177092 and Contig5482) (Fig.2.10. and Fig.2.11, Supplementary Table S2.4.). Defensins are involved in innate immunity and have been described from the midgut of *Ornithodoros moubata* (Nakajima *et al.*, 2002), as well as the salivary glands of *A. americanum* (Todd *et al.*, 2007). However, microplusin and defensin proteins have only been localized previously to the fat body, ovaries and hemocytes in *R. microplus* female ticks (Fogaça *et al.*, 2004). Therefore, this transcript may represent a novel salivary gland-specific defensin in *R. microplus* (Fig.2.10.). An additional novel *R. microplus* transcript (Contig5501) sharing ~30% sequence similarity with invertebrate astakine cytokines was uniquely up-regulated in salivary glands. A similar protein sharing 38% sequence identity was previously identified from the sialome of *Amblyomma variegatum* (Ribeiro *et al.*, 2011). Astakines are well described effectors in hematopoiesis in crustaceans, whereas related vertebrate prokineticin homologues have also been implicated in smooth muscle contraction (Lin *et al.*, 2011). A putative serine proteinase (Contig486) was identified as a transcript that may be involved in amino acid transport and metabolism, however this transcript showed high sequence identity (~80%) to a factor D-like transcript identified from *Dermacentor variabilis* that has been suggested to have antimicrobial activity (Simser *et al.*, 2004).

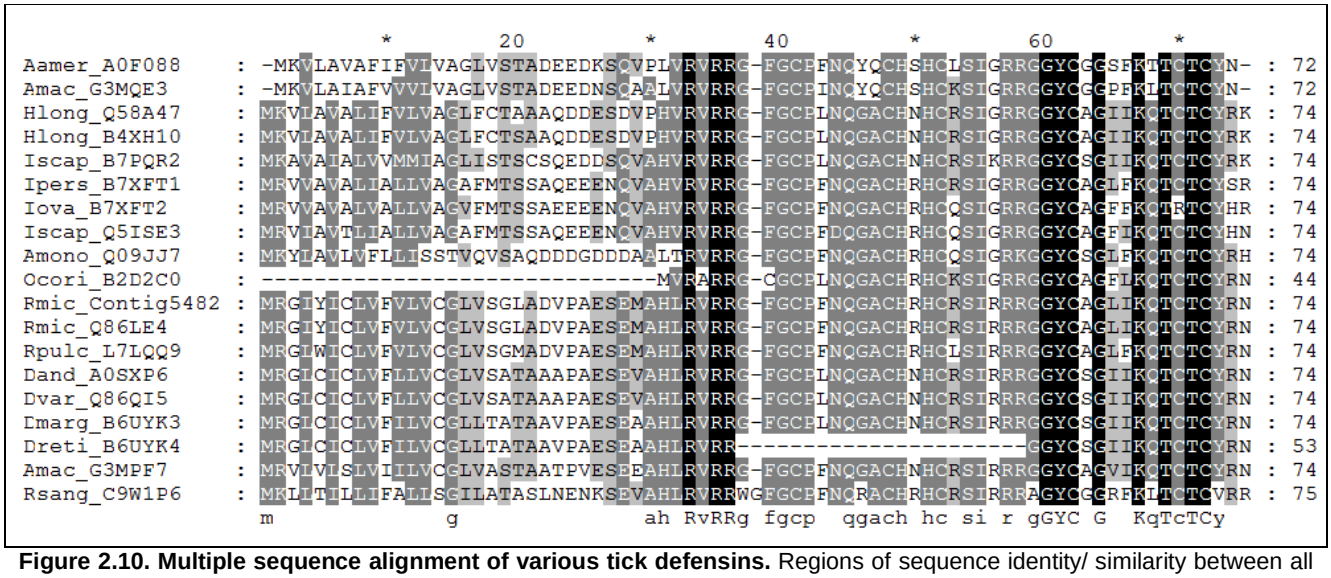

Figure 2.10. Multiple sequence alignment of various tick defensins. Regions of sequence identity/ similarity between all sequences are indicated in black and grey, while conserved residues are indicated below. The salivary gland-specific sequence (Contig5482) identical to the defensin from *R. microplus* (Q86LE4) is indicated, along with other hard and soft tick defensins. The description, accession number and sequence of each tick transcript used is included in Supplementary Material S1.

2.4.6.1.2. Transport and metabolism

A group consisting of six transcripts with putative functions in amino acid transport and metabolism, representing 3.5% of the salivary gland-specific transcripts, was identified (Fig. 2.9.B, Supplementary Table S2.8.). Of these, two transcripts encoding putative amino acid and peptide converting enzymes were highly up-regulated (Supplementary Table S2.4.): an aspartate amino transferase (Contig1049), as well as an angiotensin-converting enzyme (ACE) (Contig1) identical to the previously characterized Bm91 protein from *R. microplus* (Riding *et al.*, 1994). This angiotensin-converting enzyme (ACE) is an exopeptidase that plays a role in mediation of extracellular volume and vasoconstriction in mammals, as well as in reproduction in insects (Isaac *et al.*, 1999; Macours *et al.*, 2004). The ACE transcript has one of the highest log expression values of the salivary gland-specific transcripts, correlating with previous findings that this enzyme represents a major membrane component of *R. microplus* salivary glands (Riding *et al*., 1994; Jarmey *et al.*, 1995). The Bm91 protein also showed an improved efficacy in subsequent vaccination trials when used in combination with Bm86 (Willadsen *et al.*, 1996).

Secreted proteases have been implicated in the acquisition of a blood meal as anti-hemostatics and anti-inflammatories, extracellular matrix degradation molecules for blood

pool formation, as well as so-called “pre-oral” digestive enzymes (Ribeiro *et al.*, 2006; Batista *et al.*, 2008). In line with the latter, putative cysteine proteases containing signal sequences for secretion from the C1 peptidase family (Contig6614 and Contig1050) were identified (Supplementary Table S2.4.). Similar proteins have only been identified from midgut tissues of *R. microplus* (Kongsuwan *et al.*, 2010).

The transcript with the highest log fold expression (3.78) encodes a putative aquaporin involved in water and small neutral solute transport (CV443183) (Supplementary Table S2.4.). Aquaporins have been shown to be closely involved in salivation (Bowman and Sauer, 2004). However, knockdown experiments in *Ixodes ricinus* did not display a significant phenotype, indicating that additional factors are present (Campbell *et al.*, 2010).

2.4.6.1.3. Structural, signal transduction and other salivary gland-specific proteins

A group of structurally related transcripts was found to be uniquely expressed in salivary glands during feeding, representing around 3.5% of the total complement of proteins unique to salivary glands (Figure 2.8.B., Supplementary Table S2.4.).

Similar to other studies in salivary glands, putative glycine-rich cuticle-related (CV437645) and cement (Contig2328) proteins were identified. These transcripts may be involved in structural interactions of the peritrophic matrix, between the cuticle and salivary gland tissues, in addition to cement cone formation during attachment (Francischetti *et al.*, 2010; Maruyama *et al.*, 2010). Glycine-rich repetitive proteins belong to a large class of heterogeneous proteins with diverse putative functions in ticks. Consequently, classification into functional groups is difficult due to their highly repetitive nature and therefore most transcripts were assigned as proteins with unknown function, though some of these proteins may play structural roles, especially in cement cone formation during attachment (Maruyama *et al.*, 2010). Two related putative mucins (CV452616 and CV444691) were also identified with salivary gland-specific expression (Francischetti *et al.*, 2010). Mucins are chitin binding domain containing proteins common to hard and soft tick species and are thought to be involved in extracellular matrix adhesion, in addition to acting as a lubricant for the mouth parts and mucosal membranes during feeding (Mans *et al.*, 2008).

Components of the peritrophic matrix (i.e. peritrophins) have been tested as possible vaccine targets in dipteran ectoparasites, however little effect on mortality or fecundity were observed

on feeding adults (Wijffels *et al.*, 1999). In ticks, an extracellular glycine-rich matrix protein (RH50) was isolated and cloned from the salivary glands of *R. haemaphysaloides* (Zhou *et al*., 2006). This protein caused only a 30.5% mortality rate in the immature life stages (nymphs) of ticks feeding on immunized rabbits (Table 1.2.). In contrast, the cement protein, 64P, isolated from *I. ricinus*, *R. appendiculatus* and *R. sanguineus*, as well as a related adhesion protein (HL34) from *H. longicornis* produced mortality rates between adult and nymphs ranging from around 30% to 80% depending on the recombinant protein constructs used for immunization (Table 1.2.) (Tsuda *et al*., 2001; Trimnell *et al*., 2002; Trimnell *et al*., 2005). Similarly, a vaccination trial using a mucin (BmA7) isolated from *R. microplus* failed to demonstrate a greater protection against infestation compared to that of Bm86 (Table 1.2.) (McKenna *et al.*, 1998). The variation in vaccine efficiency observed for the above mention trials may be due to the fact that mucins, like glycine-rich proteins, belong to large functionally redundant protein families that would negatively influence vaccine efficacy by possibly "rescuing" the affected function (Guerrero *et al*., 2012).

A putative chitinase (Contig3556), an enzyme usually involved in molting, was also identified (Supplementary Table S2.4.). This enzyme also plays a role in degradation of chitin present in the gut lining and has also been implicated to play a role in digestion (You and Fujisaki, 2009). Similar enzymes have been identified from other sialomic data assembled for other ixodid species and have been suggested to perform an antifungal or housekeeping function in the cuticular structure (Francischetti *et al.*, 2010). However, limited protection against *H. longicornis* infestation was observed in mice vaccination trials using a chitinase (CHT1) protein (Table 1.2) (You and Fujisaki, *et al*., 2009).

A signal transduction transcript unique to salivary glands was identified, sharing sequence similarity to mitogen-activated protein kinase kinase kinase 1 (MEKK1) (Contig4877) (Supplementary Table S2.4.). Similar transcripts have been described from sialomic data of other tick species (Francischetti *et al.*, 2010). However, this transcript has not been previously described for *R. microplus* and represents a novel pathway present in the salivary glands of this species. MEKK1 is a signal transducer protein of c-Jun N-terminal protein kinase (JNK) and extracellular signal-regulated kinase (ERK) pathways that are involved in cytoskeleton regulation, cell migration and focal adhesion (Uhlik *et al.*, 2004).

A number of putative proteins involved in protein synthesis and trafficking (CK177403, Contig5601, CV454094 and Contig470) were also highly up-regulated in salivary glands that have been previously described from sialomic data of other ixodid ticks, including *R. microplus* (Supplementary Table S2.4.) (Francischetti *et al.*, 2010). This is expected as continuous synthesis and secretion of compounds, for example anticoagulants, is required for continued feeding.

2.4.6.2. Unique transcripts highly regulated in adult female midgut

The midgut represents the first major internal tick tissue barrier encountered by ingested blood and microbes. Transcriptional analysis indicated that 310 transcripts are unique to the midgut, representing 2.3% of the total regulated genes (Table 2.3., Supplementary Table S2.5.). Unfortunately over 50% of midgut-specific transcripts could not be functionally annotated (Fig. 2.9.C.), while 123 annotatable transcripts were highly up-regulated (Table 2.3. and Supplementary Tables S2.5. and S2.8.). The major functional classes relate to lipid transport and metabolism (7.1%), amino acid transport and metabolism (6.1%), signal transduction (5.8%), protein modification and turnover (5.8%), as well as defense mechanisms (4.8%) (Fig. 2.9.C.).

2.4.6.2.1. Defense mechanisms and anti-hemostasis

To date, numerous proteins involved in management of oxidative stress, defense against microbial invasion, inhibiting host immune responses and maintaining the fluidity of the blood meal have been described from the midgut of various tick species, including *R. microplus* (Uhlik *et al.*, 2004; Horn *et al.*, 2009; Mercado-Curiel *et al.*, 2011). In this study, a total of 18 midgut-specific transcripts with putative defense functions were identified representing the largest subset of defense transcripts for all tissue comparisons (Supplementary Tables S2.5. and S2.8.).

Of these, 11 transcripts involved in anti-coagulation belonging to four families of protease inhibitors were identified: the reversible papain-like cysteine protease (cystatin) inhibitor family (Contig5662, CV444905 and Contig1698), the Kunitz-type serine proteinase inhibitors (TC22004, CK192299 and CK192837), serpins (Contig1086, CV442792 and Contig165) and trypsin inhibitor-like (TIL) proteins (CV443795 and CK188782) (Supplementary Table S2.5.).

These protease inhibitors have been recently identified from the mialome of *D. variabilis* and have been described from tick sialome data (Anderson *et al.*, 2008; Francischetti *et al.*, 2010). Two *R. microplus* trypsin inhibitor proteins (BmTI and BmLTI) were tested in cattle vaccination trials and produced vaccination efficiencies of 72.8% and 32%, respectively (Table 1.2.) (Andreotti *et al*., 2002; Andreotti *et al*., 2012). An N-terminal synthetic fragment of BmTI (BmTI-A) failed to confer significant protection (18.4% efficacy) compared to the native protein in cattle pen trials (Table 1.2.).(Andreotti *et al*., 2007). This may be due to synthetic protein representing a less immunogenic region of the native BmTI protein and therefore more research is necessary to identify the exact region that is responsible for protective immunity.

Two additional transcripts related to the immunomodulatory lipocalin family of histamine binding proteins (CK173245 and TC21607) were also identified (Supplementary Table S2.5.). These proteins are a ubiquitous class of proteins involved in tick feeding and have been identified in the midgut of ixodid tick species, including *R. microplus* (Anderson *et al.*, 2008; Kongsuwan *et al.*, 2010).

Four putative antimicrobial transcripts that share sequence similarity with soft tick antimicrobial proteins were identified in midgut tissues (Contig4731, Contig4907, Contig5243 and Contig5493) (Lai *et al.*, 2004). Moreover, two transcripts (Contig5243 and Contig4731) shared sequence identity (> 68%) with vitellin-degrading cysteine endopeptidase (VTDCE) previously identified from *R. microplus* that has been shown to have antimicrobial activity (Fig.2.11.) (Oldiges *et al*., 2012). This endopeptidase has however been tested in cattle vaccination trials with limited efficacy (Table 1.2.) (Seixas *et al*., 2008).The antimicrobial peptide, microplusin, was previously localized to the fat body, hemocytes and ovaries of engorged females, but not the midgut (Fogaça *et al.*, 2004). Therefore, the midgut-specific transcripts (Contig4907 and Contig5493) and the salivary gland-specific transcript (CK177092) identified in this study represent a unique set of related proteins that are tissue-specific (Fig.2.11). Interestingly, no defensins were identified in the midgut, which correlates with previous findings by Fogaça *et al.* (2004). Since a microplusin homolog was identified that has salivary gland-specific expression this protein serves as a good example of differential expression of distinct subsets of transcripts from the same protein family in different tissues where they may perform similar functions (Fig.2.11).

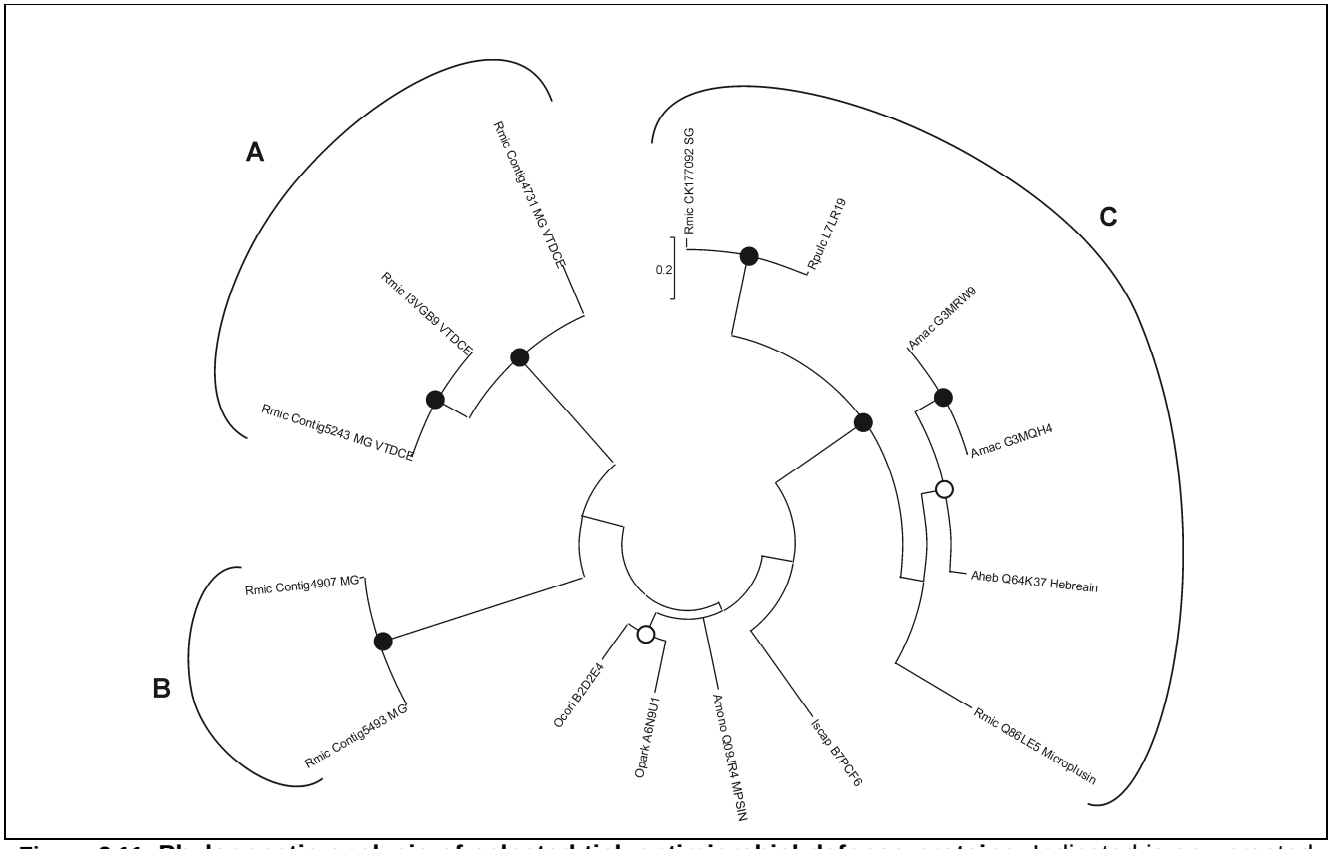

Figure 2.11. Phylogenetic analysis of selected tick antimicrobial defense proteins. Indicated is an unrooted neighbor-joining radiated phylogram with clades representing midgut-specific (MG) vitellin-degrading cysteine endopeptidase-like (A) and antimicrobial (B) transcripts, as well as other microplusin/hebreain-like transcript homologues (C) that include a salivary gland-specific (SG) transcript (CK177092). All homologues used in this analysis (including accession numbers, descriptions and sequences) are included in Supplementary Material S1. Confidence levels for 1,000 bootstraps are indicated by black (above 90%) and open dots (above 75%). The 0.2 scale bar indicates 20% amino acid divergence distance.

It has been suggested that digested blood is a source of oxidative stress since heme (a pro-oxidant) is released following hemolysis (Citelli *et al.*, 2007). As such, glutathione S-transferases or GSTs (Contig8822, Contig336 and Contig706) and cytochrome P450 enzymes (Contig894, U92732.1 and CV443756) were up-regulated in midgut tissues (Supplementary Table S2.5.). These enzymes are known to be involved in detoxification and management of oxidative stresses and have been found to be highly expressed during feeding in previous comparative studies of *R. microplus* larvae and adults on different cattle breeds (Rodriguez-Valle *et al.*, 2010). Furthermore, GSTs have also been shown to play a vital role in acquired acaricide resistance in *R. microplus* larvae (Saldivar *et al.*, 2008). *R. microplus* derived GST has been found to confer reasonable protection against infestation in cattle vaccination trials as a single (57% efficacy) or combinatorial (~30-60%) vaccine

preparation (Table 1.2.) (Parizi *et al*, 2011; Parizi *et al*., 2012). Therefore, identifying and targeting other components of the detoxification system might yield new and possibly more efficient candidates for vaccine development.

2.4.6.2.2. Nutrient transport and metabolism

Catabolism and acquisition of nutrients for basic metabolism and oogenesis from the blood meal are essential functions of the digestive tract. It therefore requires expression of a number of genes involved in the processing and transport of proteins, carbohydrates and lipids.

2.4.6.2.2.1. Proteins and amino acids

Numerous putative proteases involved in protein and amino acid metabolism were identified including: chymotrypsin-like serine proteases (Contig598, Contig3850 and Contig1821), asparaginyl peptidases or legumain-like protease (Contig1863), cathepsin peptidases (Contig6100, Contig953, Contig3558, Contig1506, Contig3719, Contig432 Contig2640 and TC15264), a elastase-like peptidase (Contig6406), a aminopeptidase (Contig5171), a serine-type endopeptidase (Contig5340), carboxypeptidases (Contig4941 and Contig5462) and a non-specific dipeptidase (Contig506) (Supplementary Table S2.5.). Considering the most prominent class of up-regulated proteases, the aspartic and cysteine (cathepsin) peptidases, these have been implicated in the hemoglobinolytic pathway in *I. ricinus* and *D. variabilis* midgut (Anderson *et al.*, 2008; Sojka *et al.*, 2008; Horn *et al.*, 2009). Similar enzymes have been implicated in the production of potent antimicrobial hemocidins in *R. microplus* (Cruz *et al.,* 2010). Proteome analysis of the midgut of partially fed female *R. microplus* ticks also identified type L and B-like cathepsins (with ~60% sequence identity to midgut-specific transcripts), as well as other metalloproteases (Kongsuwan *et al.*, 2010). Metalloproteases isolated from *I. ricinus* and *H. longicornis* were evaluated with vaccination trials in rabbits and found not to confer significant protection against infestation (Table 1.2.) (Decrem *et al*., 2008; Imamura *et al*., 2009). Similar results were obtained in cattle trials using an aspartic proteinase (BYC) that also failed to confer protection against *R. microplus* infestations (Table 1.2.) (da Silva Vaz *et al*., 1998, Leal *et al*., 2006). However, a serine protease isolated from *H. longicornis*, called Longistatin, was shown to have a protective efficacy of over 72% in mouse vaccination (Table 1.2.) (Anisuzzaman *et al*., 2012). It is therefore unclear if proteases are

good targets for vaccine development and immunogenicity may depend on the physical properties of individual proteins and/or their exposure (cytosolic or secreted) to host immune responses.

A putative pantetheine hydrolase (CV440582) involved in cellular recycling of pantothenic acid (vitamin B5) and oxidative stress was also highly up-regulated (Supplementary Table S2.5.) (Pitari *et al.*, 2000). This enzyme type has been identified in other ixodid ticks from comparative sialomic data (Francischetti *et al.*, 2010). However, this protein is novel transcript for the midgut of *R. microplus*.

Novel putative amino acid converting enzymes involved in both anabolic and catabolic processes have been identified as highly up-regulated in midgut tissues that may function in asparagine (Contig1748), cysteine (Contig8186), glycine (Contig2855), ornithine (Contig6808), methionine (Contig3884) and arginine (Contig8859) metabolism (Supplementary Table S2.5.). Similar enzymes have been identified from other tick sialomes and ESTs for *R. microplus* (Francischetti *et al.*, 2010). This may provide the first evidence for amino acid synthesis and conversion in *R. microplus* midguts, in addition to those acquired from the blood meal.

2.4.6.2.2.2. Carbohydrates

A group of novel midgut up-regulated enzymes involved in carbohydrate metabolism were identified (Supplementary Table S2.5.): glucosylceramidase (Contig7972) involved in sphingolipid metabolism (Hannun and Obeid, 2008), a hexokinase (Contig245B) that functions in fructose and mannose metabolism (glycolysis and gluconeogenesis) (Wilson, 2003), putative alpha-L-fucosidases (Contig8580 and Contig4273) that hydrolyze the carbohydrate moieties in glycoproteins (Johnson and Alhadeff, 1991), as well as glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase (CV448648) involved in glycoprotein synthesis (Ju *et al.*, 2002). Similar enzyme activities were identified from the midgut homogenates of the blood-feeding phlebotomine sand fly, *Lutzomyia longipalpis* (Gontijo *et al.*, 1998). A putative dihydropyrimidine dehydrogenase (Contig4532) that is involved in pyrimidine base degradation was also highly up-regulated in midgut tissues.

Additional novel transporters were identified that were highly up-regulated: a putative sialic acid cotransporter, sialin (membrane glycoprotein HP59, Contig6465), as well as a non-specific monocarboxylate carbohydrate transporter (CV455491). The former is involved in monosaccharide import across the lysosomal membrane and may function in the clearance of free cytosolic oligosaccharides derived from the breakdown of glycoproteins and other catabolic products (Fu *et al.*, 2001; Winchester, 2001). These proteins have not been previously described in ticks.

2.4.6.2.2.3. Lipids

Some 22 midgut-specific transcripts were identified that have putative functions in lipid metabolism and represent the largest subset of this class for all comparisons (Supplementary Table 2.8.). It has been suggested that secreted phospholipases may play a role in hemolysis of the ingested blood cells (Zhu *et al.*, 1997; Zhu *et al*., 1998). In this regard, putative lipases (Contig2297, TC17614 and Contig1290) were highly up-regulated in the midgut (Supplementary Table S2.5.). Other putative enzymes identified to be involved in lipid metabolism were: a sphingomyelin phosphodiesterase (Contig7995), a phosphatidate phosphatase (TC22152) and a prosaposin (Contig1729) that is involved in lipid degradation, as well as a number of short chain alcoholdehydrogenases (Contig2638, Contig6832 and Contig6561), a diacylglycerol acyltransferase (Contig2086), a phosphoethanolamine N-methyltransferase (Contig2302), an acyl-Co-A ligase (Contig3708) and a acyl-CoA elongase (Contig2303) involved in fatty acid biosynthesis (Supplementary Table S2.5.).

Additional novel lipid carrier proteins were identified, including a carrier protein apolipophorin (Contig156) with the highest $\log_2$ fold expression of midgut-specific transcripts (5.59) (Supplementary Table S2.5.). This glycoprotein is involved in the transport of various lipids in the hemolymph as well as heme (Duncan *et al.*, 1999; Weers *et al.*, 2006). A related transcript, vitellogenin (Contig8127), was also up-regulated in midgut tissues (Supplementary Table S2.5.). Vitellogenin is a major storage protein in the tick hemolymph and it has been shown to be up-regulated in adult females in response to mating and blood feeding to repletion for use as a precursor in egg yolk protein vitellin during oogenesis (Donohue *et al.*, 2008). This protein was previously identified in the midgut and fat body of *Haemaphysalis longicornis* and has been shown to play a role in heme sequestration (Thompson *et al.*, 2007; Boldbaatar *et al.*, 2010). Vitellogenin expression was also found to be affected in response to

ubiquitin-63E knockdown in gene expression microarrays of female *R. microplus* ticks (Lew-Tabor *et al.*, 2011). A vaccination trial in sheep using a components of vitellin (GP80 and VIT87) was able to confer protection against *R. microplus* infestation (~68% efficacy) (Table 1.2) (Tellam *et al*., 2002). Several proteins sharing sequence similarities to the Niemann-Pick C family proteins (NPC1 and 2) from hard and soft ticks (TC17851, Contig1508, CV443743 and Contig727) were highly expressed in midguts (Supplementary Table S2.5.). These proteins have been proposed to be involved in intracellular cholesterol cycling and have been identified from midgut transcripts of *D. variabilis and I. ricinus* (Vanier *et al.*, 2004; Anderson *et al.*, 2008; Horácková *et al.*, 2010). A unique cytosolic fatty acid-binding protein (Contig4249), not previously described for *R. microplus*, was also highly up-regulated and may play a role in intracellular transport of long-chain fatty acids.

In a related comparative study between the midgut transcriptomes of *R. microplus* and an endemic species *R. decoloratus*, lipid metabolism was identified as a highly conserved attribute in basic tick biology that may be explored for potential cross-species vaccine design (van Zyl *et al*., 2013, submitted). In this regard, seven of the abovementioned transcripts (TC22152, Contig2638, Contig6832, Contig6561, Contig2302, Contig3708 and Contig8127) that are specifically up-regulated in female *R. microplus* midguts are shared with *R. decoloratus*. The significance of the latter in tick biology and cross-species vaccine design will be the subject for future studies.

2.4.6.2.3. Midgut-specific transport proteins

During digestion a large amount of heme is produced and as a result high levels of cytotoxic iron are released into the midgut. Furthermore, *R. microplus* lacks the biosynthetic pathway for heme production and therefore acquires it from the blood meal (Braz *et al.*, 1999). A novel ferritin transcript (Contig3919) similar to secreted ferritin 2 of *I. ricinus* (54% identity) was identified among the unique up-regulated midgut transcripts (Fig. 2.12., Supplementary Table S2.5.). Ferritin is a storage protein that sequesters ferric iron for heme and iron metabolism. This protein has been cloned from the gut of *O. moubata* and *I. ricinus* (Kopácek *et al.*, 1999). More recently, ferritin has also been cloned from *R. microplus*, as well as *R. annulatus* and vaccination trials in cattle showed a 64% and 72 % vaccine efficacy and protection against tick infestation (Table 1.2.) (Hajdusek *et al.*, 2010). Similarly, a trial in rabbits using recombinant protein showed a promising efficacy (98%) and protection against infestation of *I.*

ricinus (Table 1.2.) (Hajdusek *et al.*, 2010). These promising results (in different species and host animals) demonstrate the suitability of this protein for further development as a potential cross-species vaccine candidate (Guerrero *et al*., 2012).

Two highly up-regulated midgut transcripts (CV439517 and Contig8877), encoding a putative calcium activated chloride channel, were identified (Supplementary Table S2.5.). This class of transporter has been implicated in calcium-dependant physiological functions such as epithelial secretion (e.g. mucus production) and membrane depolarization for smooth muscle contraction (Hartzell *et al.*, 2005). A number of additional calcium transport and -binding proteins were also identified (Contig7035, Contig728, CV438087 and Contig8732) (Supplementary Table S2.5.).

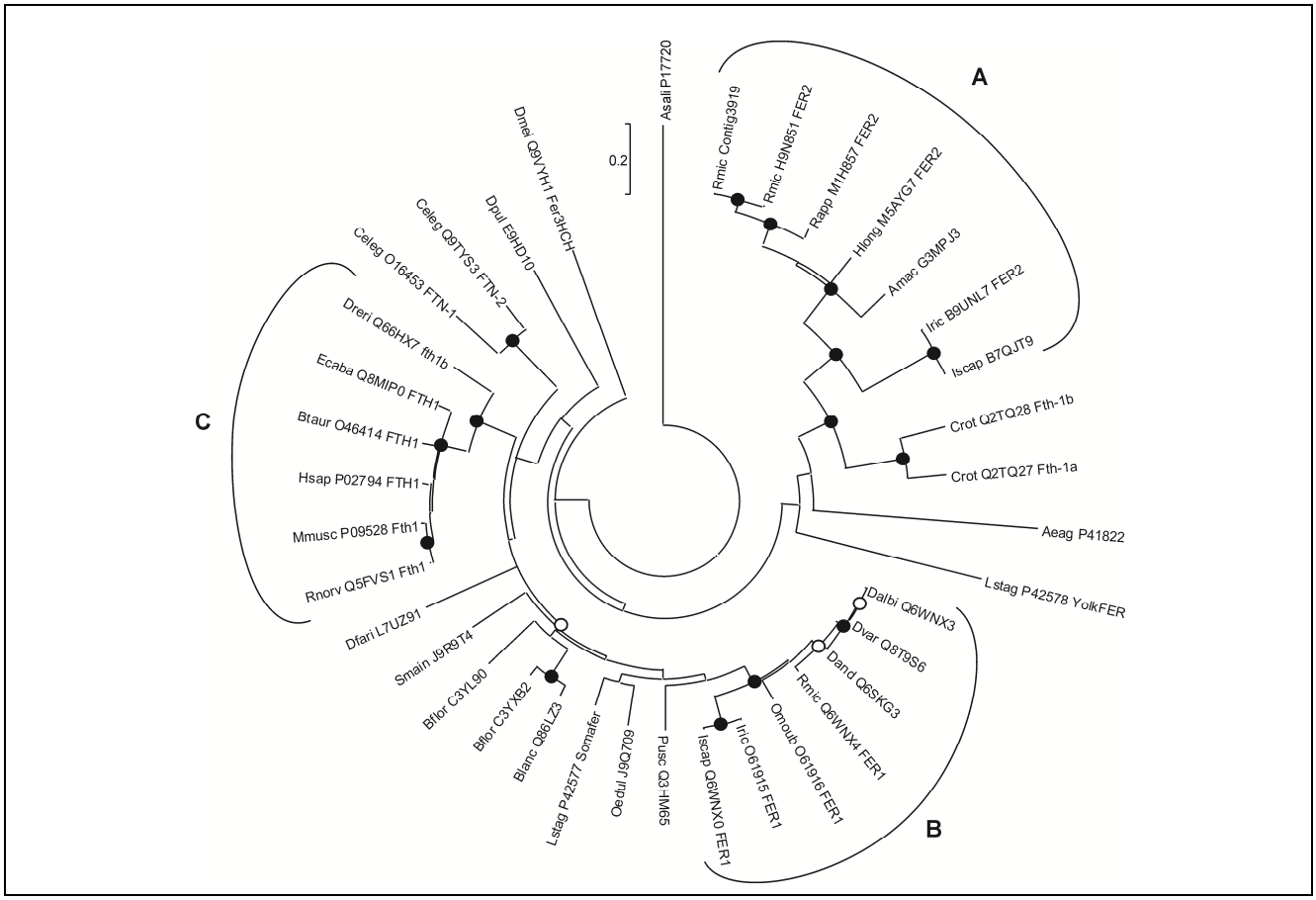

Figure 2.12. Phylogenetic analysis of selected members of the ferritin family. Indicated is an unrooted neighbor-joining radiated phylogram with clades representing secreted (A) and cytosolic (B) tick ferritins, as well as vertebrate homologues (C). The midgut-specific ferritin 2 (Contig3919) identified in this study is indicated in clade A. All homologues of the ferritin family utilized in this comparison included eukaryotic species from vertebrates, invertebrates and arthropods (accession numbers, descriptions and sequences are included in Supplementary Material S1). Confidence levels for 1,000 bootstraps are indicated by black (above 90%) and open dots (above 75%). The 0.2 scale bar indicates 20% amino acid divergence distance.

2.4.6.2.4. Structure, signal transduction and other midgut-specific proteins

Putative mucin (Contig2856), chitin binding peritrophin (TC22078) and microtubule-associated (Contig5040) transcripts were up-regulated in midgut tissues (Supplementary Table S2.5). Mucins from the midgut tissues of *I. ricinus*, *H. longicornis* and more recently from *D. variabilis,* have been described (Matsuo *et al.*, 2003; Anderson *et al.*, 2008). These proteins form part of the peritrophic matrix lining the epithelium of the midgut where they serve as a mucosal barrier (Bolognesi *et al.*, 2008). Microtubule-associated proteins on the other hand are accessory anchor proteins of microtubules involved in microtubule assembly and stability (Gundersen and Cook, 1999).

Some 15 putative signal transduction transcripts were highly up-regulated in midgut tissues (Supplementary Table S2.5.). Of these two proteins that are uniquely expressed in midgut tissues, namely ATAQ (Contig3639) and Bm86 (Contig8501) were identified (Supplementary Table S2.5.). Bm86 and ATAQ are both glycoproteins of unknown function containing epidermal growth factor domains that have been localized to the midgut tissues (ATAQ also localized to the malpighian tubules) of *R. microplus* (Nijhof *et al.*, 2010). To date, Bm86 remains the only protein antigen that has been successfully used in the production of a commercial tick vaccine (Chapter 1).

Additional signal transduction transcripts that were identified included: putative leucine-rich repeats and immunoglobulin-like domains-containing (LIG) proteins (Contig6280, Contig4072, Contig4369 and Contig3670), a plexin-A1 receptor (Contig376) and a putative low-density lipoprotein receptor-related protein (Contig3838) (Supplementary Table S2.5.). Immunoglobulin-like domains-containing (LIG) proteins have been described for *R. microplus* and found to be up-regulated in *Babesia bovis* infected *R. microplus* larvae (Heekin *et al*., 2012). As LIG-1 proteins are also implicated in a feedback loop to negatively regulate epidermal growth factor receptors via an associated tyrosine kinase (Ghiglione *et al.*, 1999), it is interesting to note that putative epithelial growth factors, granulins (Contig1195 and Contig7270), were also up-regulated in midgut tissues (Supplementary Table S2.5.). Plexins are a large family of transmembrane receptors that are implicated in semaphorin regulated growth inhibitory pathways and have not been described for ticks (Turner *et al.*, 2004). These aforementioned transcripts may form part of a regulatory pathway for growth/development of midgut tissues of adult females during feeding that can be further elucidated in future studies.

Transcripts encoding acetylcholinesterases (Contig4226 and Contig8297) were identified that have been characterized for *R. microplus* and implicated in acquiring acaricide resistance (Baxter and Barker, 1998; Guerrero *et al.*, 2012). A calmodulin binding protein (Contig473) and a putative GTP-binding Rab protein (Contig2938), were also highly up-regulated in midgut tissues (Supplementary Table S2.5.). Calmodulin proteins have been identified from midgut tissues of *D. variabilis*, as well as *R. microplus* and are well known effectors in signal transduction mechanisms, affecting a number of cellular processes including membrane trafficking mediated by Rab GTPases (Stenmark and Olkkonen, 2001; Anderson *et al.*, 2008; Rachinsky *et al*., 2008). These proteins have also been suggested to play a role in smooth muscle contraction of the midgut during feeding (Anderson *et al.*, 2008).

Other putative proteins that are highly up-regulated in midgut tissues were: DNA metabolizing enzymes such as deoxiribonuclease type II (DNase II) (Contig1765 and Contig3627) and secreted ribonuclease T2 (Contig3372), a transcription factor (CV448758) and a caspase (Contig4156) involved in programmed cell death (Supplementary Table S2.5.). DNAse type II enzymes are conserved enzymes within phagolysosomes involved in degradation of macromolecular DNA engulfed from apoptotic cells, as well as microbes and have been implicated in innate immunity in *Drosophila* (Seong *et al.*, 2006). These transcripts have not been previously described for *R. microplus*.

2.4.6.3. Unique transcripts highly regulated in adult female ovaries

For the perpetuation of a species, the production of viable gametes is of paramount importance. Therefore, tight control of the meiotic cell cycle is required to ensure the production of viable gametes for fertilization. For female ixodid ticks, fertilization and stimulation (via secreted factors like voraxin) by the males, induce a rapid engorgement phase that causes the female to increase her body weight dramatically and finally drop from the host to produce and lay eggs (Kaufman, 2007). Transcriptional analysis of *R. microplus* female ovaries identified 417 unique transcripts (Tables 2.3., Supplementary Tables S2.6. and S2.8.). The major functional classes that could be identified according to KOG classification related to transcription (7.4%), protein modification and turnover (6.7%), signal transduction (4.6%) and cell cycle control (3.6%), while approximately 52.8% of transcripts could not be functionally assigned (Fig. 2.9.D.). A total of 72 annotatable transcripts were highly up-regulated (Tables 2.3., Supplementary Tables S2.6. and S2.8.).

2.4.6.3.1. Chromatin structure, replication, cell cycle control and signal transduction

Following DNA replication (or S phase), the meiotic cell cycle development occurs in two consecutive cell divisions called M phases (Nebreda and Ferby, 2000). Transcriptional analysis identified some highly up-regulated ovary-specific transcripts that are involved in chromosomal organization. These include several nucleosome core histones of the H2A (TC21623, Contig4221 and Contig2123), H3 (Contig6410, Contig5900) and H4 (Contig5362) families, a putative maintenance of chromosomes (SMC) ATPase (CV457670) and microcephalin (Contig6899) (Supplementary Table S2.6.). These proteins are involved in maintenance of the nuclear architecture, especially during DNA replication (S phase) in other organisms such as *Drosophila* (Kornberg and Lorch, 1999; Hirano, 2005). Two groucho-related transducer proteins (CK189050 and Contig4005) were identified, presumably involved in transcriptional regulation by interaction with the histone core in response to cyclin-dependent kinases (Turki-Judeh and Courey, 2012). In this regard, a putative cyclin-dependent kinase (CV446249) was also identified (Supplementary Table S2.6.).

Various proteins serve as checkpoints during cell division and tightly control progression through the cell cycle. Related transcripts were identified such as a B-type cyclin (CV451547), a serine/threonine protein kinase (Contig347) and a polo-like protein kinase (Contig4236), as well as a putative coiled-coil domain-containing protein (Spindly) (Contig6020) that where ovary-specific (Supplementary Table S2.6.). Cyclins play key roles in cell cycle regulation during early mitotic divisions in *Drosophila*, where B-type cyclins are involved in activation of several key proteins involved in spindle formation and progression through metaphase and anaphase (McCleland *et al.*, 2009; Yoshitome *et al.*, 2012). In this regard, the related protein Spindly is involved in chromosome segregation during mitosis/meiosis by mediating the recruitment of dynactin to the kinetochore during spindle formation (Chan *et al.*, 2009). Aurora kinases and polo-like kinases can associate with the centrosome and are involved in regulation of spindle formation via activation of proteins such as Spindly and histone phosphorylation during oogenesis (Ding *et al.*, 2011; Song *et al.*, 2012 Yoshitome *et al.*, 2012). An additional putative repressor of embryonic development via the Wnt/beta-catenin pathway, protein bicaudal C homolog 1 (Contig383), was also found to be highly up-regulated (Supplementary Table S2.6.). All of the abovementioned form a complex system involved in

control of cell division during mitosis/meiosis (oogenesis) in ovaries that have not been described before in the cattle tick.

2.4.6.3.2. Transcription and control

Transcriptional initiation and control can be achieved via expression of ovary-specific transcription factors and degradation of the mRNA by the endogenous RNAi machinery, respectively. In this regard, zinc-finger proteins represent the largest family of DNA-binding transcription factors present in a number of bilaterians, including *Homo sapiens, Drosophila melanogaster, Caenorhabitis elegans* and more recently in the arthropod *Daphnia pulex* (Seetharam *et al.*, 2010). Numerous putative zinc-finger proteins, such as C2H2-type zinc-finger proteins (CV457291, Contig4738 and CK173279), were identified that were highly up-regulated in ovaries (Supplementary Table S2.6.). Putative RNA processing proteins involved in post-transcriptional gene regulation were also identified as highly up-regulated in ovaries, similar to transcripts previously identified from *R. microplus* ESTs (Kurscheid *et al.*, 2009) (Supplementary Table S2.6.). These include an RNA-directed RNA polymerase-like protein (Contig7796), as well as argonaut 2 (Contig1465) and argonaut 4 (Contig7471 and CK178055) proteins that are main components of the RNA interference (RNAi) silencing complex (RISC). A putative LSM14 homolog A (Contig1423) protein, involved in the formation of P-bodies for storage of non-translating mRNAs, was also identified (Supplementary Table S2.6.). In addition, a putative proliferating cell nuclear antigen (PCNA)-associated factor (Contig5833) and a DNA excision repair protein (Contig7899) were identified (Supplementary Table S2.6.). These proteins are important processing factors involved in post-replication repair (Essers *et al.*, 2005). Many of the abovementioned transcripts have not been described in *R. microplus* (or other ticks) and future research will offer new insights into transcriptional and translational control in ticks.

2.4.6.3.3. Metabolism and other ovary-specific proteins

Only a few highly up-regulated transcripts have been identified that are involved in amino acid, carbohydrate and lipid metabolism (Supplementary Table S2.6.). These include several ovary-specific proteases with limited description in *R. microplus*. Firstly, an astacin (Contig1441) that has been shown to be expressed in *R. microplus* female reproductive tissues (Barnard *et al.*, 2012). These enzymes were proposed as novel targets for vaccine development supported by significant phenotypes raised with gene knockdown, particularly

related to egg production (Barnard *et al.*, 2012). Secondly, are novel metalloproteases such as the thrombospondin type-1 domain-containing (ADAMTS)-like protein (Contig8445) suggested to play a role in modulating microfibril functions, as well as a neprilysin (Contig6628) similar to *Drosophila* spp. Neprilysin 2 that has been suggested to play a role in signaling during embryogenesis (Bland *et al.*, 2007; Bader *et al.*, 2012). Thirdly, a cathepsin L1 cysteine peptidase (Contig1209) was identified that has only been described in midgut tissues of *R. microplus* (Kongsuwan *et al.*, 2010). Finally, together with other serine proteases, a transcript encoding putative oviductin (Contig6991) was up-regulated in ovaries (Supplementary Table S2.6.). This secreted protease is involved in egg envelope glycoprotein alteration for fertilization in the oviduct epithelia of amphibians (Hiyoshi *et al.*, 2002). However, its role in tick oogenesis remains to be elucidated.

Two novel up-regulated carbohydrate metabolizing enzymes were identified: an alpha 1,3-fucosyltransferase enzyme (Contig2393) and an N-acetyllactosaminide beta-1,3-N-acetylglucosaminyltransferase (Contig46A) (Supplementary Table S2.6.). These enzymes are involved in N-glycan synthesis of glycoprotein moieties and unsaturated fatty acids biosynthesis, respectively (Fabini *et al.*, 2001; Okajima *et al.*, 2008).

During tick oogenesis and egg maturation, major egg yolk proteins (vitellogenin) are produced by the fat body and the midgut (Tufail and Takeda, 2009; Boldbaatar *et al.*, 2010). These hemoglycolipoproteins are taken up together with lipid-binding lipophorin into the ovaries by low-density lipoprotein receptors via receptor-mediated endocytosis (Tufail and Takeda, 2009). A putative low-density lipoprotein receptor-related protein (Contig276) was highly up-regulated in ovaries (Supplementary Table S2.6.). These results correlated well with transcripts involved in lipid transport and metabolism that were up-regulated in midguts, where related proteins were also up-regulated (Sections 2.4.6.2.2.3. and 2.4.6.2.4.). Additional related and up-regulated 3-ketoacyl-CoA synthases (CK18577, Contig97 and Contig190A) were identified that are involved in long chain fatty acid synthesis (Supplementary Table S2.6.).

Several transporter proteins were identified that were highly up-regulated in ovaries and included two novel ABC transporters (Contig2240 and Contig1691) (Supplementary Table S2.6.). ABC transporters are proteins involved in detoxification processes and have been identified from midgut tissues of female *R. microplus*, where transporter activity has been

implicated in acaricide resistance (Pohl *et al.*, 2011; Pohl *et al.*, 2012). Other stress proteins that were identified include a glutathione S-transferase (GST) (Contig1826) and a copper/zinc superoxide dismutase (Contig166) (Supplementary Table S2.6.). The latter enzyme is known to remove naturally occurring superoxide that can cause oxidative stress and has also been proposed to be involved in heme trafficking in ticks (Anderson *et al.*, 2008).

Three putative ovary-specific mucin proteins (Contig1170, Contig2770 and Contig344) were also identified (Supplementary Table S2.6.). Mucins are a large family of proteins that play various physiological roles including lubrication (e.g. for passage of the food through the gut), protection against bacterial invasion and protection of epithelial cells from digestive proteases (Tellam *et al.*, 1999). In this case these mucins may serve a protective function, in addition to lubrication of the mucosal epithelia in the oviducts. Tissue-specific mucins have been identified for all the tissues tested in this study, illustrating the functional diversity of this protein family in each tissue.

2.4.7. Gene expression of transcripts occurring in all adult female tissues during feeding

A key objective of this study was to identify proteins and potential biochemical processes that are shared between all the different adult tissues to identify conserved proteins/ molecular systems that might offer new insights into basic tick biology, as well as identify potential targets for vaccine development. Following transcriptional analysis 588 expressed transcripts were identified that were shared among all of the tissues tested (Figure 2.9.A., Table 2.3., Supplementary Table S2.7.). Considering transcripts that have a minimum intensity above the threshold of 1,000 (M-value > 0), only 4.4% of the total transcripts used in microarray analysis showed transcriptional regulation (Table 2.3.). Of these transcripts, 38.8% could not be functionally annotated (Fig. 2.9.E.). The major functional classes that could be identified involved protein modification and turnover (11.2%), translation, ribosomal structure and biogenesis (10.2%), as well as RNA processing (6.3%) and intracellular trafficking and transport (5.8%) (Fig. 2.9.E.). Only 90 annotatable transcripts with minimum two-fold threshold intensity (Cy5 > 2,000) were identified (Table 2.3., Supplementary Tables S2.7. and 2.8.).

2.4.7.1. Metabolism, energy production and transport

Shared transcripts were identified that are related to amino acid, nucleotide, carbohydrate and lipid metabolism (Supplementary Table S2.7.). Only two transcripts were identified that are involved in amino acid and nucleotide metabolism, respectively: a phosphoglycerate dehydrogenases (Contig387 and Contig8440) involved in L-serine biosynthesis and a dUTP diphosphatase (Contig8942) involved in the biosynthesis of dUMP. Transcripts encoding several putative enzymes involved in carbohydrate metabolism that were identified included: GDP-L-fucose synthase (Contig4405) and GDP-mannose 4,6 dehydratase (Contig497) involved GDP-L-fucose biosynthesis and polysaccharide breakdown, a glucan 1,3-alpha-glucosidase (Contig835) involved in glycan metabolism for glycoprotein synthesis and a tau-protein kinase (Contig8022) involved in glycogen metabolism. Lastly, putative enzymes identified that are involved in lipid metabolism included: a 3-hydroxyacyl-CoA dehydrogenase (Contig1246) involved in cholesterol degradation and fatty acid beta-oxidation and acetyl-CoA C-acetyltransferases (Contig8253 and Contig2916) involved in ketone body metabolism. The large number of transcripts identified, unique or shared, across all tissues involved in carbohydrate and lipid metabolism, may imply that metabolic energy is mainly derived from carbohydrate and fatty acid sources. This observation is supported by results obtained for midgut-specific transcription in this study, as well as from transcriptome analysis between midgut tissues of *R. microplus* and *R. decoloraus* were Contig2916 was conserved (including other previously mentioned midgut-specific transcripts) (van Zyl *et al*., 2013, submitted). The latter observation is interesting, since vertebrate blood (as the primary source of nutrients) is richest in the protein hemoglobin (14-16%) in addition to lipids inclusions, carbohydrates and cholesterol (Sonenshine, 1991). However, the energy (ATP) yield obtained from metabolism of lipids is more than double that of proteins (Livesey, 1984). Therefore, it may be possible that metabolism (as seen from the number of proteases up-regulated in the midgut and secreted by the salivary glands) of proteins as a source of nutrients is localized, while lipid metabolism may present the major source of metabolic energy throughout the tick (as seen from the number of enzymes, transport proteins and receptors up-regulated).

In terms of transport, a putative sodium-independent sulfate anion transporter of the SLC26A/SulP transporter family (CV447239) was identified as a shared transcript amongst all the tissues tested. Inorganic sulphate plays a role in cell growth and development, biosynthesis and detoxification (via sulfation), cell matrix synthesis and membrane maintenance (Markovich, 2001). Sulfate anion transporters are ubiquitous and have been

shown to be involved in sulfate ion uptake (sulphate homeostasis), however its precise physiological role in ticks remains to be elucidated (Dorwart *et al.*, 2008).

Transcripts related to mitochondrial transport and energy metabolism were identified and these include components associated with oxidative phosphorylation: a cytochrome c oxidase (Contig2837), NAD(P)(+) transhydrogenase (Contig4420) and an ADP/ATP translocase 2 (Contig4681). A putative isocitrate dehydrogenase (Contig424), a component of the citric acid cycle, was also identified as a shared transcript (Supplementary Table S2.7.). Cytochrome c oxidases and other associated proteins involved with energy metabolism have been identified from the mialome of *D. variabilis*, as well as sialomes of both hard and soft tick species (Anderson *et al.*, 2008; Francischetti *et al.*, 2010; Kongsuwan *et al.*, 2010).

A number of shared transcripts encoding components of the ubiquitin/proteasome pathway, involved in the degradation of unneeded or damaged proteins, were identified: two proteins involved in ubiquitination of target proteins for degradation, a RING-box protein 1A (Contig5214) and a ubiquitin-protein ligase (Contig8175), as well as several subunits of the proteasome endopeptidase complex (Contig2948, Contig903 and Contig887) (Supplementary Table S2.7.). Subunits of the proton-pumping V-ATPase complex were also identified (Contig8631, Contig2275 and Contig8181), which are involved in acidification of lysosomal lumen necessary for digestion of macromolecules or exocytosis-mediated secretion (Bowman and Sauer, 2004; Mindell, 2012). Similar transcripts have only been identified from comparative sialome data in other ticks, including *R. microplus* ESTs (Francischetti *et al.*, 2010). Other components of the proteasome, such as ubiquitin (63E) have been studied *in situ*, following gene knock-down and gene expression profiling for *R. microplus* (Lew-Tabor *et* al., 2011). However, the latter afforded limited protection against infestation of *R. microplus* (55% efficacy) and *R. appendiculatus* (~20% efficacy) ticks in cattle vaccination trials (Table 1.2.) (Almazán *et al*, 2010; Almazán *et al*, 2012). Nevertheless, the aforementioned gives insight into the systems involved in processing of endogenous proteins via the proteasome complex and may offer additional targets for vaccine development.

2.4.7.2. Transcription, translation and protein synthesis control

Following transcriptional analysis, some key shared transcripts were identified that function in gene expression and control (Supplementary Table S2.7.). Of these, some novel transcripts

involved in control of transcription activation that were shared between female tissues include: a RuvB-like DNA helicase (Contig5083) that is part of the NuA4 histone acetyltransferase complex involved in transcription activation via modification of core histones H4 and H2A (Doyon *et al.*, 2004; Lu *et al.*, 2009). Also a DPY30 domain-containing protein (Contig346) that is part of the mixed lineage leukemia protein -1 (MLL1) core complex involved in methylation of histone H3 (Vardanyan *et al.*, 2008; Patel *et al.*, 2011), as well as an arginine N-methyltransferase (Contig137) that is the main enzyme involved in mono-methylation of histone H4 were identified (Boulanger *et al.*, 2004; Bachand, 2007). Additional transcription factors BTF3 (Contig1649) and putative X-box-binding protein 1 (Contig6772) were identified (Liou *et al.*, 1990; Zheng *et al.*, 1990). These proteins have not been characterized for *R. microplus* and together with transcripts specifically up-regulated in other tissues (i.e. ovaries), gives new insight into the complex mechanisms involved in gene regulation in ticks.

Alternative splicing of mRNA is regarded as one of the main steps in regulation of gene expression and five transcripts encoding splicing factors of the SR family (Contig825, Contig8997, Contig1956, Contig5832, Contig8773 and Contig5369) and a thioredoxin-like protein (Contig5832) were identified (Supplementary Table S2.7.). These proteins are essential in constitutive pre-mRNA splicing and can also act as regulators in other aspects of mRNA metabolism (Long and Caceres, 2009). A related U2 small nuclear ribonucleoprotein (snRNP) (Contig1192), a component of the spliceosome complex, was also identified (Supplementary Table S2.7.). Similar proteins have only been identified from sialome data that include ESTs from *R. microplus* and gives new insights into mRNA maturation in ticks (Francischetti *et al.*, 2010).

Assembly of a functional ribosome is vital for successful protein synthesis. In this regard, a number of ribosome associated proteins were identified that included components of the 40S (Contig7181 and EW680164.1) and 60S (Contig1238, Contig3228, EW680050.1, Contig6473, Contig1078, Contig9000, CK177858 and Contig7496) ribosomal subunits, as well as the 29S (TC20332) and 39S (Contig2946) mitochondrial ribosomal subunits. Additional transcripts encoding subunits of the H/ACA small nucleolar ribonucloeprotein complex (Contig1577) involved in pseudouridylation of rRNA and the eukaryotic translation initiation factor 3 complex (Contig2574) that associates with the 40S ribosomal subunit for initiation of translation, were also found to be shared (Pestova *et al.*, 2001; Watkins and Bohnsack, 2012). These proteins have been identified from sialome and midgut proteome data of *R.*

microplus and are expected foundational proteins for cells (Francischetti *et al.*, 2010; Kongsuwan *et al.*, 2010). A peptide derivative of a ribosomal protein (P0) from *R. sanguineus* has recently been tested in small scale vaccination trials and afforded strong protection against tick infestation (~90% efficacy) in rabbits (Table 1.2.) (Rodriguez-Mallon *et al*., 2012). The latter provides strong proof for the possibility of targeting intracellular soluble proteins in rational vaccine design, contrary to current views prioritizing antigens that are membrane-associated or membrane-bound (Guerrero *et al*., 2012).

Proper folding of translated proteins is predominantly mediated by chaperones of which the alpha subunit of the nascent polypeptide-associated complex (Contig8810) was identified from shared transcripts (Supplementary Table S2.7.). This complex functions as a molecular chaperone that generally associates with newly synthesized nascent polypeptides from the ribosomes to prevent inappropriate interactions with cytosolic proteins such as signal recognition particles (SRP) (Preissler and Deuerling, 2012). In this regard, a putative SRP14 protein (Contig4224) was identified from the shared transcript that has not been previously described for *R. microplus* (Supplementary Table S2.7.). These proteins are involved in polypeptide translocation to the endoplasmic reticulum for intracellular trafficking (Preissler and Deuerling, 2012).

Additional shared molecular chaperones (Contig5499, Contig210, Contig2441, Contig2805, Contig790, Contig2759 and Contig2634) were identified (Supplementary Table S2.7.). These molecular chaperone proteins are required for proper protein folding during translation and similar chaperones have been identified in other ixodid ticks, as well as sialomes, transcriptome and midgut proteome data for *R. microplus* (Guilfoile *et al.*, 2004; Anderson *et al.*, 2008; Francischetti *et al.*, 2010; Kongsuwan *et al.*, 2010; Rodriguez-Valle *et al.*, 2010; Yébenes *et al.*, 2011). A novel putative mitochondrial chaperone involved in import and insertion of inner membrane proteins, TIM13 (Contig6255), was also identified as a shared transcript (Supplementary Table S2.7.).

Other putative transcripts identified that are involved in protein folding and processing are: a stress-induced phosphoprotein 1 (Contig5025), an endoplasmic reticulum resident protein (Contig2747), a peptidyl-prolyl cis-trans isomerase (Cyclophilin A) (Contig9031), a calnexin (Contig1372) and a calreticulin (Contig390). Cyclophilin A has been identified from proteomic analysis in the midgut of partially fed female *R. microplus* ticks, while calreticulin has been

shown to be ubiquitously expressed in all tissues and life-stages of *R. microplus* (Ferreira *et al.*, 2002; Kongsuwan *et al.*, 2010). However, vaccination trials in sheep using a homolog of the calreticulin protein only inferred limited protection against for *Haemaphysalis qinghaiensis* tick challenge (15.6% mortality) (Table 1.2.) (Gao *et al.*, 2008).

2.4.7.3. Structure, signal transduction and defense

Structural transcripts encoding nucleosome assembly protein 1-like 1 (Contig1194), myosin-2 essential light chain (Contig3160), myosin regulatory light chain (Contig3933), Moesin/ezrin/radixin homolog 1 (CK173010), beta-centractin (Contig7803), actin-related protein 3B (Contig1435), dynein light chain type 2 (Contig3260) and actin-related protein Arp2/3 complex (Contig3798) were identified as shared among all tissues tested (Supplementary Table S2.7.). These proteins form part of the cytoskeleton and are involved in a number of cellular processes including cell division and especially intracellular vesicle motility.

A related set of signal transduction transcripts were also identified that are shared between tissues: a stromal membrane-associated protein 1 (Contig426), a myosin-light-chain kinase (Contig615) and a growth hormone-inducible transmembrane protein (Contig2242), as well as a negative regulator of apoptosis Bax inhibitor 1 (Contig3339) (Supplementary Table S2.7.).

2.5. Conclusion- Tick biology and future perspectives

Transcriptional profiling of feeding adult *R. microplus* female tissues identified distinct subsets of genes that aid in blood meal acquisition (salivary glands), digestion (midgut) and reproduction (ovaries) (Table 2.3., Supplementary Table S2.7.). For salivary glands, a large repertoire of anti-hemostatics and immunomodulatory transcripts maintain the fluidity of the blood meal, while additional secreted proteases aid in blood pool formation and pre-digestion of the blood meal prior to ingestion. The midgut in turn is highly specialized to break down the components of the blood meal with wide arrays of proteases involved in digestion, where additional metabolic enzymes and transporters enable the engorging female to acquire the necessary nutrients in preparation for reproduction. Some additional highly up-regulated transcripts are also involved in alleviation of oxidative stress, defense against microbial invasion and host immune responses, as well as maintenance of blood meal fluidity via anti-

coagulants. As the female prepares for egg production, unique genes are highly up-regulated to control cell cycle development, DNA replication, post-transcriptional and post-translational modification. Some transcripts related to metabolism, transport and signaling were also differentially expressed. Fig. 2.13. summarizes the overall findings.

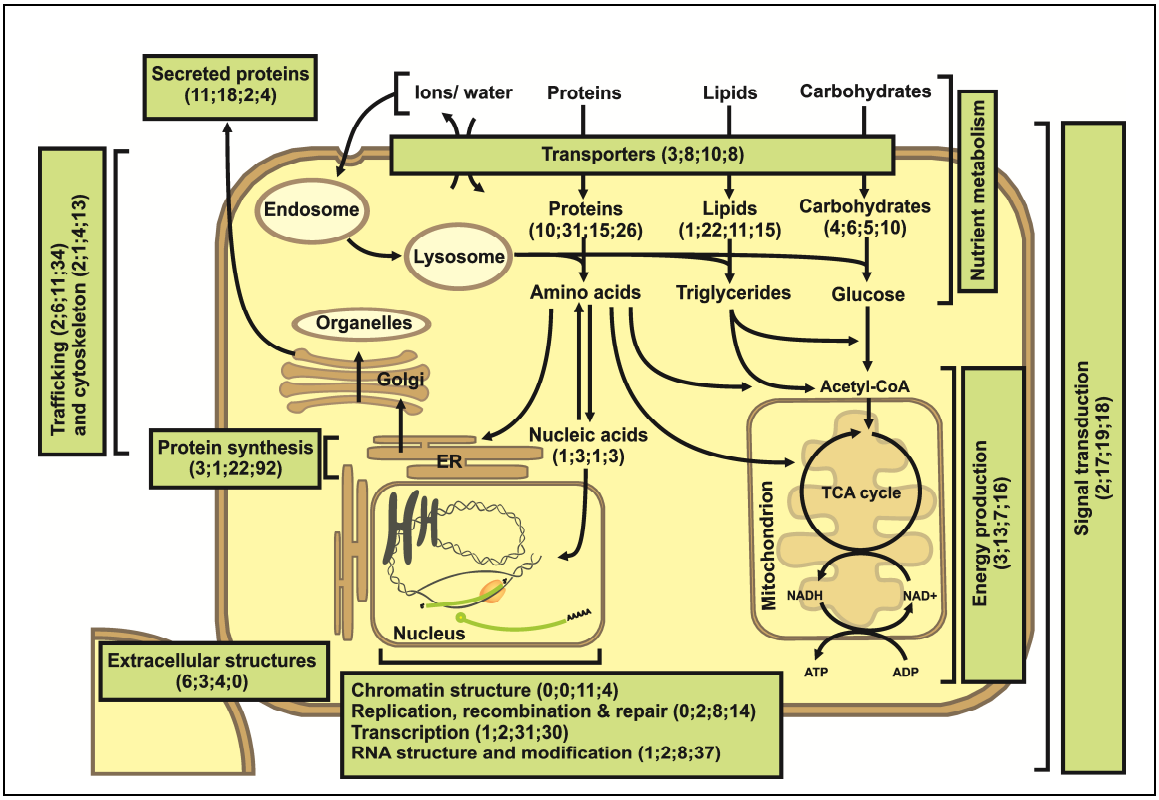

Figure 2.13. Overview of regulated transcripts in the tissues of feeding adult female *R. microplus* ticks. A simplified cell and biological pathways with key processes in blocks are indicated, as well as the relative number of transcripts (in brackets) involved in each process representing salivary glands, midguts, ovaries and shared between all tissues, respectively. The key functional processes represented are: secreted proteins (including anti-coagulants and antimicrobials) that could also include enzymes involved in nutrient acquisition (proteases and lipases); nutrient metabolism that includes both intracellular and extracellular enzymes and transport proteins; enzymes and proteins involved in energy production; enzymes and proteins involved in chromosome structure, replication, transcription, as well as RNA processing and modification; proteins and enzymes involved in protein synthesis, proteins involved in intracellular trafficking and the cytoskeleton, extracellular structures involved in cell-cell contact; receptors, enzymes and accessory proteins involved in signal transduction events throughout the cell. ER, endoplasmic reticulum; TCA, tricarboxylic acid.

Global comparison of transcripts shared between all tissues revealed a broader functional distribution (according to KOG annotation) than any single tissue comparison (Supplementary Table 2.8.). These include components involved in protein, carbohydrate and lipid metabolism necessary for biosynthetic pathways and energy production that indicate fundamental processes that are ubiquitous in all tissues during feeding. Notable are transcripts involved in signal transduction events that control the cell cycle, transcription and translation, as well as pathways involved in proteolysis and lipid metabolism, making them an important component of basic tissue biology and possible targets for future vaccine development (Fig. 2.13.). The data expands therefore our current understanding of tissue-specific and basic tick biology, introducing a large number of previously undescribed proteins and highlighting conserved processes. Furthermore, a hypothesis is formed in regards to lipids as a major source for cellular energy (possibly via β-oxidation) that is supported by previous findings from other comparative studies. Though numerous significantly regulated transcripts were described in this study (1,707 transcripts), it must be noted that due to the stringent selection criteria (M-value > 0, Cy5 intensity > 1,000 and *P* values ≤ 0.001) many transcripts that may only have basal expression relative to the reference pool has been excluded. Also transcripts that are shared between individual tissues (i.e. salivary gland and midgut) were not considered in this study and may be an additional source for future investigation and vaccine target identification (Fig.2.9.).

As is the case with other comparative studies, numerous genes that were expressed in *R. microplus* female tissues during feeding could not be functionally annotated. Further similarity searches against the *Ixodes scapularis* genome database could not confer more informative annotation of unknown genes, highlighting the uniqueness of these transcripts (Hill and Wikel, 2005). Moreover, the vast majority of the predicted genes currently available for *R. microplus* in the BmiGI (v2.1) and the Cattle Tick sequence databases remain un-annotated (Wang *et al.*, 2007; Bellgard *et al.*, 2012). Therefore, a meta-analysis approach that combines all available sequence databases (nucleotide, protein and structural) and extensive manual curation is needed. Such an approach has been successfully employed in the annotation of various tick sialomes (Francischetti *et al.*, 2010). However, a unified nomenclature for sequence annotation is necessary to avoid confusion between similar entries. In this study, final annotation was based on reviewed sequences published in the Uniprot protein and Baunschweig enzyme (BRENDA) databases (Apweiler *et al.*, 2011; Scheer *et al.*, 2011).

A central database containing all available tick sequences (Genome, ESTs, transcriptome, sialome and mialome) would greatly simplify comparative analysis but is still lacking. In this regard, the recently established Cattle Tick Database could become an invaluable resource, as the basis for a systematic attempt at annotating the full complement of genes and proteins of *R. microplus* (Bellgard *et al.*, 2012). Further, application of high-throughput techniques such as deep RNA sequencing (RNA-seq), as well as interactome analysis, will enable verification of open reading frames and aid in functional annotation of transcripts that share little to no sequence identity with other organisms (Brückner *et al.*, 2009; Wang *et al.*, 2009).

In this chapter, a microarray platform was designed and successfully applied for the analysis of gene expression in the cattle tick *R. microplus*. A catalogue of tissue-specific and shared genes were identified in major tissues involved in feeding and reproduction of adult *R. microplus* females using available sequence data and transcriptome analysis. This study presented here is the first global transcriptomic analysis via DNA microarrays of *R. microplus* female tissues fed on South African cattle breeds and represents an additional resource that can be further exploited to study proteins and pathways that may be useful for future tick control.

In the following chapter transcriptional profiling of the immature stages, larvae and nymph, will be discussed and compared to adult tissue data to identify shared transcripts. From these results a reverse vaccinology approach will be followed to identify targets that may be developed further as potential vaccine candidates.

2.6. References

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Chapter 3

Transcriptional profiling of the immature life stages and adult tissues of *R. microplus* and *in silico* screening for novel tick vaccine antigens.

3.1. Abstract

To date, few examples of transcriptome analysis of the larval stages of *R. microplus* are available and from these no data for the nymphal stage is available (Saldivar *et al*., 2008; Rodriguez-Valle *et al*., 2010). The latter does not only limit our understanding of the biology of the immature life stages, but also has a major impact on vaccine development since targeting proteins that are expressed throughout the life cycle of the tick would be preferable targets for anti-tick vaccine design. Therefore in this study, the following aims are addressed. Firstly, the expression profiling of the immature life stages of *R. microplus*, larvae and nymphs, was performed using a previously designed custom oligonucleotide microarray (Chapter 2) to identify unique transcripts that are up-regulated during feeding. Secondly, a comparative study between the transcriptomes of the immature life stages and adult female tissues to identify unique and/ shared transcripts. Global gene expression indicated 85 transcripts as shared between all life stages, along with a number of transcripts that were life stage specific or shared between the life stages tested. Finally, bioinformatic and immune-informatic analysis of the transcriptome data was performed to identify a set of potential antigens for future evaluation as anti-tick vaccines, as well as assessing the applicability of reverse vaccinology in rational anti-tick vaccine discovery.

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3.2. Introduction

3.2.1. Candidate discovery for ant-tick vaccine development

The development of a universal vaccine that can target different life stages and species of ticks is a highly desirable feature. Such a vaccine would rely on highly conserved tick proteins with limited antigenic variation that can elicit a sustained immune response with cross-reactivity between different tick species (Parizi *et al*., 2012). However, this may be an unattainable goal at present for several reasons. Firstly, genetic variability between strains/populations of ticks in different geographic areas negatively influences vaccine efficacy as seen for Bm86 in *R. microplus* infestations (de la Fuente *et al*., 2000). Secondly, the target requirements to elicit a sufficient protective response with memory in host animals are unclear. Thirdly, the current repertoire of vaccine candidates that has been tested in animal trials is limited (Table 1.2, Chapter 1). The extent to which the first constraints will influence a vaccine's efficacy in the field will only be apparent following trials in host animals. The invaluable information garnered from such trials will also further expand our knowledge in regards to the necessary requirements for an antigen to confer protection. To date, proteins involved in adhesion, invasion, secretion, signaling and evasion of host responses, as well as lipoproteins are regarded as key players in the host-pathogen/host-parasite interface and therefore good vaccine candidates (Vivona *et al*., 2008). Therefore, the expansion of the current candidate repertoire to include such candidates could be of great importance to overcome current limitations.

3.2.1.1. Antigen discovery: Conventional approaches

Classical vaccines are subdivided into living or non-living preparations. Live vaccines consist of an attenuated form of a pathogen that induces immunity by mimicking a natural infection. In contrast, a non-living vaccine uses a chemically inactivated whole pathogen or utilizes isolated components of a pathogen (or parasite) as a subunit vaccine, to infer protective immunity (Flower, 2008). These traditional methods for vaccine development involve a range of immunological- (i.e. isolated serum), biochemical- (i.e. biochemical fractionation of pathogenic components) and microbiological (i.e. *in vitro* cultivation of pathogens) methods. These methods have been applied with great success in the development of vaccines against pathogenic viruses, like *Vaccinia virus* (smallpox) and poliovirus (*Picornaviridae*), as well as

bacteria such as *Yersinia pestis* (plague) and *Basillus anthracis* (Anthrax) (Rappuoli and Bagnoli, 2011). Classical biochemical fractionation was used initially to identify the vaccine antigen Bm86 from *R. microplus* midgut tissues, as well as other protective antigens (Willadsen and McKenna, 1991). However, classical approaches may fail for pathogens/parasites with variable surface antigens (i.e. *Plasmodium* spp.) or lack of identifiable immuno-dominant protective antigens, as well as for those that cannot be easily cultured *in vitro* or isolated from source material (Serruto and Rappuoli, 2006; Rappuoli and Bagnoli, 2011).

3.2.1.2. Antigen discovery: DNA technologies and in silico approaches

The advent of recombinant DNA technologies has afforded new possibilities in the field of vaccinology for identification, production and isolation of vaccine antigens. Recombinant techniques have several advantages over classical methods, such as production of large quantities of pure antigen, production of improved antigens with better presentation and immunogenicity, as well as increased safety since large volumes of pathogenic material for study and vaccine production is no longer required (Rappuoli and Bagnoli, 2011).

In this regard, recombinant techniques were used to produce the first pure subunit vaccine derived from a surface antigen (HBsAg) of the Hepatitis B virus (*Hepadnaviridae*) in *Saccharomyces cerevisiae* (Valenzuela *et al*., 1982). Previously, this antigen could only be isolated from infected patient serum, as it was impossible to culture the pathogen *in vitro*. Since then this subunit vaccine has been produced commercially along with other recombinant vaccines directed at human papillomavirus (HPV) (*Papillomaviridae*), porcine circovirus type 2 (PCV2) (*Circoviridae*) and Classical Swine Fever (CSF) (*Porcilis pesti*) (Ohlschlager *et al*., 2009; Rappuoli and Bagnoli, 2011; Cox, 2012).

Another advance that has influenced vaccine candidate discovery is the sequencing and assembly of pathogen/parasite (prokaryotes and eukaryotes) genomes. The first genome was completed in 1995 for *Haemophilus influenzae* and the number has grown considerably to well over 10,000 entries that are currently available on GenBank (http://www.ncbi.nlm.nih.gov./genome/) for eukaryotes, prokaryotes and viruses (Fleischmann *et al.*, 1995). Currently, nine genomes are available for invertebrate vector species that include ticks (i.e. *I. scapularis*), lice (i.e. *Pediculus humanus*), mosquitoes (i.e. *Anopheles*

gambiae, *Aedes aegypti* and *Culex quinquefasciatus*), flies (*Glossina morsitans*) and other biting insects (i.e. *Rhodnius prolixus*) (Megy *et al*., 2012). In cases where a genome is lacking, transcriptome analyses via DNA microarrays or RNA-Seq allows for the identification of expression patterns throughout the life cycle of the pathogen/parasite and in combination with proteomics, the associated protein expression levels (Brückner et al., 2009; Wang et al., 2009).

The availability of these resources has opened new possibilities for large scale vaccine discovery using modern *in silico* methods. The latter has been termed reverse vaccinology, where genomic information is used in vaccine discovery, rather than starting with the organism itself (Rappuoli, 2000).

3.2.2. Reverse vaccinology

The first reverse vaccinology approach was performed in 2000, using the completed genome of the causative agent of bacterial septicemia and meningitis, *Neisseria meningitides* serotype B (Pizza *et al*., 2000). As previous classical and recombinant methods have failed to produce a functional vaccine, this seminal work led to the development of a multivalent recombinant vaccine that has since reached phase III testing in clinical trials (Bambini and Rappuoli, 2009; Rappuoli and Bagnoli, 2011). The latter gave proof of principal for this approach to antigen discovery, especially for other organisms where conventional methodologies have been unsuccessful. Recent examples for other pathogen/parasite species that have employed a reverse vaccinology strategy include *Streptococcus pyogenes*, *Leishmania* spp. and *Herpes simplex virus* (HSV1 and HSV1) (John *et al*., 2012; Sharma *et al.*, 2013; Xiang and He, 2013).

To identify promising anti-tick vaccine candidates in a reverse vaccinology approach, *in silico* analysis is the central step. This usually entails the prediction and localization of encoded proteins within the given parasite genome that is usually followed by analysis of their expression profiles and the subcellular localization of the target proteins within a cell (Flower 2008; Flower et al. 2010). In the case of *R. microplus*, a fully annotated genome is lacking and only an assembled sequence database consisting of ESTs is currently available (Wang *et al*. 2007).

3.2.2.1. *In silico* prediction of proteins

The *in silico* prediction of protein coding frames from genomic or transcriptomic data is an important step for the identification of vaccine candidates and can hamper downstream analyses if incorrect open reading frames are used (Rappuoli and Bagnoli, 2011). As only assembled ESTs are currently available for *R. microplus*, the occurrence of truncated transcripts, as well as incorrect assembly of contiguous sequences (contigs) could have negative downstream effects on predicted data (Nagaraj et al., 2007). In this regard, translation pipelines such as the prot4EST tool are invaluable to predict putative open reading frames for further *in silico* analysis (Wasmuth and Blaxter, 2004). This tool performs similarity searches using BLASTp against the SwissProt/TrEMBL protein database to identify the most likely protein coding sequences from the translated nucleotide sequences (in all 6 reading frames). Following BLAST analysis, protein sequences with high sequence identity to database entries are assembled, while the longest uninterrupted reading frame is used for protein sequences that share limited to no sequence similarity with database entries (Wasmuth and Blaxter, 2004).

As whole antigen prediction is best used in conjunction with other methods such as membrane topology and subcellular localization prediction, both approaches are used in a reverse vaccinology strategy and was consequently used in this study (Vivona *et al.*, 2008). An obstacle faced during prediction of subcellular compartmentalization or membrane-association is the under-estimated complexity of the cell, and the lack of tools for the prediction of transient, permanent or multiple localizations, as well as localization to organelles or multi-protein complexes (Flower *et al.*, 2010). To date, SignalP remains one of the best methods for signal sequence prediction, as it uses both neural networks and hidden Markov models to allow discrimination between uncleaved signal anchors and cleaved signal peptides (Flower, 2008, Petersen *et al*., 2011). The more conventional approach, which is based on the assumption that the subcellular localization of protein homologs is similar, is still being with success (Doderer *et al*., 2006; Salsman *et al*., 2008). Briefly, proteins are localized using global homology searches (such as BLAST) or protein motif/family-based identification (such as PROSITE and Pfam) (Sigrist *et al.*, 2010; Punta *et al.*, 2011). In addition to localization, membrane topology also needs to be evaluated for proteins to ensure that exoplasmic regions are selected for subsequent expression of recombinant proteins. In this regard, TMHMM is a powerful program that makes use of a hidden Markov model, with a prediction accuracy of 97-98% for transmembrane helices and the ability to discriminate

between soluble and membrane proteins with both specificity and sensitivity better than 99% (Sonnhammer et al. 1998; Krogh et al. 2001).

3.2.2.2. *In silico* prediction of tick antigens

As stated previously, components of the innate and adaptive immune system interplay extensively to confer protective immunity and is mediated by antibodies (i.e. IgG), cytokines, B- and/or T-cells (Fig. 1.3, Chapter 1) (Zepp, 2010). Protective immunity against ticks was previously demonstrated and it was shown that Bm86 induces a strong IgG-mediated response (Kemp *et al.*, 1989; Rand *et al.*, 1989; Vargas *et al.*, 2010). Studies in *B. taurus* and *B. indicus* cattle showed a strong adaptive immune response, suggesting that an acquired T-cell response is critical to the development of tick-specific IgG, as well as development of host resistance to infestation (Piper *et al.*, 2009).

As recognition of antigenic epitopes by T-cells, B-cells and soluble antibodies form the basis of the immune response, a number of *in silico* epitope predictors have been designed, and used with great success for bacterial and viral pathogens (Flower, 2008; Vivona *et al.*, 2008; Bagnoli and Rappouli, 2011). However, identification of anti-tick vaccine candidates is limited due to the fact that the major histocompatibility alleles used for epitope screening is limited to human or murine alleles, as very limited data is available for bovine alleles (de Groot *et* al., 2006; Nene *et al*., 2012). Furthermore, tick proteins are most likely a product of divergent or convergent evolution since they lack obvious sequence similarity to most available sequences found in databases. Considering that most algorithms still rely on sequence alignment to identify sequence similarities or motifs characteristic of antigens (Flower *et al.*, 2010), an alignment-free approach such as VaxiJen, may lead to the identification of truly novel tick protective antigens. VaxiJen was developed to allow antigen classification solely based on the physicochemical properties of proteins without recourse to sequence alignment (Doytchinova and Flower 2007a; Doytchinova and Flower 2007b). Although never applied to ectoparasites, VaxiJen has shown impressive prediction accuracy of up to 70-89% for bacterial, viral and tumor antigens and 78-97% accuracy for endoparasitic and fungal antigens (Doytchinova and Flower, 2007b; Flower *et al.*, 2010).

3.2.3. DNA microarrays and immature life stages in ticks

In silico tools alone cannot determine the expression state, as well as the accessibility of a protein to elicit a favorable immune response. For proteins to be accessible, they should be expressed during a reasonable period of the life cycle and secreted or presented on external surfaces of the parasite (Flower, 2008). Although the function a protein performs in the parasite is irrelevant to its status as an antigen, targeting a protein of vital function during vaccination remains sensible. Furthermore, as it is known that membrane fractions from *R. microplus* and not secreted proteins confer protection to cattle, emphasis will be placed on membrane-associated proteins in this study (Opdebeeck *et al.* 1988; Jackson and Opdebeeck 1990; Guerrero *et al*., 2012a). However, selected examples are currently available for soluble (and cytosolic) proteins that have been shown to confer protection in vaccination trials (Table 1.2.). In terms of transcriptome analysis, DNA microarrays have been successfully applied in tick research especially in the case of adult female *R. microplus* (Chapter 2). However, few examples of DNA microarray analysis of the immature life stages of *R. microplus* are currently available. These include investigation of acaricide-induced gene expression in larvae (Saldivar *et al*., 2008), as well as responses of larvae to different cattle breeds (Rodriguez-Valle *et al*., 2010). Only a single microarray analysis has been performed on the salivary glands of feeding *I. scapularis* nymphs (McNally *et al*., 2012). In this study, numerous genes were up-regulated between fed and unfed ticks (587 transcripts), a third of which could not be functionally annotated. The largest subset of annotatable transcripts identified, were involved in metabolism, protein synthesis and protein modification. No similar data is currently available for *R. microplus* and therefore, the transcriptional profiling of the *R. microplus* nymph life stage will be completely novel and invaluable for the understanding of its biology and vaccine design. In summary, DNA microarray analysis has been shown to be a valid high-throughput approach to identify transcripts that are involved in tick feeding. High-throughput analysis of gene expression across all life stages will therefore allow rapid expansion of our current understanding the biology of the immature life stages of *R. microplus*, as well as facilitate the identification shared transcripts among tissues and life stages that will offer novel candidates for further *in silico* (immuno-informatic) analysis.

In this study, the following aims are addressed. Firstly, the expression profiling of the immature life stages of *R. microplus*, larvae and nymphs, was performed using a previously designed custom oligonucleotide microarray (Chapter 2) to identify unique transcripts that are up-regulated during feeding. Secondly, a comparative study will be performed between the

transcriptomes of the immature life stages and adult female tissues to identify unique and/ shared transcripts. Global analyses of gene expression indicated that 85 transcripts were shared between all life stages, along with a number of transcripts that were life stage specific or shared between all the life stages tested. Finally, bioinformatic and immune-informatic analysis of the transcriptome data was performed to identify a set of potential antigens for future evaluation as anti-tick vaccines, as well as assessing the applicability of reverse vaccinology in rational anti-tick vaccine discovery.

3.3. Materials and methods

3.3.1. Tick rearing, sample collection, RNA extraction and microarray analysis

All tick rearing, sample collection, RNA extraction and final microarray analysis were performed as previously outlined in Sections 2.2.1. to 2.2.5. (Chapter 2). Test groups for the current study consisted of immature life stages collected for partially fed larvae (day 4) and nymphs (day 7), including the adult female tissues (salivary gland, midgut and ovary) collected as previously described from partially fed females (day 20) (Chapter 2), from two biological replicates.

3.3.2. Microarray data analysis, functional annotation and bioinformatic analysis

Data extraction and data normalization was previously performed as described in Section 2.2.6. (Chapter 2). The subsequent $\log_2$ fold change values for each group pair-wise comparison were calculated as described previously, using an empirical Bayes ANOVA to identify significantly differentially expressed transcripts with a *P* value adjusted for multiple comparison false discovery rates. Comparisons with *P* values ≥ 0.01 were ignored for this the purposes of this study. To identify transcripts that are life stage specific, only genes with M values > 0, a Cy5 intensity > 1,000 and a $\log_2$ fold expression of one or greater in a single life stage relative to all other life stages and adult tissues examined, were considered. All transcripts with a $\log_2$ fold expression higher than 2 were considered as highly up-regulated under the current chosen conditions. Transcripts with M values > 0 and Cy5 expression intensity > 1,000, in all of the life stages and adult tissues tested, were considered shared (non-differentially expressed) under the current chosen conditions. As before, all transcripts with expression intensities higher than 2,000 were considered strongly shared.

Functional annotation of transcripts was performed as described in Section 2.2.6. (Chapter 2), using the desktop cDNA Annotation System (dCAS) software (v.1.4.3) (Guo *et al.*, 2009). Final functional annotation was based on a consensus between two or more databases (E values ≤ 1×10^{-6}) and classification into eukaryotic orthologous group terms for gene ontology, as described before (Section 2.2.6., Chapter 2).

To enable identification of protein coding sequences that can be used for further bioinformatic and immuno-informatic analysis, prot4EST was used to obtain the best putative reading frame for all assembled contigs (Wasmuth and Blaxter, 2004). Additional alignments were performed as before with the Clustal W program (http//:www.ebi.ac.uk/clustalw/) and amino acid sequences/ alignments were edited with the Genedoc multiple sequence alignment editor and shading utility (http//:www.psc.edu/biomed/genedoc/, ver. 2.6.002). For prediction of membrane spanning transcripts the TMHMM web server (http://www.cbs.dtu.dk/services/TMHMM/, v.2.0) was used and secreted transcripts were identified using the SigP server (http://www.cbs.dtu.dk/services/SignalP-3.0/, v.3.0). Final classification of transcripts was as either non-secreted (including truncated transcripts lacking identifiable signal sequences), membrane-associated and secreted soluble transcripts (that may include GPI-anchored sequences), based on their SigP and TMHMM results. For further identification of potentially GPI-anchored proteins, a GPI anchoring site had to be predicted by at least 2 of the following four programs: PredGPI (http://gpcr.biocomp.unibo.it/predgpi/pred.htm), GPI-SOM (http://gpi.unibe.ch/), MemType2L (http://www.csbio.sjtu.edu.cn/bioinf/MemType/) and BigPI (http://mendel.imp.ac.at/gpi/gpi_server.html).

For immuno-informatic analysis, the web-based VaxiJen tool (http://www.ddg-pharmfac.net/vaxijen/vaxijen/vaxijen.html), applying the parasite model, was used to rank proteins according to their likelihood of being potential antigens. Using the median values of previously calculated normalized Cy5 intensities (Chapter 2) as an indicator for gene expression, a screening of the *R. microplus* transcriptome was performed using an intensity threshold of 1,000 as a minimum requirement for the “presence” or “absence” of a gene within any given life stage, tissue or comparison. For Bm86, the only commercially available anti-tick vaccine, a score of 0.7698 was obtained. Therefore only proteins with a VaxiJen score of above 0.7 were considered as potential antigens. Transcripts identified from transcriptome analysis using a minimum threshold intensity of 1,000 ($M > 0$) were also screened for potential antigens conserved across immature life stages and adult female tissue.

3.4. Results and Discussion

3.4.1. Global gene expression in feeding *R. microplus* immature life stages and adult female tissues

Using the same custom oligonucleotide microarray chip designed in Chapter 2, transcriptional profiling of the immature life stages (larvae and nymphs) of feeding *R. microplus* was performed. For all the following comparisons, transcripts with *P* values ≤ 0.01 were not considered to provide a sufficient level of confidence for the data analyzed and were omitted. The intensity and $\log_2$ expression values applicable to all life stages and adult female tissue comparisons were used to determine transcripts that are life stage specific or shared within life stages.

The global expression analysis identified 4660 transcripts differentially expressed among all adult tissues and immature life stages above threshold, representing ~35% of the total number of contigs on the array. However, it was found that only around 6% (2,102 transcripts) of the full complement of genes tested was significantly regulated in any life stage or comparison (Table 3.1., Figure 3.1.A.).

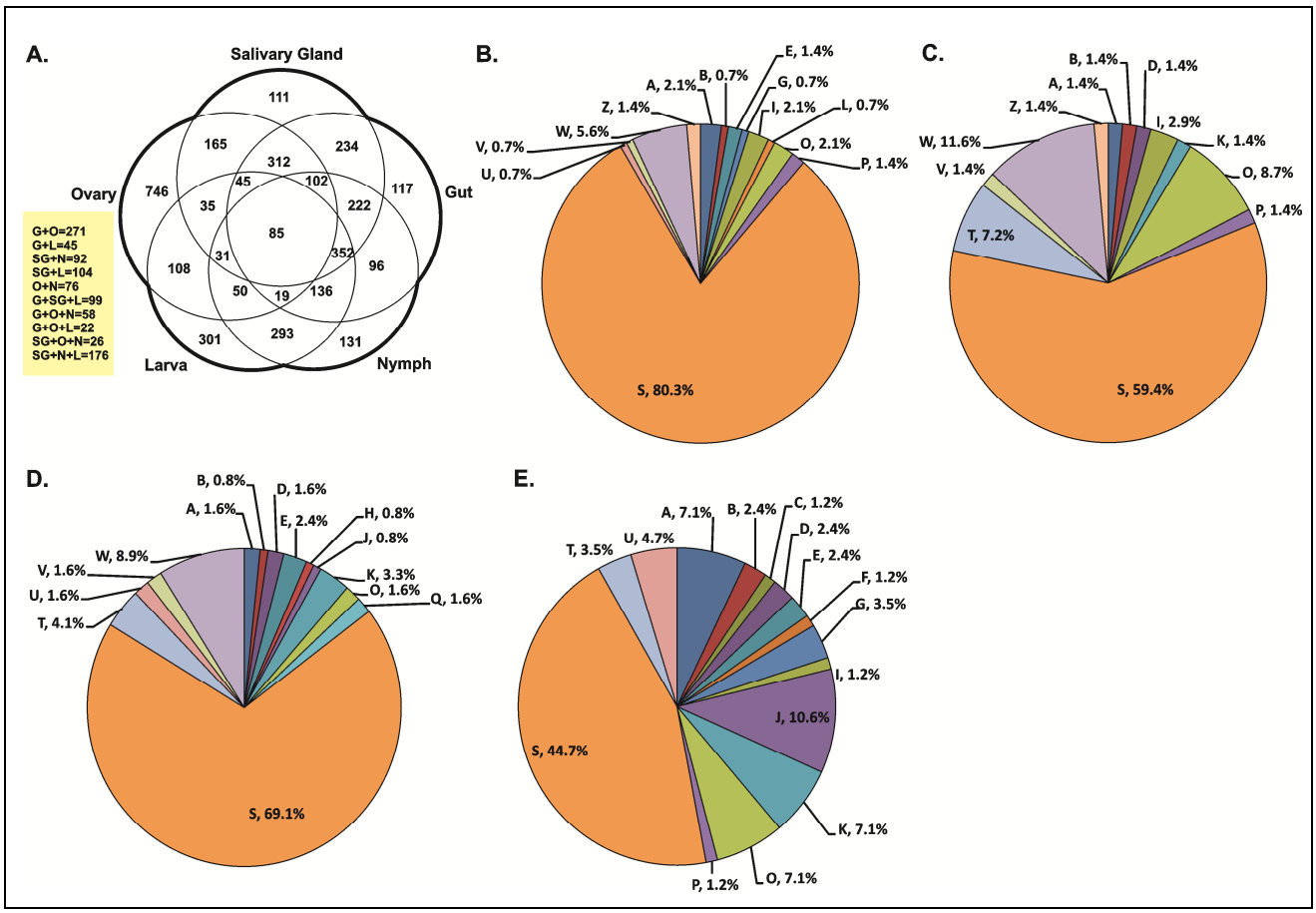

Figure 3.1. Distribution and classification of genes regulated between immature life stages and tissues of adult female *R. microplus* ticks. Indicated are the numbers of unique genes shared between larvae, nymphs, salivary glands, midguts and ovaries with M-values > 0, an intensity threshold above 1,000 and *P* values ≤ 0.01. (A) Venn diagram indicating the number of genes that are unique to or shared between the different life stages and adult tissues (M-values > 0, intensity threshold above 1,000). Regulated transcripts in all life stages and adult tissues are classified according to their eukaryotic orthologous functional groups (KOGs). The percentages of unique transcripts regulated in (B) larvae, (C) nymphs, (D) shared between immature life stages and (E) shared between all life stages and tissues of adult female ticks during feeding are indicated. The functional classifications that are represented include: A- RNA processing and modification; B- Chromatin structure and dynamics; C- Energy production and conversion; D- Cell cycle control, cell division, chromosome partitioning; E- Amino acid transport and metabolism; F- Nucleotide transport and metabolism; G- Carbohydrate transport and metabolism; H- Coenzyme transport and metabolism; I- Lipid transport and metabolism; J- Translation, ribosomal structure and biogenesis; K- Transcription; L- Replication, recombination and repair; M- Cell wall/membrane/envelope biogenesis; N- Cell motility; O- Posttranslational modification, protein turnover, chaperones; P- Inorganic ion transport and metabolism; Q- Secondary metabolites biosynthesis, transport and catabolism; S- Function unknown (also includes transcripts with only general functional predictions); T- Signal transduction mechanisms; U- Intracellular trafficking, secretion, and vesicular transport; V- Defense mechanisms; W- Extracellular structures; Y- Nuclear structure; Z- Cytoskeleton.

Table 3.1. Summary of unique transcripts expressed in immature life stages and adult female tissues of feeding *R. microplus* (*P* ≤ 0.01). Number of transcripts determined using an intensity threshold of a 1,000 and an average absolute two-fold change relative to other tissues.

	Regulated	Fold change [b]/ Intensity	Larvae	Nymph	Larvae and Nymph	All life stages and tissues
Regulation of significant genes [a]	Up	>3	31	11	8	
		>2	51	22	19	
		>1	93	44	62	
	No change	>5,000*				16
		>2,000*				24
		>1,000*				85
Total regulated genes above intensity threshold [c]	4,660		301	131	293	85
Total regulated genes with *P* value ≤ 0.01 [d]	2,102		142	69	123	85*
% Regulated genes [e]	15.6		1.1	0.5	0.9	0.6

[a.] Data corresponds to fold change values using an empirical Bayes method to identify differentially expressed transcripts with a *P* value ≤ 0.001 adjusted for multiple comparisons false discovery rates.

[b.] Fold change corresponding to the $\log_2$ expression ratio of genes with at least a greater than 2 fold absolute expression for any give tissue comparison.

[c.] Total number of genes regulated above an intensity threshold of 1,000 and an M-value (Cy5/Cy3) > 0.

[d.] Total number of genes regulated with a *P* value ≤ 0.01 adjusted for multiple comparisons false discovery rates.

[e.] Percentage of genes regulated from 13,456 total transcripts used on the microarray.

[*.] Number of non-differentially expressed genes for all tissue comparisons based on M-value (Cy5/Cy3) > 0 and a minimum threshold intensity of 1,000.

A set of 85 transcripts was found to be expressed in all three tissues (Supplementary Table 3.6). Of these, 24 transcripts were identified with more than double the intensity threshold for expression. A number of life stage specific transcripts were also identified that were uniquely up-regulated in larvae (142 transcripts), nymphs (69 transcripts) and both immature life stages (123 transcripts) (Fig.3.1.A., Table 3.1., Supplementary Tables S3.3. to S3.5.).

3.4.2. Gene expression during feeding in immature life stages

For newly hatched larvae and molted nymphs a number of physiological and behavioral changes are expected as the tick develops from a juvenile to an adult. Therefore genes involved in tick attachment, feeding and development should be up-regulated for the immature ticks to acquire the metabolites and energy necessary for advancement to the next stage of its life cycle. As discussed previously, the cattle tick is a one-host tick with only two immature developmental stages (larval and nymph) preceding the adult stage (Chapter 1, Section 1.1.3.). To understand the biological processes/ components essential for these developmental stages the complement of genes that are uniquely expressed and shared between the immature stages will need to be evaluated. The following sections will deal with these various classes of transcripts identified in this study.

3.4.2.1. Unique transcripts highly regulated in larvae

In this study, 142 transcripts were identified that were larvae-specific (above threshold and *P* value ≤ 0.01), of which 80.3% could not be functionally annotated or classified into a specific ontology (Fig. 3.1.B., Tables 3.1. and Supplementary Table S3.3. and S3.6.). Using a minimum two-fold absolute expression, relative to other tissues, these tissue-specific transcripts represent 0.7% of the total complement of transcripts analyzed (Table 3.3., Supplementary Table S3.6.). Some of the major functional classes according to KOG that were represented related to extracellular structures (5.6%), lipid transport and metabolism (2.1%), posttranslational modification and protein turnover (2.1%) (Fig.3.1.B.). In general, transcriptional profiling showed a greater number and diversity in functional classes of transcripts identified specific to larvae-specific compared to nymph-specific transcripts.

3.4.2.1.1. Structural proteins

Structurally related transcripts were found to be uniquely expressed in larvae during feeding, representing around 5.6% of the total complement of proteins unique to this life stage (Figure 2.8.B., Supplementary Table S3.6).

Similar to previous transcriptome studies of *R. microplus* larvae, putative glycine-rich cuticle-related (Contig3247, Contig3043, Contig5146, Contig272, Contig2866 and Contig5475), cuticle (Contig1610) and cement (Contig2328) proteins were highly up-regulated (Rodriguez-Valle *et al*., 2010). As stated previously, these transcripts may be involved in structural interactions of the peritrophic matrix or cuticle, in addition to cement cone formation during attachment (Francischetti et al., 2010; Maruyama et al., 2010). Previous immunization of rabbits with a similar glycine-rich protein isolated from *R. haemaphysaloides* only induced a 30.5% mortality rate in nymphs, with no effect on adult stages (Table 1.2.) (Zhou *et al*., 2006). These results are reflected by the current data as this class of proteins are the most abundantly expressed (highly up-regulated) in the immature life stages, therefore necessary for development, as compared to adult tissues (Chapter 2). In contrast, a ~62% mortality in adult females and ~47% mortality in nymphs were observed following vaccination of Guinea pigs with truncated constructs of a cement protein (64TRP) previously isolated from *R. appendiculatus* (Table 1.2) (Trimnell *et al.*, 2002). Similar protection was demonstrated against infestation of *I. ricinus* and *R. sanguineus* following vaccination (Table 1.2) (Trimnell *et al.*, 2005). However, as stated before there are potential difficulties in targeting large protein families of functionally redundant proteins and target “rescue” by other family members may account for the low efficiencies in vaccination trials (Guerrero *et al*., 2012a).

3.4.2.1.2. Metabolism and transport

Three highly up-regulated transcripts involved in lipid transport and metabolism were identified that included: a retinaldehyde-binding protein (Contig4499) and two 3-ketoacyl-CoA synthases (Contig3162 and Contig3581) (Supplementary Table S3.6.). Retinoic acid is an active metabolite in the vitamin A pathway required for growth and development and is esterified to fatty acids to form a retinyl ester that binds to retinol-binding protein-1 (CRBP1) for storage in lipid droplets within the cell (Kumar *et al*., 2012). It is therefore interesting to note the significant up-regulation of 3-ketoacyl-CoA synthases that are involved in long chain fatty acid synthesis. Similar transcripts were also shown to be specifically up-regulated in

ovaries tissues of adult females (Chapter 2, section 2.3.6.3.3.). An additional putative enzyme, glycerate kinase (TC22153) that produces 2-phosphoglycerate from D-glycerate in glucose metabolism, was highly up-regulated in feeding larvae (Supplementary Table S3.6.) (Kehrer *et al*., 2007).

During digestion of the blood meal, large amounts of heme are produced that result in high levels of cytotoxic iron to be released into the tick midgut. As previously discussed, *R. microplus* also lacks the biosynthetic pathway for heme production and therefore acquires it from the blood meal (Braz *et al.*, 1999). In this regard, two novel ferric-chelate reductase homologs (Contig2382 and Contig1755) were also identified that reduces endosomal ferric iron (Fe^{3+}) to its ferrous (Fe^{2+}) form prior to transport into the cytosol. A similar protein was shown to be important iron metabolism of the intracellular parasite *Leishmania amazonensis* (Flannery *et al*., 2011). In ticks this released iron is scavenged by iron binding ferritin proteins that transport them throughout the cell, as well as to other tissues (Hajdusek *et al*., 2009). The latter proteins were shown to be highly up regulated in female midgut tissues (Chapter 2). Together all these transcripts appear to form a unique set of proteins essential for nutrient acquisition in larvae.

3.4.2.2. Unique transcripts highly regulated in nymphs

Transcriptional analysis identified 69 unique transcripts expressed in *R. microplus* nymphs (Tables 3.1., Supplementary Tables S3.2. and S3.6.). Using a minimum two-fold absolute expression, relative to other tissues, nymph-specific transcripts represent ~0.3% of the total complement of transcripts analyzed (Table 3.3., Supplementary Table S3.6.). The major KOG functional classes that were identified related to extracellular structures (11.6%), posttranslational modification and protein turnover (8.7%), as well as signal transduction mechanisms (7.2%) (Fig.3.1.B.). A total of 14 annotatable transcripts were highly up-regulated (log_2 expression ≥ 2), while 59.4% of the transcripts could not be annotated (Tables 3.1., Supplementary Tables S3.2. and S3.6., Fig.3.1.B.).

3.4.2.2.1. Structural proteins, protein synthesis and control

Similar to larvae, a distinct subset of putative glycine-rich cuticle-related transcripts (CK188336, Contig7988, Contig309 and Contig3760), along with a mucin (CK178060) and a cell adhesion fasciclin protein (Contig2455) were identified that are unique to nymphs (Tables

3.1., Supplementary Tables S3.2. and S3.6.). Together these transcripts represent the largest subset of nymph-specific transcripts (Supplementary Tables S3.2. and S3.5., Fig.3.1.B.). Glycine-rich and mucin proteins are a common features in most tick species and have previously been shown in this study to be up-regulated in all adult female tissues (Chapter 2) (Mans *et al*., 2008). Other highly up-regulated structural proteins included: a putative myosin regulatory light polypeptide (Contig1654) implicated in cytokinesis, receptor capping, and cell locomotion, as well as a putative histone (CK182426) involved in transcription regulation (Komatsu *et al*., 2000; Angelov *et al*., 2003). Microarray analysis of the salivary glands of *I. scapularis* nymphs demonstrated comparable trends in expression of similar structurally related proteins during feeding, further illustrating the importance of these transcripts to the immature life stages (McNally *et al*., 2012).

Two unique transcripts encoding putative proteins involved in protein folding were up-regulated in nymphs that included: a subunit of a molecular chaperone (AA257938.1), as well as a protein disulfide-isomerase (Contig1289) involved in rearrangement of disulfide bonds (Brackley and Grantham, 2008). Similar transcripts were also shown to be up-regulated in *I. scapularis* nymphs during feeding (McNally *et al*., 2012). An additional unique transcript encoding a putative non-specific serine/threonine protein kinase SMG1 (CV452362) was identified that is involved in mRNA surveillance for degradation of erroneous mRNA species following transcription (Denning *et al*., 2001). The latter has not been previously described for *R. microplus*.

3.4.2.2.2. Metabolism, transport and signal transduction

Transcripts related to lipid transport and metabolism, as well as signal transduction events were highly up-regulated during feeding of *R. microplus* nymphs (Supplementary Tables S3.2. and S3.6.). A unique lipid carrier protein with the highest $\log_2$ fold expression of nymph-specific transcripts (5.48), hemelipoglycoprotein (Contig2405), was identified that shares sequence identity to a homolog from *D. variabilis*. This glycoprotein was previously identified in hemolymph extracts of *D. variabilis* immature (larval and nymph), as well as adults (male and female) stages and is involved in the storage and transport of various lipids, as well as heme (Guddera *et al*., 2001). Furthermore, it was shown to be up-regulated in response to feeding in both larvae and adult *R. microplus* ticks using DNA microarray analysis (Rodriguez-Valle *et al*., 2010). Similar proteins have since been identified in both hard and soft ticks and

have been suggested to be conserved for Ixodida (Donohue *et al*., 2008). Two related transcripts encoding a 3-ketoacyl-CoA synthases (CV445795) and a ferric-chelate reductase 1 (Contig3680), similar to that identified for larvae, was also uniquely up-regulated in nymphs (Supplementary Tables S3.2. and S3.6.). The latter illustrates that distinct transcript sets are expressed for each stage that performs similar processes shared between the immature stages and other adult female tissues (i.e. 3-ketoacyl-CoA synthases).

A putative acetyl cholinesterase (Contig7464) was highly up-regulated (log_2 expression of 5.14) in feeding nymphs (Supplementary Tables S3.2.). This protein type was identified from adult tissues (Chapter 2) and has been characterized for *R. microplus* and implicated in acquiring acaricide resistance (Baxter and Barker, 1998; Guerrero et al., 2012b).

3.4.2.3. Tissue-specific expression of transcripts during feeding of immature life stages

Identification of transcripts distinctly shared between an immature life stage and an adult tissue, will likely identify proteins that are related to specific tissue function for that life stage. Surprisingly no such significant shared transcripts were identified between larvae and a particular adult female tissue, but may be due to the stringent selection criteria chosen for data analysis. In contrast, a number of transcripts were identified that were previously seen as tissue-specific following analysis of tissues in feeding females (Chapter 2). The transcripts highly up-regulated in adult midgut and salivary glands (log_2 expression ≥ 2) that are shared with the nymphs will be discussed in the following sections (Supplementary Table S3.3.).

3.4.2.3.1. Nymph and adult female salivary glands

A set of 7 transcripts were identified that are salivary gland-specific and shared between nymphs and adult females (Supplementary Tables S2.3. and S3.3.B.). These include putative secreted defense proteins, such as serine protease inhibitors (Contig6586, Contig688 and CK178656) and histamine binding proteins (Contig2493 and Contig2592). As described before for adults, serpins are involved in anti-coagulation and immunosuppression, while tick histamine binding proteins are involved in sequestration of effector molecules from host defenses (i.e. histamine, serotonin, complement and immunoglobulins) during feeding (Francischetti *et al.*, 2010). Similar transcripts were also found to be differentially expressed in

the salivary glands of feeding *I. scapularis* nymphs (McNally *et al*., 2012). Serpins have been explored as a source for novel tick vaccines, but only limited efficacy was achieved in immunization experiments (Table 1.3.) (Sugino *et al.*, 2003; Imamura *et al.*, 2005; Prevot *et al.*, 2007).

Three additional salivary gland-specific transcripts were identified that were shared between nymphs and adults (Supplementary Tables S2.3. and S3.3.B.) and include: a putative chitinase (Contig3556) functioning in antifungal or housekeeping of the cuticular structure (You and Fujisaki, 2009; Francischetti *et al.*, 2010), as well as a putative phospholipase A2 (CV454720) that may play a role (along with a previously identified aquaporin in adults) in salivation or in pre-digestion of ingested blood cells (Zhu *et al.,* 1997; Zhu *et al*., 1998; Bowman and Sauer, 2004).

3.4.2.3.2. Nymph and adult female midgut

Some 13 midgut-specific transcripts were identified that are shared between nymphs and adult females (Supplementary Tables S2.4 and S3.3.A). Of these, several putative proteases involved in protein and amino acid metabolism were identified that included: chymotrypsin-like serine proteases (Contig598 and Contig3850), a cathepsin peptidase (Contig6100) and a serine-type endopeptidase (Contig5340). These proteases play a major role as digestive enzymes in the acquisition of a blood meal and have been identified in several tick species, including *R. microplus* (Anderson *et al.*, 2008; Sojka *et al.*, 2008; Horn *et al.*, 2009; Kongsuwan *et al.*, 2010). Two other putative enzymes, a phosphoethanolamine N-methyltransferase (Contig2302) and a gamma-interferon-inducible lysosomal thiol reductase (Contig861), were also identified that are involved in phospholipid metabolism and lysosomal protein degradation reactions, respectively.

Two putative lipid binding and carrier proteins that are unique to both the midgut of nymphs and adult females were identified that included: a apolipophorin (Contig156) involved in the transport of lipids and heme in the hemolymph (Duncan *et al.*, 1999; Weers *et al.*, 2006), as well as a Niemann-Pick C2-like protein (Contig1508) proposed to function in intracellular lipid cycling and innate immunity (Horácková *et al.*, 2010). An additional putative calsequestrin (Contig728) involved in calcium binding and transport was also shared between adult midguts and nymphs (Supplementary Tables S2.4. and S3.3.A.).

Since digested blood is a source of oxidative stresses, many enzymes have been identified from comparative studies in *R. microplus* that are involved in detoxification and management of oxidative stresses (Citelli *et al.*, 2007; Rodriguez-Valle *et al.*, 2010). In this regard, two glutathione S-transferases (Contig706 and Contig8822), previously identified as midgut-specific, were shown to be now shared between nymph and female midgut tissues (Supplementary Tables S2.4. and S3.3.A.). These enzymes have recently been shown to be significantly up-regulated in feeding *I. scapularis* nymphs following transcriptional profiling (McNally *et al*., 2012). Furthermore, GSTs have also been shown to play a vital role in acquired acaricide resistance in *R. microplus* larvae (Saldivar *et al.*, 2008). However, these proteins offer limited protection against *R. microplus* infestation in vaccination trials, in spite of it being present in both adult and immature life stages (Table 1.2.).

3.4.2.4. Transcripts unique to individual immature life stages and shared with all adult female tissues

Transcripts were identified that were previously shown to be expressed in all adult tissues (Chapter 2). However, a limited number of these transcripts have shown distinct expression in only a single immature live stage with two-fold or higher expression (Supplementary Table S3.4.). In this regard, two putative membrane proteins that were shared between larvae and all adult female tissues were up-regulated and included: a novel highly up-regulated subunit of the sodium/potassium-transporting ATPase complex that is involved in cation (K^+ and Na^+) exchange across the plasma membrane (Contig3592), as well as a mannose-specific lectin ERGIC-53 (Contig101) that forms part of the cargo receptor complex involved in the sorting or recycling of proteins and lipids between the endoplasmic reticulum and the Golgi (Sun *et al*., 1998; Nufer *et al*., 2003). Membrane proteins such as these transcripts could be considered for evaluation as novel vaccine targets as they are present in at least one immature stage and shared among all adult tissues.

In contrast, no highly up-regulated transcripts were obtained for shared tissue transcripts in nymphs, though 3 transcripts encoding two enzymes (Contig8440 and TC16293) and a molecular chaperone (Contig2634) were identified as up-regulated with $\log_2$ fold expression values around 1 (Supplementary Table S3.4).

3.4.2.4.1. Transcripts shared between immature life stages

Some 123 transcripts were identified as specific to the immature life stages (above threshold and *P* value ≤ 0.01), of which 69.1% could not be functionally annotated or classified into a specific ontology (Fig. 3.1.C., Tables 3.1. and Supplementary Tables S3.4. and S3.6.). Considering transcripts regulated above an absolute fold expression of 2, only 0.45% of the total complement of transcripts analyzed on the microarray were represented (Table 3.3., Supplementary Table S3.6.). The major functional classes identified according to KOG classification related to extracellular structures (8.9%), signal transduction mechanisms (4.1%), transcription (3.3%) and amino acid transport and metabolism (2.4%) (Fig.3.1.C., Supplementary Table S3.6.).

3.4.2.4.2. Structural proteins

As expected for the immature life stages, several structurally related transcripts (10 transcripts) were highly up-regulated as they develop to adults, representing the major up-regulated functional class of this comparison (Fig. 3.1.C, Supplementary Tables S3.4. and S3.6.). Putative proteins related to the cuticle (CK192286, Contig5775, Contig5078, Contig8769 and Contig7012) and mucins (CK178059, Contig1434 and CK191373) were identified, as well as a novel sarcoglycan (CK1800069) component of the dystrophin-glycoprotein complex that connects the cytoskeleton of muscle fibers to the extracellular matrix (Greener *et al*., 2000; Chen *et al*., 2006). Another putative structural protein, skeletor (Contig375), was also highly up-regulated and is involved in spindle formation during cell division (Walker *et al*., 2000). Excluding mucin and cuticle-related proteins, no similar transcripts were identified in other comparative studies of the immature or adult life stages of other ticks.

3.4.2.4.3. Other shared proteins

Highly up-regulated transcripts related to protein degradation, synthesis and co-factor biosynthesis were identified as shared between immature life stages and included: a secreted carboxypeptidase A2 (Contig8918), a 60S ribosomal proteinL18 (Contig8918) and a decaprenyl-diphosphate synthase subunit (Contig5698) (Supplementary Table S3.4 and S3.6). For antimicrobial defense a highly up-regulated microplusin-like protein (CV445617) was identified that is shared between immature life stages (Supplementary Table S3.4 and

S3.6). Similar transcripts were found to be up-regulated during feeding in adult female salivary gland and midgut tissues (Chapter 2). From the data it is clear that distinct sets of antimicrobial defense proteins are up-regulated in adult tissues and the immature life stages of *R. microplus* (Fig. 3.2.). Transcripts related to protein degradation, synthesis and defense have been shown to be up-regulated in other comparative studies for *R. microplus* larvae and *I. scapularis* nymphs (Rodriguez-Valle *et al*., 2010; McNally *et al*., 2012).

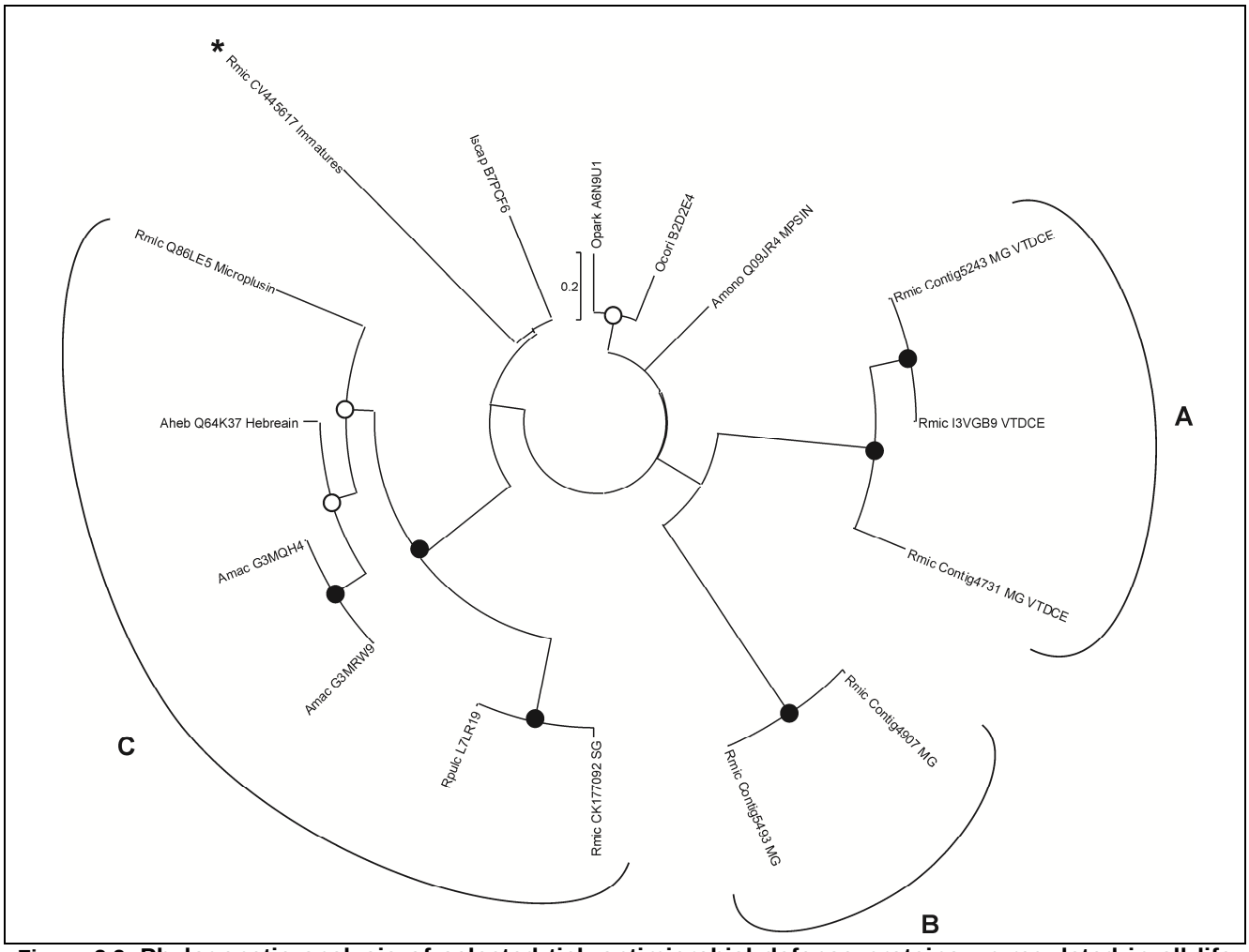

Figure 3.2. Phylogenetic analysis of selected tick antimicrobial defense proteins up-regulated in all life stages during feeding. Indicated the unrooted neighbor-joining radiated phylogram with clades representing midgut-specific (MG) vitellin-degrading cysteine endopeptidase-like (A) and antimicrobial (B) transcripts, as well as other microplusin/hebreain-like transcripts vertebrate homologues (C) that include salivary gland-specific (SG) (CK177092) and immature life stage-specific (CV445617, asterisk) transcripts. All homologues utilized, as well as accession numbers, descriptions and sequences are included in Supplementary Material S1. Confidence levels for 1,000 bootstraps are indicated by black (above 90%) and open dots (above 75%). The 0.2 scale bar indicates 20% amino acid divergence distance.

3.4.2.5. Gene expression shared between immature life stages and selected adult tissues during feeding

Of the 2,102 transcripts that were found to be up-regulated (above threshold and *P* value ≤ 0.01), around 1,768 transcripts were shared between one or both immature life stages and selected adult female tissues (Tables 3.1). Together these transcripts represent 13.1% of the total complement of transcripts analyzed in microarray analysis (Table 3.3). The bulk of these transcripts are shared between the immature life stages and selected adult tissues, representing functions that may be either tissue-specific or ubiquitous across life stages and tissues for *R. microplus*. In regards to the former, if a transcript is shared between a particular adult tissue and the immature life stages, it may imply that that target transcript is expressed in the equivalent tissue of larvae and nymphs. Results from these comparisons may provide targets expressed in all life stages that may be effective for further vaccine development. These comparisons will be discussed in the following sections.

3.4.2.5.1. Shared transcripts between larvae, nymphs and adult salivary gland tissues

Ten transcripts were identified that are uniquely up-regulated in the salivary glands of immature and adult stages of *R. microplus* (Supplementary Table S3.4.C.). Seven transcripts were highly up-regulated (log_2 expression ≥ 2) and 6 of these transcripts have been previously identified as tissue-specific in adult females (Chapter 2, Supplementary Table S2.3.). Of these transcripts, two putative proteases were identified that were highly up-regulated in the salivary glands and included: a secreted cathepsin from the peptidase C1 family (Contig6614) and a serine proteinase of the peptidase S1 family (Contig486). The cysteine protease has previously been implicated in blood meal acquisition as a possible pre-oral digestive enzyme, while the latter serine proteinase was suggested to also play a role in antimicrobial defense due to its high sequence identity to a factor D-like transcript identified from *Dermacentor variabilis* (Chapter 2). An additional putative enzyme from the endoplasmic reticulum, a flavin-containing monooxygenase (FMO) (EW679909.1), was also up-regulated (Supplementary Table S3.4.D.). These enzymes are known to function in catabolism of foreign chemicals, including drugs and pesticides (Hodgson *et al*., 1995; Phillips and Shephard, 2008)

Protease inhibitors related to thyropins (Contig300) that inhibit cysteine proteases and act as binding partners for heparin, as well as alpha-2-macroglobulins (Contig2131 and Contig1745) involved in clearance of exogenous proteases and innate immunity were highly up-regulated (Anatriello *et al.*, 2010; Francischetti *et al.*, 2010). An additional signal transduction similar to mitogen-activated protein kinase kinase kinase 1 (MEKK1) (Contig4877) was also up-regulated and similar transcripts have been identified from other tick sialomic data, however not for *R. microplus* (Francischetti *et al.*, 2010). This transcript is involved in signal transduction events related to regulation of the cytoskeleton, cell migration and focal adhesion (Uhlik *et al.*, 2004).

3.4.2.5.2. Shared transcripts for midgut tissues

Around 8 highly up-regulated transcripts were identified that might be midgut-specific for *R. microplus* ticks as they occur in all the life stages tested (Supplementary Table S3.4.B.). Most of these transcripts have been identified as tissue-specific for feeding *R. microplus* females (Chapter 2). In this regard, four highly up-regulated putative metabolic enzymes were identified that include: an acid phosphatase (Contig392), a gluconolactonase (Contig7035) involved in modulation of Ca^{2+}-dependent enzymes, signaling and cellular processes (Marques *et al*., 2013), as well as two highly up-regulated proteases (Contig953 and CV437104). A previously described carrier protein, vitellogenin (Contig8127), was one of the highest up-regulated midgut-specific transcripts (log_2 expression of 5.03) identified for this comparison and has been stated to play a vital role in heme sequestration (Thompson *et al.*, 2007; Boldbaatar *et al.*, 2010). A similar transcript was found to be up-regulated from gene expression microarrays of *R. microplus* larvae and components of this protein has been tested in vaccination trials with reasonable efficacy (~68%) (Table 1.2.) (Tellam *et al.*,2002; Rodriguez-Valle *et al.*, 2010). The latter supports the rationale for targeting proteins that are needed in more than one life stage that perform vital functions, as vitellin is a major storage and transport protein of lipids and heme in ticks (Logullo *et al*., 2002; Donohue *et al.*, 2008).

Two additional defense transcripts, a serpin (Contig1086) and a Kunitz-type serine proteinase inhibitor (CK192837), were highly up-regulated in midguts and are involved in anti-coagulation. Similar inhibitors have been identified from mialomic data of adult ticks, as well as transcriptomic data of immature life stages (larvae and nymphs) (Anderson et al., 2008, Kongsuwan *et al*., 2010; McNally *et al*., 2012). A unique glycoprotein, nidogen (Contig4613),

was also up-regulated that may play a structural role in the cell-extracellular matrix (Kruegel and Miosge, 2010). An extracellular matrix protein related to collagen (P29) was tested in vaccination trials in rabbits, but gave limited efficacy (40-56%) in protection against *H. longicornis* infestations (Table 1.2) (Mulenga *et al*., 1999). However, matrix proteins do fulfill the vaccine antigen criteria of cell surface exposure and therefore candidates should be evaluated on a case by case basis.

3.4.2.5.3. Shared transcripts between larvae, nymphs, salivary glands and midgut tissues

Twenty-four transcripts were identified that are shared between the immature life stages and the salivary gland and midgut tissues of adult female ticks (Supplementary Table S3.4.D.). Of these only three transcripts were highly up-regulated (log_2 expression ≥ 2) and encoded: a putative actin regulatory protein villin (Contig4355) that is involved in multiple epithelial cell functions (i.e. cell morphology, motility and apoptosis) (Khurana and George, 2008), a subunit of the gamma-aminobutyric acid receptor (Contig1328) a plasma membrane ligand-gated ion channel involved in neurotransmission (Buckingham *et al*., 2005), as well as a putative carboxylesterase (Contig191). A related esterase enzyme was found to be up-regulated in previous transcriptional analysis of *R. microplus* larvae and these enzymes have been implicated in a variety of processes including digestion, reproduction, metabolism and resistance to pesticides (Rodriguez-Valle *et al.*, 2010; Sun *et al*., 2011; Guerrero *et al*., 2012b). The shared receptor and ion channel proteins may be good examples of novel targets that might be investigated further for the development of new generation acaricides.

The remaining up-regulated transcripts (log_2 expression ≥ 1) have putative functions in cell structure, nutrient metabolism, protein synthesis and signaling (Supplementary Table S3.4.D.).

3.4.2.5.4. Shared transcripts between larvae, nymphs, salivary glands and adult ovaries

A small group of transcripts where up-regulated that are shared between the immature life stages, as well as salivary gland and ovary tissues in adult females (Supplementary Table S3.4.G.). Due to the more stringent criteria applied to the study of feeding adult female tissues (Chapter 2), these transcripts have not been discussed previously. Among these

transcripts only one putative protein, an innexin (Contig862), was highly up-regulated with a $\log_2$ expression of 2.79 (Supplementary Table S3.4.G.). This protein is a multi-pass membrane protein of the pannexin protein family that forms gap junctions between epithelial cells for intercellular transport and communication (Norman and Maricq, 2007; Tepass, 2012). A similar protein was previously identified from the sialome data of *I. scapularis* and has since been identified from sialomic data in other tick species, including *R. microplus* ESTs (Valenzuela *et al*., 2002, Francischetti *et al*., 2010). Another cell junction related single-pass membrane protein, neurexin-4 (Contig7873), was also found to be up-regulated for this comparison (Supplementary Table S3.4.G.) (Littleton *et al*., 2011; Tepass, 2012). However, as these proteins occur between cells and therefore are not exposed to interact with host immune responses, their efficacy as targets for vaccine development will have to be demonstrated.

3.4.2.5.5. Gene expression of shared transcripts between all life stages and female adult tissues during feeding

One of the main objectives of this study was to identify proteins and processes that are shared between all the life stages of feeding *R. microplus* ticks as they likely are conserved and therefore important for basic tick biology. By extension these proteins and processes may also present excellent targets for rational vaccine design. Consequent transcriptional analysis showed that 85 transcripts were ubiquitously expressed among all of the life stages and tissues tested (Figure 3.1.A., Table 3.1., Supplementary Table S3.5.). Furthermore, all of these transcripts have been previously shown to be expressed in all adult female tissues (Chapter 2). Considering transcripts with a minimum intensity threshold of 1,000 (M-value > 0), only 0.6% of the total transcripts tested in microarray analysis showed transcriptional regulation (Table 3.1.). Of these transcripts, 44.7% could not be functionally annotated (Fig. 3.1.E.). The major functional classes that were identified related to translation, ribosomal structure and biogenesis (10.6%), protein modification and turnover (7.1%), amino acid transport and metabolism (7.1%), transcription (7.1%) and intracellular trafficking and transport (4.7%) (Fig. 2.9.E.). However, only 13 annotatable transcripts with a minimum two-fold threshold intensities (Cy5 > 2,000) were identified (Tables 3.1., Supplementary Tables S3.5. and 3.6.).

In this regard, a shared transcript encoding a putative cytochrome c oxidase subunit (Contig2837) was identified that is a component of the mitochondrial respiratory chain involved in energy metabolism (Supplementary Tables S3.5.). This protein has been identified from the mialomes and sialomes of both hard and soft tick species (Anderson *et al.*, 2008; Francischetti *et al.*, 2010; Kongsuwan *et al.*, 2010). Furthermore, this protein and other components of the respiratory chain have been shown to be up-regulated from previous transcriptome analyses in immature life stages of *R. microplus* (larvae) and *I. scapularis* (nymphs) (Rodriguez-Valle *et al*., 2010; McNally *et al*., 2012).

Ribosomes form a core component in protein synthesis and it is not surprising that a number of shared ribosome associated proteins were identified that included components of the 40S (Contig7181 and EW680164.1) and 60S (CK177858, Contig1107B, Contig3228, Contig9000, EW680050.1) ribosomal subunits (Supplementary Table S3.5.). An additional transcript encoding a subunit of the H/ACA small nucleolar ribonucloeprotein complex (Contig5596) involved in pseudouridylation of rRNA was identified as shared among all life stages (Watkins and Bohnsack, 2012). These proteins have been previously identified from the sialome and midgut proteome data of *R. microplus* (Francischetti *et al.*, 2010; Kongsuwan *et al.*, 2010). Furthermore, similar components were found to be up-regulated during feeding of *I. scapularis* nymphs (McNally *et al*., 2012). A related putative signal recognition particle (SRP14) protein (Contig4224) was also identified that is involved in translocation of newly synthesized polypeptides to the endoplasmic reticulum for intracellular trafficking (Supplementary Table S3.5.) (Preissler and Deuerling, 2012). In the case of secreted and membrane proteins, many post-translational modifications occur following translocation of the polypeptide chain into the endoplasmic reticulum lumen and sorting via the Golgi network. Around 50% of all known proteins are glycosylated and in this regard a putative glycoprotein 6-alpha-L-fucosyltransferase (Contig1596) was identified as a shared transcript that is involved in the addition of fucose to the peptide chains of glycoproteins (Takahashi *et al*., 2009).

Degradation of unneeded or damaged proteins is an essential component of cell maintenance and 2 shared transcripts encoding components of the ubiquitin/proteasome pathway were identified that encoded two proteins involved in ubiquitination of target proteins for degradation, a RING-box protein 1A (Contig5214) and a ubiquitin-protein ligase (CV446781) (Supplementary Table S3.5.).

3.4.3. Conclusions for the biology of the immature life stages

Following transcriptional profiling of the immature life stages, comparative studies between the immature and adult life stages resulted in the identification of distinct subsets of transcripts preferentially up-regulated between juvenile and adult ticks. Some of these transcripts were also regulated at very high levels (log_2 expression ≥ 2) and therefore represent a core of components/processes that are likely essential for basic tick cell biology and survival (Fig.3.3).

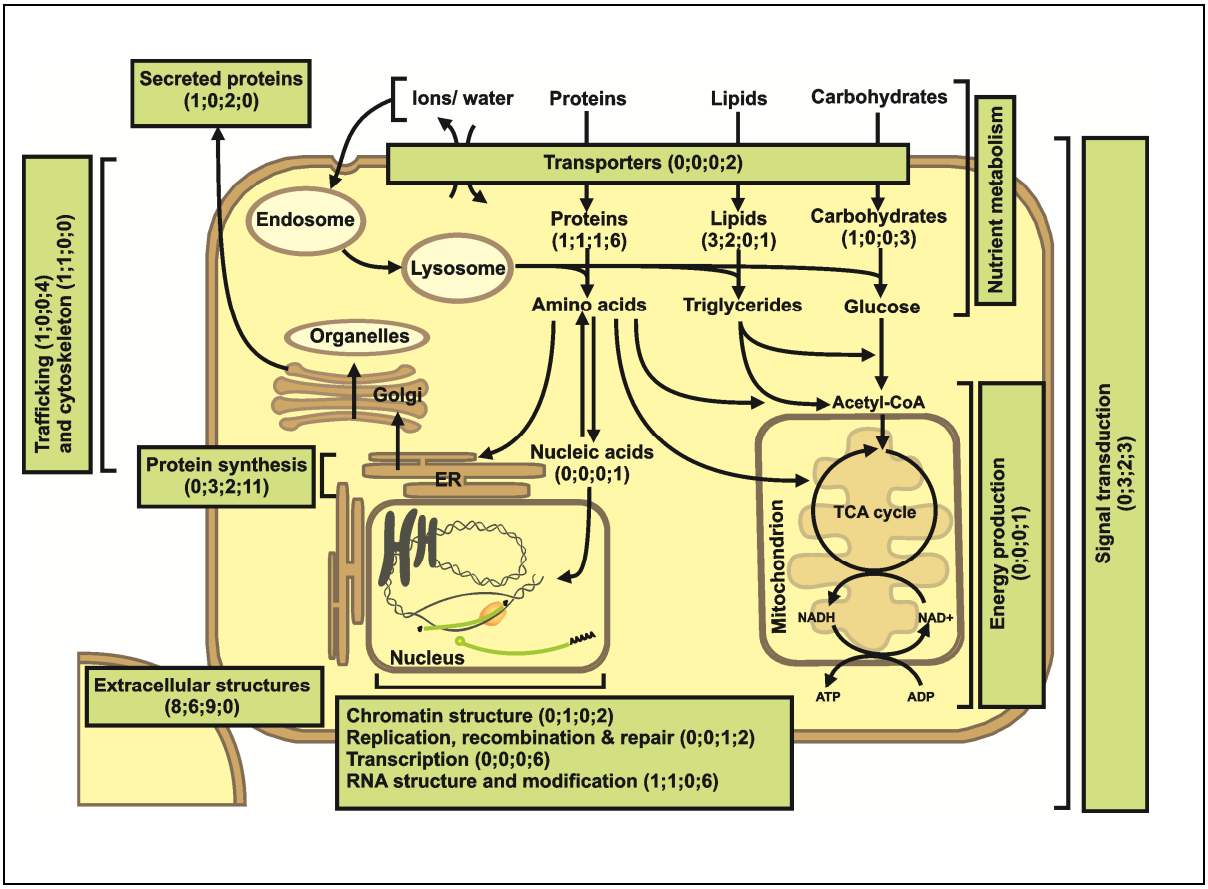

Figure 3.3. Overview of regulated transcripts in immature life stages and adult female tissues of feeding *R. microplus*. A simplified cell and biological pathways with key processes in blocks are indicated, as well as the relative number of transcripts (in brackets) involved in each process representing larvae, nymphs, shared in immature life stages only and shared between all life stages, respectively. The key functional processes represented are: secreted proteins (including anti-coagulants and antimicrobials) that could also include enzymes involved in nutrient acquisition (proteases and lipases); nutrient metabolism that includes both intracellular and extracellular enzymes and transport proteins; enzymes and proteins involved in energy production; enzymes and proteins involved in chromosome structure, replication, transcription, as well as RNA processing and modification; proteins and enzymes involved in protein synthesis, proteins involved in intracellular trafficking and the cytoskeleton, extracellular structures involved in cell-cell contact; receptors, enzymes and accessory proteins involved in signal transduction events throughout the cell. ER, endoplasmic reticulum; TCA, tricarboxylic acid.

As was the case for adult tissue comparisons, though numerous significantly regulated transcripts were identified in this study (2,102 transcripts), not all these transcripts were considered further due to the stringent selection criteria used in this study (M-value > 0, Cy5 intensity > 1,000 and *P* values ≤ 0.01). Many transcripts may have been omitted that only have basal expression levels (i.e. M-value = 0), resulting in the small number of transcripts representing biologically essential processes (i.e. energy metabolism) (Fig. 3.3.). Furthermore, only adult tissue transcripts that are shared with the immature stages are examined in this study. Therefore, the full complement of adult "inter-tissue" and tissue-specific transcripts are not discussed here (Fig. 3.1. A.). In this regard, a great deal of the tissue-specific and related transcripts has already been dealt with in Chapter 2. It must also be stated that all tissue and life stage comparisons in this study have been viewed and described individually. However, combining all transcripts identified for a particular stage (i.e. salivary glands) within all its corresponding life stage and tissue comparisons (i.e. salivary gland and ovary, salivary gland and midgut, etc.), the full set of transcripts and their corresponding functional contributions for that stage will be more apparent. The latter will be covered in a future study.

For this study, the largest subset of highly up-regulated transcripts encoded structurally related proteins that are likely essential for development of the immature life stages (Fig.3.3.). The same trend has been observed previously for other transcriptome analyses involving the immature life stages of ticks (Rodriguez-Valle *et al*., 2010; McNally *et al*., 2012). The remainder of these highly up-regulated transcripts identified are mostly devoted to nutrient transport and metabolism and protein synthesis, emphasizing the drive to obtain the necessary components and energy required by immature ticks to develop through the life cycle (Fig.3.3.). When considering all the life stages and adult tissues as a whole from the current data, it is clear that core processes related to energy production, protein degradation (i.e. components of the proteasome) and especially protein synthesis (i.e. ribosomes) are conserved and therefore essential for all the life stages tested.

There is however an urgent need to expand the current repertoire of available candidates for further exploration of tick biology, as well as for evaluation as anti-tick vaccine candidates. The available transcriptomic data for all the life stages and adult female tissues represents an invaluable resource that can be used for data mining to identify new candidates using modern

in silico methods for putative antigen discovery. This reverse vaccinology approach to antigen discovery was explored in the following sections.

3.4.4. Immuno-informatic analysis of transcriptome data and identification of putative vaccine antigens

To expand the current repertoire of targets for anti-tick vaccine development a reverse vaccinology approach similar to that proposed by Rappuoli and Bagnoli (2011) was followed using the transcriptome data obtained in this study and is summarized in Fig.3.4.

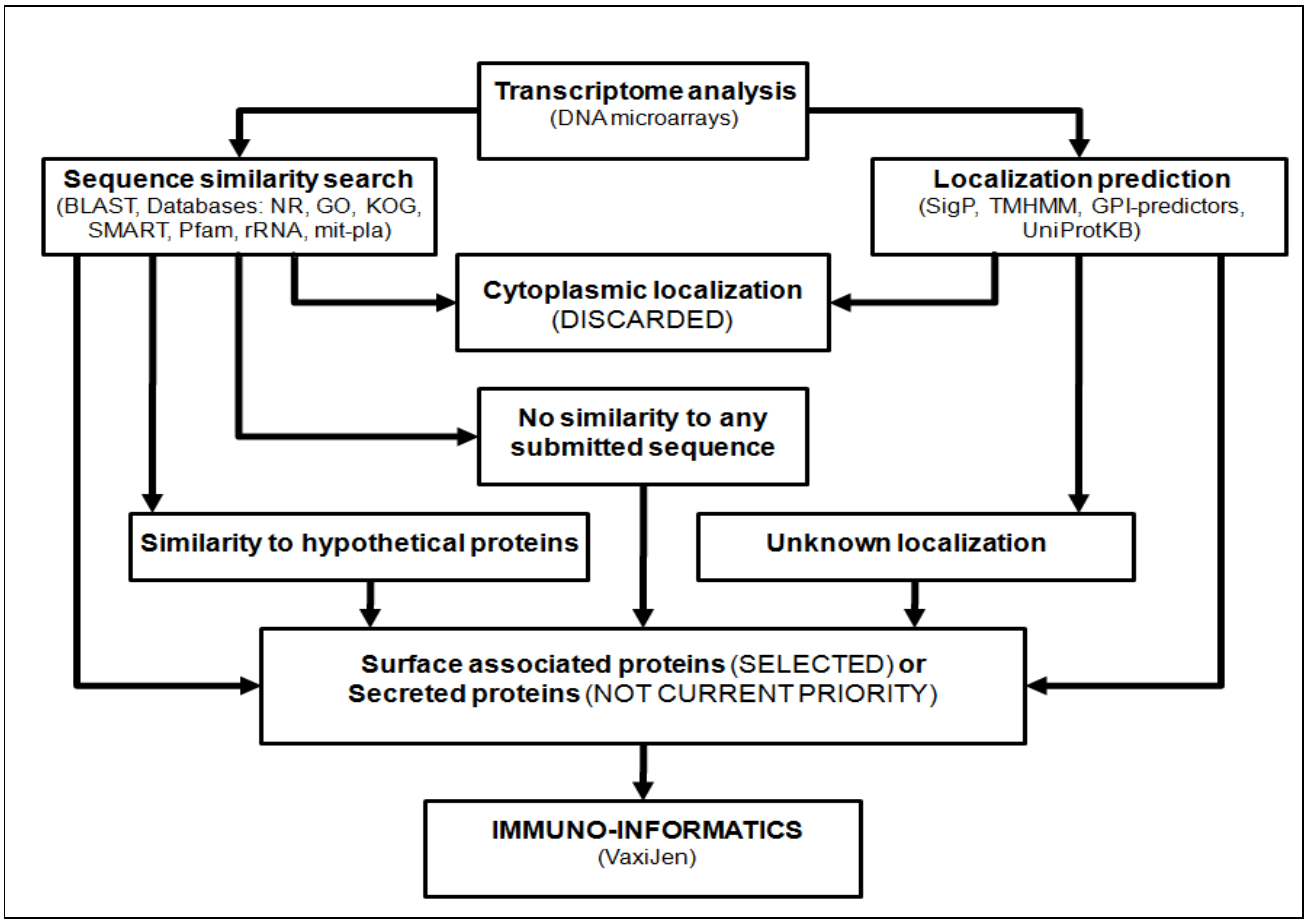

Figure 3.4. Strategy used for the identification of anti-tick vaccine candidates using a functional genomics and *in silico* reverse vaccinology approach. Transcriptome data for *R. microplus* is used to identify transcripts that are expressed throughout the life cycle of the cattle tick. The resultant nucleotide sequences are subjected to BLAST searches against non-redundant (NR), gene ontology (GO and KOG), protein structure (SMART and Pfam), rRNA and mitochondrial plastid (mit-pla) databases for sequence annotation and functional classification. Protein localization was performed using *in silico* predictors for secretion (SigP), membrane topology (TMHMM and GPI-predictors) and results compared to data available in the UniProtKB protein database. Final probable antigenicity was determined using the VaxiJen program.

3.4.4.1. Large-scale in silico reverse vaccinology screening of transcriptome data for *R. microplus*

Using signal intensity from the current transcriptional data as an indicator for gene expression, a screening of all 13,456 transcripts represented on the microarray for *R. microplus* was performed using a minimum threshold intensity of 1,000. The overall distribution of transcripts showed that the majority of genes regulated above the intensity threshold were shared among all the life stages and tissues (Fig. 3.5., Table 3.2.). Interestingly, the second largest set of transcripts identified was unique to ovaries (Fig. 3.5.). Previous results obtained for adult ovary tissues showed a similar trend (Chapter 2).

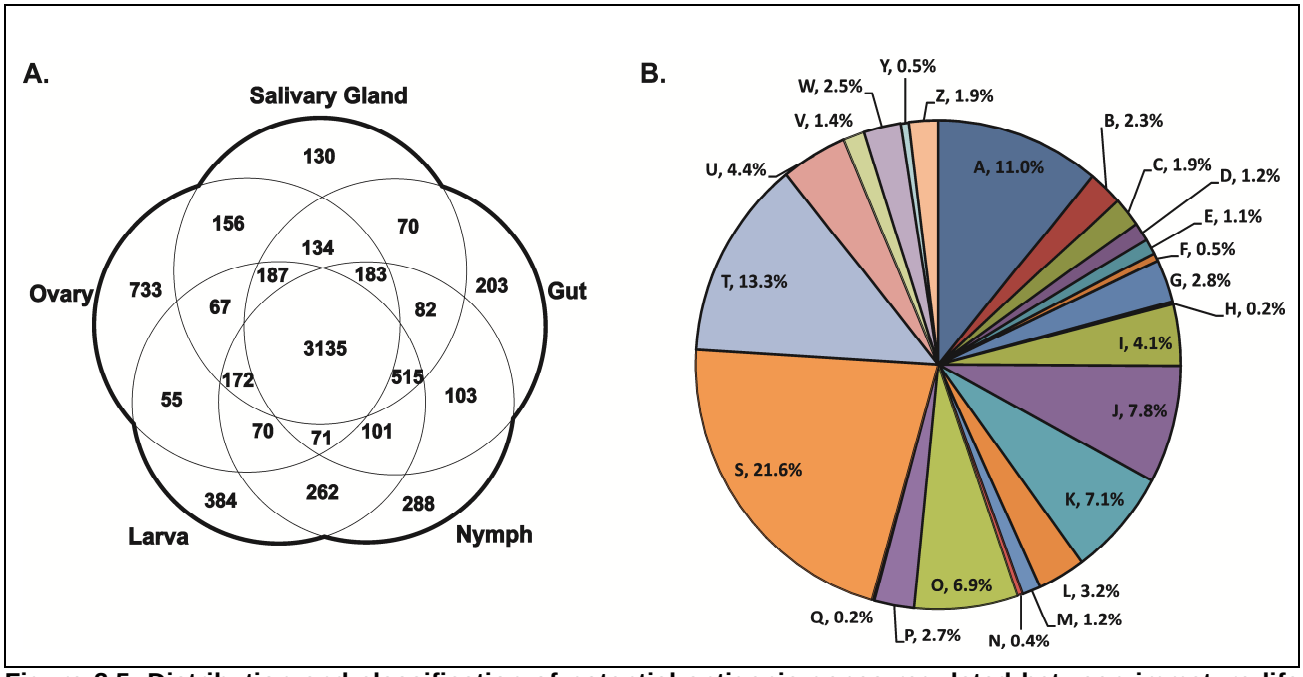

Figure 3.5. Distribution and classification of potential antigenic genes regulated between immature life stages and adult female tissues of *R. microplus* ticks. Indicated is the number of unique genes shared between immature life stages and adult tissues with an intensity threshold above 1,000. (A) Venn diagram indicating the number of genes that are unique to or shared between larvae, nymphs, salivary glands, midguts and ovaries. (B) Indicated are the percentages of genes shared between all immature life stages and adult tissues above threshold, with a VaxiJen score greater than 0.7. Transcripts are classified according to their eukaryotic orthologous functional groups (KOGs): A- RNA processing and modification; B- Chromatin structure and dynamics; C- Energy production and conversion; D- Cell cycle control, cell division, chromosome partitioning; E- Amino acid transport and metabolism; F- Nucleotide transport and metabolism; G- Carbohydrate transport and metabolism; H- Coenzyme transport and metabolism; I- Lipid transport and metabolism; J- Translation, ribosomal structure and biogenesis; K- Transcription; L- Replication, recombination and repair; M- Cell wall/membrane/envelope biogenesis; N- Cell motility; O- Posttranslational modification, protein turnover, chaperones; P- Inorganic ion transport and metabolism; Q- Secondary metabolites biosynthesis, transport and catabolism; S- Function unknown (also includes transcripts with only general functional predictions); T- Signal transduction mechanisms; U- Intracellular trafficking, secretion, and vesicular transport; V- Defense mechanisms; W- Extracellular structures; Y- Nuclear structure; Z- Cytoskeleton.

Table 3.2. Summary of predicted antigenic transcripts for selected tissue and life stage comparisons. Indicated is the number of transcripts identified from microarray analysis, as well as the number of transcripts that are predicted antigens with their predicted cellular fate.

Tissue and life stage comparison	Total number of transcripts [a]	VaxiJen score >0.7 [b]	Non-secreted intracellular [c]	Membrane-associated [d]	Secreted soluble [e]
All life stages	3135	566	398	130	38
L+ all tissues	187	33	23	8	2
N+ all tissues	183	20	15	3	2
L+G+SG	86	20	16	3	1
L+G+O	26	3	3	0	0
L+O+SG	67	10	5	5	0
N+G+SG	81	15	14	1	0
N+G+O	73	11	9	1	1
N+O+SG	64	15	8	1	1
L+G	44	9	7	2	0
L+O	55	9	5	3	1
L+SG	94	24	14	5	33
N+G	103	15	8	6	4
N+O	124	21	16	2	2
N+SG	72	20	1	6	1
Total [f]	4394	791	542	176	86

[a.] Total number of transcripts expressed above an intensity threshold of 1,000

[b.] Total number of transcripts predicted to have a protective antigen probability score higher than 0.7 using the VaxiJen server.

[c.] Total number of transcripts that have no identifiable membrane spanning regions or signal sequences. This includes transcripts that are cytosolic, as well as truncated transcripts.

[d.] Total number of transcripts that have predicted membrane spanning regions. These include transcripts that have or lack identifiable signal sequences.

[e.] Total number of transcripts that have predicted signal sequences, however lack any identifiable membrane spanning regions. This also includes transcripts that may be C-terminally truncated and GPI-anchored.

[f.] Total number of transcripts per separation class.

3.4.4.1.1. Identification of putative vaccine antigens

For proteins to be accessible as vaccine antigens, they should be expressed during a reasonable period of the life cycle and secreted or presented on membranes or external surfaces of the parasite (Flower, 2008). Therefore, considering transcripts that were up-regulated in at least one immature life stage and an adult tissue, expression analysis indicated that 33% of the total complement of sequences was regulated above the intensity threshold (Fig. 3.5, Table 3.2.). The resulting transcripts were subjected to further *in silico* analysis to identify protein coding sequences (with Prot4EST), secretion signals (SigP), membrane topology (TMHMM) and probability as putative antigens using the VaxiJen web server. A total of 791 transcripts were predicted to be potential protective antigens with VaxiJen scores greater than a 0.7 cut-off value (Table 3.2.).

The comparison that contained the largest subset of probable antigens comprised of 566 transcripts that were shared between all life stages and tissues considered in this study (Table 3.2.). Sequence annotation and functional classification according to eukaryotic gene ontology (KOG), indicated that almost 22% of these antigenic transcripts could not be functionally annotated (Fig. 3.5.). However, some of the major functional classes that were represented by annotatable sequences included: transcripts involved in RNA modification and processing (11%); translation, ribosomal structure and biogenesis (7.8%); transcription (7.1%) and posttranslational modification, protein turnover, chaperones (6.9%). The largest subset of annotatable transcripts identified (13.3%) relate to signal transduction mechanisms (Fig. 3.4). The latter highlights the essential role signal transduction plays in basic metabolism and cellular function. Two smaller classes that were identified contained transcripts related lipid transport and metabolism (4.1%), as well as intracellular trafficking (4.4%) (Fig. 3.5.).

Analysis of the membrane topology of the 566 predicted antigens expressed in all the life stages and adult tissues showed that 398 transcripts had no identifiable membrane spanning regions or signal sequences for secretion (Table 3.2.). However, 130 transcripts could be identified that showed membrane topology in regards to TMHMM or GPI-anchor sequence prediction analysis. Further analysis of the predicted antigenicity scores for these transcripts showed that more than 50% obtained a similar or better score than that predicted for the Bm86 antigen at 0.7698. Following signal peptide prediction, 89 transcripts were identified to be putatively secreted. Of the latter, only 38 transcripts were identified that had no predicted

membrane localization for this comparison (Table 3.2.). An additional 20 membrane proteins were predicted from the various comparisons with the nymphs. In contrast, 26 additional transcripts were identified as putatively membrane-associated from the remaining comparisons with larvae (Table 3.2.). Overall, 176 putative membrane-associated proteins were identified, with or without identifiable signal peptides, for all the comparisons considered (Table 3.2.). The probability that many potential antigenic membrane transcripts were not identified by the *in silico* tools used cannot be disregarded. However, it is expected as the sequence data used in this study was derived from assembled ESTs that contain truncated transcripts, as well as incorrect assembly of the contiguous sequences (contigs) that could affect downstream predictions (Nagaraj *et al.*, 2007).

Considering only transcripts that were expressed throughout all the tissues and life stages tested, manual inspection of the data and comparison to homologous sequences described in the UniProtKB protein database was performed and 18 putative secreted membrane-associated transcripts were identified with probable antigen scores greater than 0.7 (summarized in Table 3.3).

Table 3.3. Predicted antigenic membrane proteins from robust large-scale transcriptome screening that are expressed in all life stages of *R. microplus*. Indicated are predicted antigenic secreted membrane transcripts that are regulated above a threshold intensity of 1,000 (VaxiJen score > 0.7).

Transcript category (KOG classification) [a]	Contig[b]	Description [c]	Predicted membrane topology [d]	VaxiJen Score [e e]	Localization according to Uniprot [f]	Putative function [g]	References
Cell cycle control, cell division, chromosome partitioning	Contig3591	Putative bladder cancer-associated protein (blcap), BLCAP family	Single-spanning	**0.9557***	Plasma membrane, multi-pass membrane protein	A possible regulator of cell proliferation, coordinated apoptosis and cell cycle progression.	Yao *et al*., 2007
Defense mechanisms	Contig5705	Putative defense protein (l(2)34Fc), Insect defense protein family	GPI	**0.8284***	Secreted	A late response immune regulated gene that may have antimicrobial activity.	De Gregorio *et al.*, 2002
Extracellular structures	Contig4466	Putative glycine rich protein, Glycine rich protein (GRP) family	Single-spanning	**1.0607***	ND	Probable extracellular structure or formation of cement cone	Francischetti *et al*., 2010
	GO248528.1	Putative glycine rich protein, Glycine rich protein (GRP) family	Multi-spanning	**0.8437***	ND	Probable extracellular structure or formation of cement cone	Francischetti *et al*., 2010
	Contig5481	Putative glycine rich protein, Glycine rich protein (GRP) family	Single-spanning	0.7624	ND	Probable extracellular structure or formation of cement cone	Francischetti *et al*., 2010
	Contig3276	Putative glycine rich protein, Glycine rich protein (GRP) family	Multi-spanning	0.7527	ND	Probable extracellular structure or formation of cement cone	Francischetti *et al*., 2010
	Contig2618	Putative glycine rich protein, Glycine rich protein (GRP) family	Single-spanning	0.737	ND	Probable extracellular structure or formation of cement cone	Francischetti *et al*., 2010
Function unknown	Contig5206	Putative keratinocyte-associated protein 2 (KRTCAP2), KRTCAP2 family	Multi-spanning	**0.8599***	Plasma membrane, multi-pass membrane protein	A component of the oligosaccharyltransferase (OST) complex.	Shibatani *et al*., 2005
	CK179775	Putative membralin (Tmem259)	Multi-spanning	**0.7732***	Plasma membrane, multi-pass membrane protein	Component of the membrane with unknown function	Chen *et al*., 2005
Inorganic ion transport and metabolism	Contig17	Putative store-operated calcium entry-associated regulatory factor (tmem66), SARAF family	Single-spanning	**0.7773***	Endoplasmic reticulum membrane, single-pass type I membrane protein	Involved in regulation of intracellular calcium stores.	Palty *et al*., 2012
	Contig185A	Putative Zinc transporter (SLC39A7), ZIP transporter (TC 2.A.5) family, KE4/Catsup subfamily	Multi-spanning	**0.774***	Plasma membrane, multi-pass membrane protein	Putative Zinc transporter that plays a role maintaining intracellular zinc homoeostasis	Taylor *et al.*, 2003; Jeong and Eide, 2013
Inorganic ion transport and metabolism	Contig166	Putative superoxide dismutase (sod1), Cu-Zn superoxide dismutase family	GPI	0.7534	Cytoplasm	Enzyme catabolises naturally occurring superoxide that can cause oxidative stress and has also been proposed to be	Anderson et al., 2008

Transcript category (KOG classification) [a]	Contig[b]	Description [c]	Predicted membrane topology [d]	VaxiJen Score [e e]	Localization according to Uniprot [f]	Putative function [g]	References
						involved in heme trafficking in ticks.	
Intracellular trafficking, secretion, and vesicular transport	CV439583	Putative transmembrane 9 superfamily member 4 (Tm9sf4), Nonaspanin (TM9SF) family	Multi-spanning	**0.8327***	Plasma membrane, multi-pass membrane protein	With TMN2 and TMN3, plays a critical role in the late stages of a nutrient-controlled pathway notably regulating FLO11 gene expression. Acts downstream of RAS2 and TOR. Essential for cell adhesion and filamentous growth.	Froquet *et al*., 2008
	Contig4076	Putative lysosome-associated membrane glycoprotein 1 (LAMP1), LAMP family	Single-spanning	0.7236	Plasma, endosome and lysosome membranes. Single-pass type I membrane protein	Involved in presentation of carbohydrate ligands to selectins	Thomas *et al.,* 2010
	CV445691	Putative Zinc transporter ZIP9 (slc39a9), ZIP transporter (TC 2.A.5) family	Multi-spanning	0.705	Plasma membrane, multi-pass membrane protein	Putative Zinc transporter that plays a role maintaining intracellular zinc homoeostasis	Jeong and Eide, 2013
Signal transduction mechanisms	Contig1594	Putative Cation-dependent mannose-6-phosphate receptor (M6pr)	Single-spanning	0.7162	Lysosome membrane, single-pass type I membrane protein.	Transport of phosphorylated lysosomal enzymes from the Golgi complex and the cell surface to the lysosomes.	Dahms *et al*., 2008
	Contig1297	Putative CD63 antigen (Cd63), tetraspanin (TM4SF) family	Multi-spanning	0.7096	Plasma, lysosome and endosome membranes. Multi-pass membrane protein.	A putative role in growth regulation and signal transduction pathways.	Maecker *et al*., 1997
Translation, ribosomal structure and biogenesis	Contig185B	Putative secreted ribonuclease kappa-A (rnaseka), RNase K family	Single-spanning	0.7251	Membrane; Multi-pass membrane protein	Endoribonuclease which preferentially cleaves ApU and ApG phosphodiester bonds, involved in rRNA transcription.	Economopoulou *et al*., 2007)

[a.] Classification of transcripts according to eukaryotic orthologous group terms for gene ontology (Tatusov et al., 2003).

[b] Assigned contiguous sequence identification for transcripts following assembly of all available expressed sequence tags and the *R. microplus* Gene Index version 2.1.

[c] The functional annotation of genes based on comparison among BLAST outputs from seven databases outlined in Section 2.2.4 (Cahpter 2). All transcript descriptions are based on consensus with database entries from Uniprot (http://www.uniprot.org/uniprot/) and BRENDA (http://www.brenda-enzymes.org/), in the case of enzymes.

[d.] Membrane topology based on results obtained from bioinformatic screening using bioinformatic tools as outlines in Section 3.2.2.

[e.] Screening for possible antigenic transcripts using VaxiJen (parasite model). Only transcripts with a reasonable annotation and scores greater than 0.7 are presented.

[f.] Putative protein localization as determined for homologous entries in the UniProt database (http://www.uniprot.org/uniprot/).

[g.] Putative functions determined for transcripts from the UniProt database (http://www.uniprot.org/uniprot/) and literature.

[h.] References are included in Chapter 3 bibliography.

* Indicates transcripts that obtained VaxiJen scores equivalent to or better than that determined for Bm86 (VaxiJen score = 0.7698). VaxiJen scores greater than 1, indicate an overfitting of the test transcript with comparable antigens available in the Vaxijen data base.

[ND.] Not determined

Inspection of the data revealed a reasonable concordance between predicted data and database entries. However, only two contrasting results were observed for proteins that have been predicted as GPI-anchored proteins (Contig5705 and Contig166), but are soluble proteins according to homologous protein database entries. These two transcripts can however be set aside for now, according to the current strategy (Fig.3.4.). Of the remaining transcripts 9 potential antigens obtained VaxiJen scores equivalent to or better than that predicted for Bm86 of ~0.77. These top performing transcripts included two glycine-rich proteins that are associated with extracellular structures and have been shown to be ineffective as vaccine candidates for immunization (Table 1.2.) (Zhou *et al*., 2006). These proteins were also shown in this study to be ubiquitous in all the life stages of *R. microplus*, with large subsets of transcripts expressed in each stage (Chapter 2 and 3). The aforementioned therefore makes these proteins functionally redundant and difficult to target for vaccination (Guerrero *et al*., 2012a). Furthermore, glycine-rich proteins are highly repetitive in glycine, serine and threonine residues, and therefore of low sequence complexity that may help to skew immuno-informatic prediction results with Vaxijen. These results however serve to illustrate the need for negatively performing antigens that can be used to train predictive software such as VaxiJen to improve their accuracy. Finally, five transcripts encoding membrane-associated proteins and one secreted transcript remain for further evaluation as vaccine candidates (Table 3.3.).

An additional immuno-informatic analysis of the transcripts previously identified as shared amongst all adult tissues and life stages using stricter selection criteria (Cy5 > 1,000, M > 0, VaxiJen > 0.7) (Section 3.3.2.6.5, Supplementary Table S3.4.), identified 6 transcripts that had calculated scores equal to or greater than the selected threshold (Supplementary Table S3.6.). However, all the transcripts identified were predicted to be intracellular with only one membrane-associated transcript (Supplementary Table S3.6.). The latter transcript also failed to obtain a Vaxijen score equal to or greater than that determined for Bm86 (Supplementary Table S3.6.). When considering the current repertoire of targets that have been evaluated through vaccination in host animal trials, it is interesting to note that reasonable vaccine efficacies were obtained for some soluble extracellular (i.e. BmTI, vitellin, ferritin 2), as well as intracellular (i.e. Ribosomal protein P0, Subolesin/Akirin) proteins (Table 1.2.). In spite of the fact that cell surface localization is thought to be a major determinant of immunogenicity (Flower, 2008), potential antigens that are soluble or intracellular, should not be completely

discarded. Potential antigens from other tissue and life stage comparisons will offer more targets for further evaluation but are not presented here.

Regardless of current constraints, 5 new potential antigens localized to the plasma membrane and one secreted transcript was identified with scores higher than Bm86 that are shared across all tissues and life stages. These putative transcripts are involved in diverse functions including ion transport, intracellular trafficking and cell cycle control (Supplementary Table S3.8).

3.4.4.1.2. Preliminary proof of concept using synthetic peptides

A preliminary proof of concept was provided by Mr. W. van Zyl (MSc., University of Pretoria) for the use of reverse vaccinology to identify novel tick antigens. Briefly, synthetic antigenic peptides were identified from predicted membrane proteins (including hypothetical proteins) selected using the current reverse vaccinology methodology and *in silico* epitope predictors. These peptides were screened for immuno-dominance in an ELISA assay using polyclonal antisera obtained from BALB/c mice immunized with a crude extract of tick midgut membrane proteins. Results indicated three immuno-dominant peptides (peptide 1 from Antigens 2, 3 and 4) that showed better recognition (*P* value < 0.001) by polyclonal antisera from BALB/c mice immunized with a crude extract of tick midgut membrane proteins, compared to previously published peptide controls for Bm86 (Fig. 3.5.). Therefore, the current methodology for vaccine discovery has merit and future studies will be conducted to further validate the identified targets in this study.

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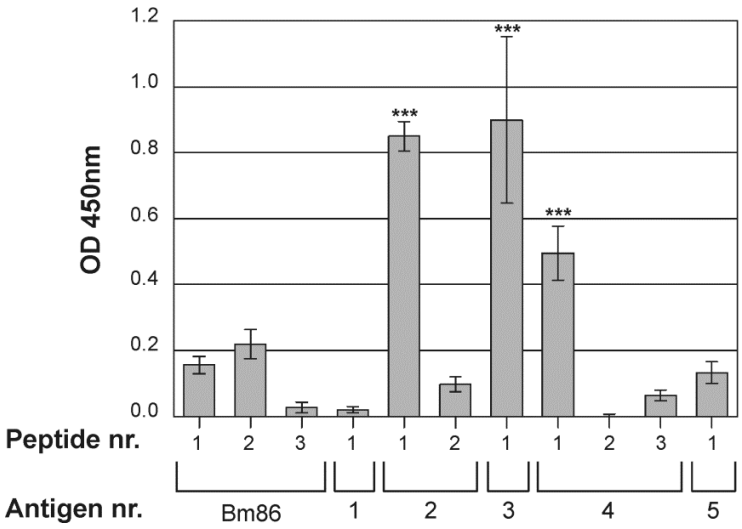

Figure 3.6. Evaluation of the reactivity of selected membrane proteins using antisera from mice challenged with crude midgut membrane proteins. Indicated are ELISA results obtained for several peptides representing predicted antigens (1-5) using antisera from BALB/c mice immunized with a crude *R. microplus* gut membrane extract. Asterisks indicate a significant difference (*P* value < 0.001) between results obtained for predicted antigen peptides compared to previously validated Bm86 epitopes that was used as positive controls.

3.5. Conclusion- From transcriptomics to reverse vaccinology

As discussed previously, transcriptional profiling of the immature life stages of *R. microplus* identified distinct subsets of up-regulated transcripts involved in extracellular structures, nutrient transport and metabolism, as well as protein synthesis (Fig.3.3.). The remainder of these up-regulated transcripts were mostly devoted to nutrient metabolism and protein synthesis, emphasizing the drive to obtain the necessary components and energy required by the immature ticks to develop through their life cycle (Fig.3.3.). When considering all the life stages and adult tissues as a whole, a clear core of processes related to energy production, protein synthesis and protein degradation is evident. As expected, these are therefore conserved processes and essential for all the life stages tested.

Applying the current transcriptome data in a reverse vaccinology screening using *in silico* tools, some 791 promising putative vaccine candidates could be identified that were selected based on the criteria of being expressed in all of the life stages of *R. microplus* at levels comparable to that of Bm86. These candidates obtained similar scores in VaxiJen, an alignment-independent computational tool that has been successfully applied in the prediction of other parasitic antigens.

However, the predicted antigens should also be available for immune surveillance, thereby giving preference to membrane- and secreted antigens. The approach for predicting subcellular localization in combination with VaxiJen also resulted in the inclusion of Bm86, shown in literature to be membrane-bound and to confer protective immunity (Rand *et al.*, 1989). This result provides a tentative proof that the proposed pipeline has some promise in identifying other potential candidates. In this regard, some transcripts that were predicted as good antigens, failed to confer protection in vaccination trials, as in the case for glycine-rich proteins (Zhou *et al*., 2006). Therefore, expanding negative antigen datasets will also assist in improving the predictive capacity of tools such as VaxiJen, which is a vital step for future studies. Most of the predicted membrane-associated vaccine candidates function in vital biological processes such as signal transduction, trafficking and transport (Fig. 3.5.). Targeting these antigens may have significant downstream effects resulting in severe impairment of biological functions in the tick.

Therefore, 6 novel target antigens are proposed that might be evaluated further for as vaccine candidates in future studies. These putative candidate proteins include: plasma membrane-associated bladder cancer-associated protein (Contig3591), a keratinocyte-associated protein 2 (Contig5206), a membralin (CK179775), a zinc transporter (Contig185A) and a transmembrane 9 superfamily member 4 protein (CV439583), as well as a secreted insect defense protein (I(2)34Fc) (Contig5705). These target proteins do not appear to be part of large protein families that could lead to functional redundancy and further sequence analysis will reveal possible significant homology to mammalian hosts (i.e. *B. taurus* or *B. indicus*). The latter will allow tailoring of target antigens for production of peptide or subunit vaccine preparations containing the most unique regions of the parasite protein to avoid possible host auto-immune responses.

A preliminary evaluation of the current methodology predicted membrane-associated antigens that were selected and the reactivity of representative epitopes for each antigen was assessed, using antisera from mice immunized with *R. microplus* midgut membrane proteins. This led to the identification of immuno-dominant antigens with higher recognition by the antisera than those previously confirmed for Bm86 (Fig. 3.6.). However, antigenicity alone does not guarantee that immunization with a particular protein will in fact confer anti-tick resistance in cattle trials. Another good example is the 5'-nucleotidase isolated from *R. microplus* midgut fractions, which strongly elicited an antibody response in sheep and cattle,

but did not confer significant protection in cattle following tick challenge (Hope *et al.*, 2010). It is interesting to note that the VaxiJen score obtained for this protein was lower than the threshold value of 0.7. Incorporation of *in silico* tools to predict the most likely antigenic epitopes for B- and T-cell recognition provide an additional avenue for rational selection of potentially immuno-dominant peptides for evaluation in vaccination trials. The latter approach was also followed in the previous study by W. van Zyl (MSc., University of Pretoria).

In this chapter, a previously designed microarray platform was successfully applied for the analysis of gene expression in the immature life stages of the cattle tick, *R. microplus*. A catalogue of life stage-specific and shared genes were identified involved in basic metabolic processes of *R. microplus*. This study presented here is the first global transcriptomic analysis via DNA microarrays of *R. microplus* nymphs and represents an additional resource that can be further exploited to study proteins and pathways that may be useful for future tick control. Further, analysis of global transcriptome data using less stringent parameters identified a total of 176 membrane-associated and 86 secreted soluble proteins from which the most promising candidates can be selected and expressed for vaccine trials. In this regard, 6 potential plasma membrane-associated antigenic transcripts were identified that could be evaluated in future vaccination trials. Both protective and non-protective antigens, evaluated during vaccination trials, will be invaluable in improving the predictive potential of VaxiJen and by extension the current strategy (Fig. 3.4). This reverse vaccinology approach offers an alternative pipeline for the identification of additional anti-tick vaccine candidates.

3.6. References

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Chapter 4

Concluding discussion

The southern cattle tick, *R. microplus*, is a major pest species of cattle and is rightfully regarded as the most economically devastating tick species in the world (Guerrero *et al*., 2006). It is a highly adaptable vector species that is infesting new geographical locations on the African continent, even displacing endemic species, spreading disease causing pathogens and rapidly acquiring resistance to chemical control (Tønnesen *et al*., 2004; Rajput *et al*., 2006; de la Fuente *et al*., 2007a; Madder *et al*., 2007; Li *et al*., 2007, Lynen *et al*., 2008; Madder *et al*., 2011; Adakal *et al*., 2013; Nyangiwe *et al*., 2013).

Since the overuse of chemical acaricides has enabled the development of acaricide-resistance in ticks, an integrated pest management system has become the best option to control tick infestations and transmission of associated pathogens. These measures include rotation of grazing lands and chemical control to lower tick burdens, as well as the inclusion of an anti-tick vaccine to alleviate the pressures enabling acquired resistance to acaricides (George *et al*., 2004; de la Fuente *et al*., 2007a; de la Fuente *et al*., 2007b). Such a strategy was successfully employed in Cuba and showed a marked decrease in the use of acaricides and the incidence of disease, resulting in significant savings for the Cuban cattle industry (de la Fuente *et al*., 1998).

The use of the midgut protein, Bm86, as an antigen in a commercial vaccine has been well documented and its efficacy in lowering tick burdens on cattle has been sufficiently demonstrated in both controlled and field trials (de la Fuente *et al*., 2007a; Merino *et al*., 2013). However, considering the vast amount of transposons and retro-transposable elements in the genome of *R. microplus* (Guerrero *et al*., 2010), as well as inter-strain sequence variation and differential control of gene expression (Kamau *et al*., 2011), acquired resistance to protein-based vaccines will be a significant obstacle in the foreseeable future. This has already been reported in the field with Bm86 resistant tick strains (de La Fuente *et al*., 2000). The latter can likely be ascribed to either antigen variability that occurred via antigenic drift (the acquisition of point mutations during replication), major recombination/ reassortment of genetic material between related strains (antigenic shift), differential gene

expression or a combination of these. Furthermore, differential gene expression of Bm86 has also been demonstrated between life stages whithin a species (i.e. Bm86 is highly expressed in the midgut tissues of adult *R. microplus*), as well as between species (i.e. higher expression of Bm86 homolog in life cycle of *R. appendiculatus* relative to *R. microplus*) (Nijhof *et al*., 2009; Bastos *et al*., 2010; Nijhof *et al.*, 2010). The latter may also contribute (along with inherent sequence variability) to the variability observed in vaccine efficiency of Bm86 for cross-species protection (Chapter 1, Table 1.2.).

Regardless of the previous successes of Bm86-based vaccines to control tick infestations, it is clear that after more than two decades these vaccines have become ineffective. Moreover, of the more than 40 unique anigens that have been identified from various tick species since Bm86, only 13 new antigens have been tested in cattle vaccination trials and only 2 of these have shown efficacies comparable to Bm86 (Fig. 4.1.). Therefore, new targets for second generation anti-tick vaccines are desperately needed.

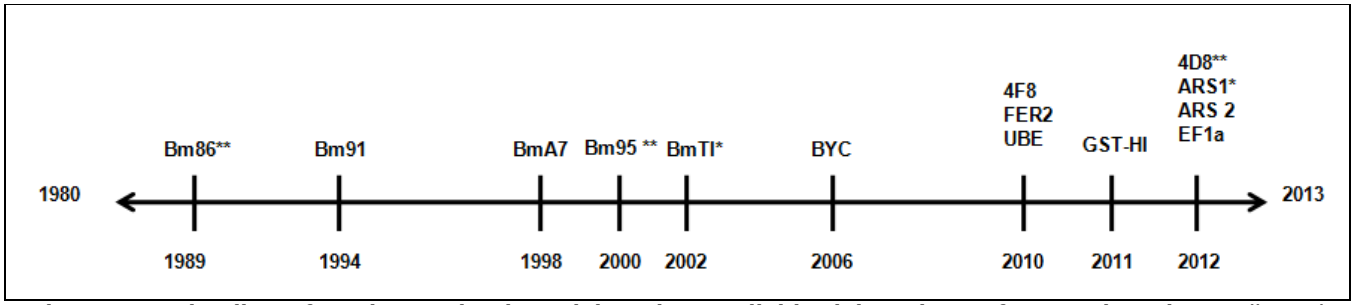

Figure 4.1. Timeline of cattle vaccination trials using available tick antigens for *R. microplus*. Indicated are the current antigens and the first year when they were applied in cattle trials to assess protection against *R. microplus* infestations. Also indicated are antigens that showed a vaccine efficiency of >70% (*) and >80% (**), respectively. Not indicated are combinations of antigens that were also applied in trials, these are available along with antigen descriptions in Table 1.2. (Chapter 1).

4.1. Transcriptional profiling of *R. microplus*

A vast amount of sequence data is currently available for *R. microplus*, in the form of a partially assembled genome and EST data (Lee *et al*., 2005; Wang *et al*., 2007; Bellgard *et al*., 2012). Therefore, a functional genomics approach is an attractive avenue for understanding how the genome and transcriptome of this organism functions. In this regard, DNA microarrays have become an an essential research tool for monitoring gene regulation and gene expression on a large scale, enabling the identification of differentially expressed

genes (under a particular set of conditions) and elucidation of cellular processes via computational biology (Jares, 2006).

Application of DNA microarray technologies to *R. microplus* research has risen dramatically in recent years, as it is a powerful tool for the study of the host-vector-pathogen interface. To date, DNA microarrays have been applied successfully in the study of gene regulation involved in tick feeding, pathogen infection, acaricide resistance, responses to gene knock-down and feeding on different cattle hosts (Saldivar *et al*., 2008; Rodriguez-Valle *et al*., 2010; Lew-Tabor *et al*., 2011; Mercado-Curiel *et al*., 2011; Heekin *et al*., 2012; McNally *et al*., 2012). This has established DNA micoarrays as a functional tool for gene discovery in *R. microplus* and was therefore applied in this study to identify transcript/proteins up-regulated in the cattle tick within key tissues and life stages during feeding (Chapter 2 and 3).

In Chapter 2, transcriptional profiling was performed on the various tissues of feeding adult *R. microplus* females using a custom oligonucleotide microarray designed from all available EST data and an assembled gene index (BmGI) for *R. microplus* (Wang *et al*., 2007). The resultant data was found to be reproducible following Pearson analysis and correlated well with real-time PCR analysis of selected transcripts. Data analysis of the adult female tissues identified 1,707 transcripts that are significantly up-regulated in these tissues and represent distinct subsets of genes that aid in blood meal acquisition (salivary glands), digestion (midgut) and reproduction (ovaries). These encoded large repertoires of anti-hemostatics and immunomodulatory proteins from salivary glands that maintain blood fluidity, while additional secreted proteases aid in blood pool formation and pre-digestion of the blood meal. In the midgut, the bulk of the up-regulated proteins were involved in nutrient transport and metabolism likely in preparation for reproduction. Finally, as the female prepares for egg production, unique genes were highly up-regulated to control cell cycle development, DNA replication and post-transcriptional and post-translational modification. A global comparison identified a set of 588 transcripts shared between all the adult female tissues and revealed a broad functional distribution of transcripts with encoded proteins involved in protein, carbohydrate and lipid metabolism necessary for biosynthetic pathways and energy production, indicating fundamental processes that are ubiquitous in all tissues during feeding. Of these, numerous transcripts involved in signal transduction events that control the cell cycle, transcription and translation, were identified making these important components of basic adult tissue biology.

In Chapter 3, additional transcriptional profiling was performed on the immature life stages, larvae and nymphs, of feeding *R. microplus* cattle ticks. Global microarray analysis across all adult tissues and life stages identified 2,102 transcripts that were significantly up-regulated during feeding in *R. microplus*. Of these transcripts as set of 334 transcripts were differentially expressed within the immature life stages, representing a set of genes unique to these stages. Within these up-regulated genes a large subset of transcripts encoded proteins involved in extracellular structures likely essential for development, while the remaining transcripts were mostly involved in nutrient transport and metabolism (protein and lipid), as well as protein synthesis. The latter identified a core of essential proteins necessary to acquire the energy needed for the immature ticks to develop through their life cycle. When considering transcripts that were shared throughout all the life stages and adult tissues a set of 85 transcripts were identified that are conserved for *R. microplus*. From functional classification it is clear that a core of processes related to nutrient transport and metabolism, as well as protein synthesis, control and degradation are highly conserved and therefore essential for all the life stages tested.

From global expression data, some complexes and/or molecules functioning in specific systems are evident. Examples of the latter are components involved in proteolysis/ proteasome complex, as well as mitoses/meiosis and lipid metabolism (Chapter 2 and 3). These biological systems offer new avenues for future studies that can be expanded by using drug-treatment and interactomics studies to elucidate pathways and identify additional targets for rational vaccine design. A component from the proteasome complex of *R. microplus*, ubiquitin, has already been assessed in cattle trials with a 55% vaccine efficacy (Table 1.2) (Almazán *et al*., 2010). Therefore, as an example, additional targets in this system that could form the basis for a multivalent vaccine that could target the pathway and thereby impair its function more effectively. Additionally, receptors and transporters that were identified during this study may also be the subject of future combinatorial and *in silico* screening for the identification of promising new generation acaricides.

However, with regards to the current array, it will be necessary to up-date and improve the design to reflect the current data available and address some limitations that have become apparent during the course of this study. Firstly, the probe content of the current array must be updated as the BmGI has grown from the original ~13,500 to include 14,586 unique

sequences (Quackenbush *et al*., 2000). The latter could be compounded by the 24,238 unique transcripts currently reported from the *R. microplus* genome sequencing, including 9,652 new transcripts identified from the Australian strain of *R. microplus* (Bellgard *et al.*, 2012). However, caution might have to be taken as it has recently been reported that the Australian strain for *R. microplus*, has been reclassified as a distinctly different species *R. australis* and the reported sequences might therefore be species specific (Labruna *et al*., 2009; Estrada-Peña *et al*., 2012). A second consideration that must be addressed is the inclusion of probe duplicates on the arrays to improve statistical analysis and facilitate validation of array results. Lastly, the inclusion of bovine derived blood leukocyte genetic markers to assess the possible contribution of contamination from host DNA that would improve the validity of microarray results (Piper *et al*., 2009; Levings and Roth, 2013). In this regard around 10 sequences sharing a high degree of sequence identity (> 1 x10^{-90}) with *B. taurus* proteins were included in the current array as probable contaminant sequences derived from EST data (data not shown). These sequences however showed no significant regulation on the tick microarray, contributing to the validity of the obtained results along with the numerous tick associated transcripts (i.e. anticoagulants, as well as proteins involved in iron, heme and lipid transport and metabolism) that were found to be up-regulated (Chapter 2 and 3). An example of a tick associated protein is the previously mentioned midgut-specific ferritin 2 that has been shown to have promise as a cross-species protective antigen (Table 1.2.) (Hajdusek *et al*., 2010).

4.2. Reverse vaccinology in anti-tick antigen discovery

For vaccine design in the post-genomic era, modern *in silico* tools offer an alternative approach that can utilize the vast amount of information stored within genomic and transcriptome data to predict novel potential vaccine candidates (Fig 4.2.).

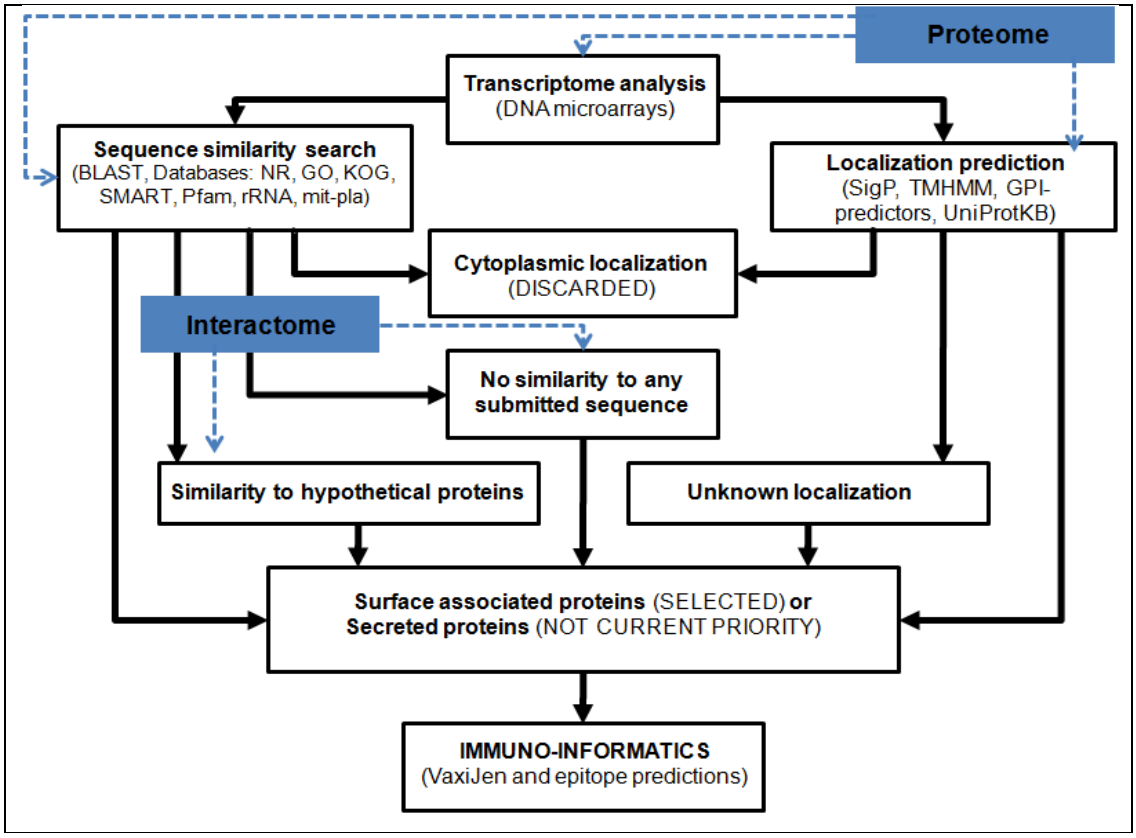

Figure 4.2. Updated strategy for *in silico* identification of anti-tick vaccine candidates using a combined functional genomics and reverse vaccinology approach. Indicated are the contributions that additional proteomics will have on confirming predicted open reading frames and expression levels of their respective encoded proteins, improving similarity searches. Insight into the interactome will greatly assist in the annotation of proteins, as protein function and tertiary structure are more conserved than primary protein sequence. Thus elucidation of biochemical pathways and functional protein complexes may be possible (Maritz-Olivier *et al*., 2012).

A robust methodology was followed for the large-scale screening of available transcriptome data to identify potential vaccine antigens (Fig. 4.2.). The *R. microplus* transcriptome data was subjected to a large-scale reverse vaccinology screening using specific *in silico* tools and 791 promising putative vaccine candidates (conserved in all tissues and life stages) with scores comparable to or higher than that of Bm86, could be identified (Chapter 3). Currently, proteins involved in key processes of the host-parasite interface for example adhesion, invasion, secretion, signaling and evasion of host responses are regarded as good targets for vaccine design (Vivona *et al*., 2008; Guerrero *et al*., 2012). Therefore, targeting these antigens may have significant downstream effects resulting in severe impairment of biological functions in the tick. Furthermore, membrane proteins are regarded as ideal vaccine candidates that will likely interact directly with host immune factors (Rappuoli and Bagnoli, 2011; Guerrero *et al*., 2012). Additionally, immunization against membrane proteins offers the possibility to interfere with the tick-pathogen interface. An example of this is Bm86 that

reduced vector reinfection with *Babesia sp.* by 76% (de la Fuente *et al*., 1998). A non-membrane example is the akirin, subolesin, that reduced vector infection by more than 98% for both *A. marginale* and *B. bigemina* in cattle vaccination trials (Merino *et al*., 2011). The latter antigen has also resently been evaluated in small-scale mouse vaccination trials for the control of other vector species such as sandflies and mosquitoes with efficacies of 72% and 92%, respectively (Moreno-Cid *et al*., 2013). However, in order to fully exploit these proteins and the various processes they mediate as tick control agents, an understanding of their molecular function and mode of action is vital.

The predicted subcellular localization of the identified antigens was determined to identify predicted antigens that are available for immune surveillance, i.e. putative membrane- and secreted antigens. This selection criteria was also based on the fact that Bm86 (which was predicted to be a protective antigen), is a membrane-bound protein that confers protective immunity (Rand *et al*., 1989). Analysis predicted a set of 176 membrane-associated and 86 secreted soluble proteins from which the most promising candidates could be selected for expression in vaccine trials. In this regard, 6 potential plasma membrane-associated/ secreted antigenic transcripts were identified that could be considered for further development. Unfortunately, due to the very high stringency adopted for previous transcriptional profiling comparisons (i.e. Cy5 > 1000, M > 0, *P* value > 0.01; Chapter 3), no additional secreted membrane-associated transcripts could be identified that are conserved across all life stages and tissues tested. However, this does not include transcripts uniquely expressed between the tissues and life stages that were not considered in the current analysis. Re-evaluation of transcriptomic data using less stringent selection criteria (i.e. M ≥ 0) and including other comparisons will expand the current candidate repertoire to include new membrane-associated/ “exposed” transcripts that could be evaluated further.

From this study, two transcripts were predicted to be good antigens (i.e. glycine-rich proteins) (Chapter 3, Table 3.3.), however similar proteins failed to confer protection in vaccination trials (Table 1.2.) (Zhou *et al*., 2006). The latter highlights the need to expand negative antigen datasets that will assist in improving the predictive capacity of immuno-informatic tools such as VaxiJen. Though only candidates that have comparable results to Bm86 were considered in the current strategy (Fig. 4.2.), the choice of protein expression system, antigen modification, vaccine formulation and use of subunits or antigenic peptides, may improve antigenicity and allow inclusion of putative antigens that scored lower than the cut-off chosen

(i.e. native versus recombinant/synthetic antigens, Table 1.2) (Reed *et al*., 2008; Almazán *et al*., 2012). Furthermore, the compliment of soluble and secreted proteins that were not considered within the current methodology still represents a viable source for novel vaccine targets. This is illustrated by vaccination trials using recombinant secreted ferritin 2 (vaccine efficacies greater than 64%), as well as a synthetic peptide conjugate of an intracellular ribosomal protein (vaccine efficacy over 90%) (Chapter 1, Table 1.2) (Hajdusek *et al*., 2010; Rodríguez-Mallon *et al*., 2012).

A complementary study involving a serum screen of antigenic peptides, derived from membrane-associated transcripts (discovered through the proposed methodology), identified immune-dominant peptides that displayed a greater recognition of antisera derived from mice challenged with isolated tick midgut, than those previously confirmed for Bm86 (van Zyl *et al*.,2013, submitted). These results provided the first tentative proof of concept for a large-scale systematic reverse vaccinology approach to tick antigen discovery using functional genomics, reported for tick research. It illustrates how a large set of sequence information (i.e. 13,456 transcripts) can be screened systematically and distilled into a smaller workable pool of potential candidates that can be evaluated in expensive animal trials. The findings of this study can be summarized in Fig.4.3.

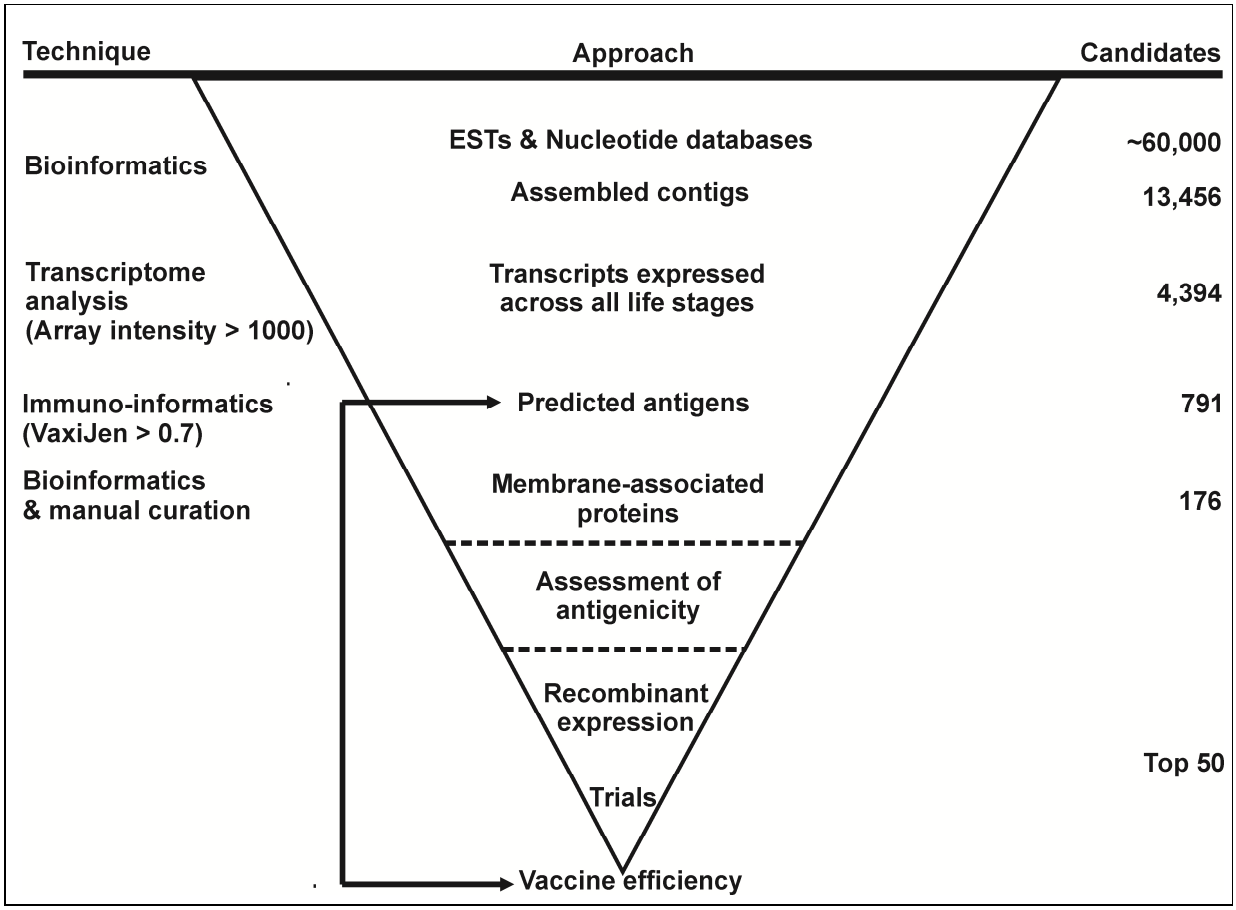

Figure 4.3. Systematic analysis and evaluation of transcripts regulated between immature life stages and adult tissues of *R. microplus* ticks. Indicated is the systematic evaluation of transcriptomic data for the identification of potential vaccine candidates using a combination of bioinformatic and immuno-informatic approaches. Indicated by the black arrow, trial data will be used to expand the available antigen database, as well as improve the current reverse vaccinology approach.

4.3. Additional limitations, considerations and future perspectives

A large number of novel proteins have been described in this work and along with known tick proteins expand our current understanding of tick biology. In spite of this, a number of factors must be taken into consideration for antigens to be developed further into viable vaccines. These include functional annotation of tick transcripts, parasite- , and host- and antigen factors.

4.3.1. Functional annotation of *R. microplus*

Functional annotation of tick transcripts remains a major hurdle in unlocking the full value of the sequence data currently available. Therefore, new tools are desperately needed to

characterize the vast amount of un-annotated genes currently available for *R. microplus* in the BmiGI (v2.1) and the Cattle Tick sequence databases (Wang *et al*., 2007; Bellgard *et al*., 2012). A similar trend was observed in this study, as numerous genes expressed in *R. microplus* during feeding could not be functionally annotated, not even from similarity searches against the *Ixodes scapularis* genome database (Hill and Wikel, 2005). The latter highlights the uniqueness of these transcripts and also stresses the need for a meta-analysis approach to sequence annotation that combines all available sequence databases (nucleotide, protein and structural) with extensive manual curation (as was used in this study). Similar approaches have been successfully employed in the annotation of various tick sialomes and other non-model organisms (Francischetti *et al*., 2010, Yandell and Ence, 2012).

To augment and facilitate functional gene annotation, new *in silico* tools have become available that can be employed for evidence-based (homology) and *ab initio* gene prediction (i.e. AUGUSTUS), using available model organisms and/or mathematical models to predict putative coding sequences (Keller *et al*., 2011; Yandell and Ence, 2012). An additional approach uses protein-protein interaction networks to functionally annotate proteins, based on sequence and/or structural homology, gene co-expression and phylogeny (Salwinski and Eisenberg, 2003; Shoemaker and Panchenko, 2007; Zhang *et al*., 2012). Combinatorial approaches have also been developed that incorporates sequence homology searches, gene expression and protein-protein data, to functionally annotate predicted proteins (i.e. CombFunc) (Wass *et* al., 2012). However, the lack of proteomic and protein interaction data for *R. microplus*, as well as the great evolutionary distance between ticks and other model organisms (i.e. *Drosophila*), severely handicaps the prediction of such protein-protein networks. An additional limitation to gene discovery and functional annotation, is the lack of a central database containing all available tick sequences (including assembled genomes, ESTs, transcriptome and proteome data) that would greatly simplify comparative analysis. In this regard, the recently established Cattle Tick Database could become an invaluable resource, as the basis for a systematic attempt at annotating the full complement of genes and proteins of *R. microplus* (Bellgard *et al*., 2012).

Application of complementary high-throughput techniques such as deep RNA sequencing (RNA-seq), as well as interactome analysis and functional proteomics, will enable verification of open reading frames and aid in functional annotation of transcripts that share little to no

sequence identity with other organisms (Fig. 4.2.) (Brückner *et al*., 2009; Wang *et al*., 2009). RNA-seq is a versatile and highly efficient technique, with a greater detection capacity than standard DNA microarrays (25% more transcripts detected). It has been successfully applied in transcriptional profiling of host immune responses to a pathogen invasion (i.e. HIV and vaccinia virus) or pathogen derived vaccine antigens (Luciani *et al*., 2012).

Limited proteome data for *R. microplus* is currently available and includes only two studies performed on whole larvae and partially fed female midgut tissues (Untalan *et al.*, 2005; Rachinsky *et al.*, 2008; Kongsuwan *et al.*, 2010). Therefore, a complementary proteomic study is currently underway on female *R. microplus* midguts to expand the current repertoire of validated protein sequences (Bosman, unpublished data). However, a major drawback to traditional proteomic methods (i.e. one-dimensional and two-dimensional electrophoresis) is the identification of membrane proteins that do not readily resolve using standard techniques. New affinity purification and high-throughput mass spectrometry techniques enable the *in vitro* determination of membrane protein topology, resolution of receptor complexes and receptor-ligand interactions, as well as elucidation of membrane-associated signaling networks (Savas *et al*., 2011).

The most frequently used interactomic research tool (*in vivo*), remains the yeast two-hybrid (Y2H) screening system that allows the detection of protein-protein interactions in their native environment (cytosolic or membrane-bound) (Brückner *et al*., 2009). This technique has previously been used for elucidation of protein-protein interactions between tick proteins such as subolesin and elongation factor 1α (EF1α) (de la Fuente *et al*., 2008), as well as tick-pathogen interactions between *B. burgdorferi* OpsA and a tick receptor for OspA (TROSPA) (Pal *et al*., 2004). A future large-scale interactomic (Y2H) screening of *R. microplus* tissues and life-stages is envisaged to assist in the elucidation of protein networks and complexes, as well as enabling functional annotation of previously un-annotatable transcripts.

To conclude, a unified nomenclature for sequence annotation is seriously lacking in current transcriptome and proteome data for ticks and is a necessity to avoid confusion between similar entries. In this study, final annotation was based on reviewed sequences published in the UniProt protein and Baunschweig enzyme (BRENDA) databases (Apweiler *et al*., 2011; Scheer *et al*., 2011).

4.3.2. Parasite factors: genetic to biological

It has been mentioned previously that inter- and intra-species differences/variation will contribute to eventual vaccine efficacy in trails. Furthermore, it was observed in this study that similar classes of proteins (sequence variants) have distinct expression patterns within tissues (i.e. anticoagulants and antimicrobials) and life stages (i.e. glycine-rich proteins and mucins) or belong to large protein families (i.e. glycin-rich proteins and serpins) (Chapter 2 and 3). The implication being that in at least the same spieces, a protein can potentially have several distinct forms with variable expression in each life stage/ tissue that may eventually cuase variable sensitivity to host immune responses (influencing vaccine efficacy). The latter may in some cases be a contributing factor in the variable vaccine efficacy obtained between different life stages within the same species. An example of this is the extracellular matrix protein, P29, from *H. longicornis* that showed a 40% mortality in larvae and a 56% mortality in nymphs, while no mortality was observed in adult ticks (Mulenga *et al*., 1999). The ineffectivity of targeting large protein families, such as glycine-rich proteins and serpins, has been demonstrated previously in vaccination trials for different tick species (vaccine efficacies lower than 43%) (Table 1.2.). This may highlight the need for the development of subunit or peptide vaccines directed at highly concerved regions (or antigenic epitopes) within the gene family to target all redundant sequences that could “rescue” targeted protein functions (Guerrero *et al*., 2012).

Another dimension that merits concideration is host immune response modulation as a result of tick secreted compounds (i.e. proteins and chemical compounds such as prostaglandins) during feeding (Willadsen and Jongejan, 1999; Brake and Pérez de León, 2012). It has been shown in *B. taurus* that host responses are modulated away from T-helper 1 (Th1) toward T-helper 2 (Th2) responses and has been implicated in promoting pathogen transmission as interferon gamma (IFNγ) responses are Th1-mediated (Brake and Pérez de León, 2012). An excellent example of this co-adaptation to disease transmission is the pathogen *B. burgdorferi* that utilizes serveral tick-derived molecules to invade and multiply within their mammalian host (de Taeye *et al*., 2013). This has implications for vaccine design, as the efficacy of a vaccine target may depend on a particular immune response that is currently being suppressed by the parasites’ immune modulators. Therefore, multi-valent vaccines targeting these immune modulators may be nessecary to circumvent parasite control over the host immune responses, especially to block dissease transmission (Brake and Pérez de León, 2012;.Guerrero *et al*., 2012).

Finally, parasite biology must also be considered in the design of anti-tick vaccines. To date, vaccination against soft tick species have been less effective as compared to trails in hard tick species, with a single promising candidate, a P-selectin-binding protein (Om44), confering ~50% vaccine efficacy in pigs (Table 1.2) (Garcia-Varas *et al*., 2010). The latter may be largely due to adaptational (biological) differences, as soft ticks require far shorter periods (minutes to a few hours) to feed from hosts compared to hard ticks (days), thus having less time to interact with host responses (Sonenshine, 1991). A different approach (i.e. chemical or biological) may therefore be necessary in this case and will require further indepth investigation and identification of alternative targets/ strategies that could be evaluated for tick control.

4.3.3. Host factors: immunity to biomarkers

An important factor to consider when designing second generation anti-tick vaccines is a full understanding of the underlying mechanisms and criteria required to confer protective immunity against tick infestation in cattle hosts. The latter is needed to enable rational selection of effective antigens that can elicit sustained immune responses in the host and require less booster immunizations (Gurerro *et al*., 2012). It has been shown that a good T-cell response is needed for production of IgG against tick antigens and new research on the determinants needed to elicit cytotoxic T-cell responses via the bovine major histocompatibility complex I (MHCI) has recently been reported (Nene et al., 2012). However, further research is required on both MHCI and MHCII to identify determinants required for T-cell activation and enable prediction of protective antigens using bovine alleles *in silico*.

Comparisons between host humoral and cellular immune responses following vaccination with either effective or ineffective vaccine antigens, would also allow the identification of host immune markers (or immunological biomarkers) that can be exploited to correlate a specific immune response to vaccine efficacy (Levings and Roth, 2013, Fellay *et al*., 2013). As an example, it was shown that protective immunity against *E. ruminantium*, involves the production of Interferon gamma (IFNγ) by activated T cells following vaccination with the major antigenic proteins (Liebenberg et al., 2012). As a result, induction of IFNγ was tested using *in vitro* assays to assess favorable host immune responses and therefore antigen suitability prior to expensive vaccination trials. Therefore studies that evaluate the entire host

response during vaccination in cattle (i.e. DNA and protein arrays) will identify possible markers for use in immune surveillance.

A final consideration regards the breed of animal used in vaccination, as variation within and between breeds will also affect the immune response elicited to a particular vaccine (Piper *et* al., 2009; Fellay *et al*., 2013). As an example, it has been shown that there are significant differences between naturally resistant *B. indicus* and susceptible *B. taurus* cattle in regards to the percentage of immune cell subsets (i.e. leukocytes, cytokines and tick-specific immunoglobulins) produced during immune challenge (Piper *et al*., 2009). In a related study, antiserum recognition of a *R. microplus* serine protease inhibitor was evaluated in these two cattle breeds (Rodriguez-Valle et al., 2012). It was shown that antiserum from *B. indicus* detected the tick antigen well, while antiserum from *B. taurus* could not. It was therefore suggested that this protein may have been a specific adaptation to feeding on resistant cattle and it may therefore be a good candidate for a biomarker of tick resistance.

4.3.4. Antigen factors: formulation and evaluation

In this study, a number of transcripts were identified with poor predicted antigenicity relative to Bm86 (Table 3.3. and Supplementary Table S3.6.). However, these poor antigens can be potentiated by adaptation of their structure, method of production, vaccine formulation and delivery.

A number of adjuvants are currently commercially available and in development that can increase the immunogenicity of a poor antigen, as well as influence the magnitude and nature of the host response elicited (McKee *et al*., 2010). The latter include adjuvants made from alum derivatives, combinatorial oil emulsions and liposomal preparations. Even choice of expression system for protein production can enhance efficiency of a target antigen, by providing post-translational modifications (i.e. glycosylation) or provide membrane products that can act as adjuvants to increase host humoral responses (i.e. *P. pastoris* and MSP1 expression systems) (Chapter 1, Table 1.2) (Rodríguez Valle *et al*., 2001; Canales *et al*., 2009; Almazán *et al*., 2012).

Cutting edge vaccine carrier systems, such as nano/ microparticles, may provide an alternative means for antigen delivery in rational vaccine design. This approach has been

successfully employed in transmission blocking of *Plasmodium berghei* from infected *A. gambiae* mosquitoes in a mouse model vaccinated with microparticles containing a vector derived antigen (Dinglasan *et al*., 2013). Furthermore, these compounds have natural immune boosting abilities, confer prolonged protection due to slow-release of antigen and have been shown to influence the type of immune response elicited, i.e. Type 1 or Type 2 (Fifis *et al*., 2004; Sloat *et al*., 2010; Dinglasan *et al*., 2013; Yue *et al*., 2012). Therefore, a microparticle encapsulated antigen can be tailored to reach a desired immune response endpoint for increased vaccine efficiency.

To date, few examples have been recorded for the application of DNA-based anti-tick vaccines in animal vaccination trials. The potential of DNA vaccines in the control of tick infestation was previously demonstrated during expression library immunization (ELI) studies in mice challenged with *I. scapularis* (Almazán et al., 2003). More recently the antigen, subolesin, has been used in an orally administered DNA vaccine that reduced larval infestation by 52% and reduced transmission of *Borrelia burgdorferi* by 40% (Bensaci *et al*., 2012). Additional undisclosed *R. microplus* antigens, were reported to confer protection against infestation (~75% efficacy) using a DNA-based vaccine in cattle trials (Guerrero *et al*., 2012). Additional advances in this field in regards to increase vaccine stability, improve delivery and priming-boosting of host responses could eventually make anti-tick DNA vaccines commercially feasible.

Evaluation of candidates, whether as individual (subunit) or as multivalent (cocktail) preparations, remains an expensive and often practically daunting task to manage, especially in large test animals (such as cattle) when space and funds are limited (Willadsen, 2008). In this regard, the simplified artificial feeding systems (i.e. specialized feeding chambers or capillary feeding) can be employed as proxies for preliminary evaluation of potential vaccine candidates *in vitro* (Kröber and Guerin, 2007; Gonsioroski *et al*., 2012; Rodriguez-Valle *et al*., 2012). Therefore, ticks fed on antisera produced from vaccinated animals that were immunized with individual or combinatorial antigen preparations, can be assessed for their feeding and reproductive potential (on a more modest scale). Consequently, only the best antigen preparations would be finally selected and evaluated in further exhaustive vaccination trials *in vivo*.

Concluding remarks

This study has established the first microarray platform for tick research in South Africa. This platform was consequently utilized to obtain transcriptional profiling data of an African cattle tick strain (the Mozambique strain) feeding on South African cattle breeds. In this regard, it is interesting to note that this strain is part of a haplotype that is phylogenetically distinct from the American and Australian strains (Oberholster, unpublished data). Additionally, this microarray platform has also been successfully applied in the transcriptome analysis of *R. decoloratus*, an endemic species for which an array platform is currently lacking (van Zyl *et al*., 2013, submitted). From this study, several target proteins were identified from the transcriptome of *R. microplus* that are currently being recombinantly expressed for evaluation in future cattle trials. Previous transcriptome studies investigated *R. microplus* larvae, whole females, as well as midgut and salivary gland tissues of adult males (Saldivar *et al*., 2008; Rodriguez-Valle *et al*., 2010; Mercado-Curiel *et al*., 2011). Therefore, this study represents the first global transcriptome analysis via DNA microarrays of feeding *R. microplus* nymphs and individual adult female tissues. This makes the data presented here an essential resource that can be further exploited to open new avenues for the study of proteins and pathways involved in basic tick biology, as well as for the identification of novel targets for future tick control.

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"Human subtlety will never devise an invention more beautiful, simpler or more direct than does nature. Because in her inventions nothing is lacking, and nothing is superfluous."

Leonardo da Vinci