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A combined functional genomics and *in silico* approach for the identification of anti-*Rhipicephalus* vaccine candidates

by

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Summary

The cattle tick, *Rhipicephalus microplus*, has a debilitating effect on the livestock industry worldwide, owing to its being a vector of the causative agents of bovine babesiosis and anaplasmosis. In South Africa, co-infestation of livestock with *R. microplus* and *R. decoloratus* occurs. An alternative to chemical control methods is sought in the form of an anti-tick vaccine. Using microarray technology, this study aimed at identifying genes that are shared between midgut tissues of adult female *R. microplus* and *R. decoloratus* ticks. In addition, results from another study were used and a reverse vaccinology pipeline was devised to identify putative novel vaccine candidates.

Using a custom oligonucleotide microarray comprising 13 477 *R. microplus* sequences, 2476 genes were found to be shared between the two abovementioned tick species. In addition, 136 were found to be more abundantly expressed in *R. decoloratus* and 1084 in *R. microplus*. Chi-square analysis revealed that genes involved in lipid transport and metabolism are significantly over-represented in *R. microplus* and *R. decoloratus*. With vaccine design in mind, considering genes that are expressed in the midgut of both tick species, 6730 genes were identified and of these, 1224 are predicted to contain membrane-spanning helices.

One major limitation to anti-tick vaccine discovery in the past has been a lack of candidates to evaluate, combined with limited knowledge of the transcriptome of *R. microplus*. This study identified a large pool of transcripts that are expressed in the midgut of both *R. microplus* and *R. decoloratus* adult females. Of these, those that are expressed in larvae, nymphs and the midgut were identified in another study and an *in silico* pipeline was used to predict membrane-bound protective antigens using an alignment-free approach, which led to the identification of seven proteins that were predicted to be both glycosylphosphatidylinositol (GPI)-anchored and more likely than Bm86 to be protective antigens. Finally, epitopes were predicted and corresponding synthetic peptides were evaluated using enzyme-linked immunosorbent assay (ELISA), resulting in the identification of three epitopes that are recognized to a greater extent than previously published Bm86 epitopes, when using murine serum raised against membrane proteins from the midgut of *R. microplus.* These results are significant because novel *R. microplus* proteins that are also present in *R. decoloratus* were identified. Trials using recombinant protein are under way and this will ultimately validate the experimental methodology discussed in this dissertation.

Finally, regardless of whether the next-generation anti-tick vaccine has been discovered, this study also led to the identification of novel reference genes that can be used for real-time PCR experiments.

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List of abbreviations

BLAST	Basic local alignment tool
bp	Base pair
CD	Cluster of differentiation
cDNA	Complementary DNA
dCAS	Desktop cDNA annotation system
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EST	Expressed sequence tag
GDP	Gross domestic product
GO	Gene ontology
GPI	Glycosylphosphatidylinositol
IFN-γ	Interferon gamma
Ig A/M/G	Immunoglobulin A/M/G
KOG	Eukaryotic orthologous groups
LIMMA	Linear models for microarray analysis
LOESS	Locally weighted scatterplot smoothing
MHC	Major histocompatibility complex
MIAME	Minimum information about a microarray experiment
msp1	Major surface protein 1
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNAi	RNA interference
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Semi-qPCR	Semi-quantitative real-time PCR
TAE	Tris acetic acid EDTA
TBS	Tris-buffered saline
TEMED	Tetramethylethylenediamine
Th 1/2	T helper 1/2
TMHMM	Transmembrane helices Markov model
Tris	Tris(hydroxymethyl)aminomethane hydrochloride

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Chapter 1
Literature Review

1.1 Introduction

1.1.1 Ticks in South Africa

In South Africa, one of the biggest problems that livestock farmers face is ticks and tick-borne diseases. The most important tick-borne diseases affecting cattle are corridor disease, heartwater, redwater and gall sickness, caused by *Theileria*, *Ehrlichia*, *Babesia* and *Anaplasma* species, respectively (Marufu, 2008; Walker et al., 2003). These and other pathogens are transmitted by various ticks, found in South Africa (Table 1.1).

Table 1.1. Ticks and tick-borne diseases that affect cattle in South Africa

Indicated are the most prominent tick-borne diseases affecting the livestock industry in South Africa (Walker et al., 2003).

Pathogen	Common and scientific names of disease	Tick vector(s)	References
Ehrlichia ruminantium	Heartwater (Ehrlichiosis or Cowdriosis)	*Amblyomma hebraeum* *Amblyomma variegatum*	(Liebenberg et al., 2012)
Theileria mutans	Corridor disease (Benign bovine theileriosis)	*Amblyomma hebraeum* *Amblyomma variegatum*	(Chaisi et al., 2012)
Theileria velifera	Corridor disease (Benign bovine theileriosis)	*Amblyomma variegatum*	(Chaisi et al., 2012)
Theileria parva	East coast fever, Buffalo, Corridor or January disease (Theileriosis)	*Rhipicephalus appendiculatus*	(Sibeko et al., 2011)
Babesia bigemina	Redwater (Bovine babesiosis)	*Rhipicephalus decoloratus* *Rhipicephalus microplus*	(Terkawi et al., 2011)
Babesia bovis	Redwater (Bovine babesiosis)	*Rhipicephalus microplus*	(Terkawi et al., 2011)
Anaplasma bovis	Gall sickness (Bovine ehrlichiosis / Anaplasmosis)	*Rhipicephalus appendiculatus*	(Pfitzer et al., 2011)
Anaplasma marginale	Gall sickness (Bovine ehrlichiosis / Anaplasmosis)	*Rhipicephalus decoloratus* *Rhipicephalus microplus* *Rhipicephalus evertsi evertsi* *Hyalomma marginatum rufipes*	(Pfitzer et al., 2011)

Ticks are classified in the class Arachnida, subclass Acari, order Parasitiformes and suborder Ixodida. The approximately 894 species are divided into three families, the Argasidae (soft ticks), Ixodidae (hard ticks) and Nuttalliellidae (Guglielmone et al., 2010; Klompen et al., 2002). To date, some 80 hard tick species have been described in South Africa (Horak et al., 2002). Ticks of economic importance (Table 1.1) in South Africa are indicated in Figure 1.1, where the evolutionary relationship between tick genera is shown. They are classified in the *Rhipicephalus* and *Amblyomma* genera. As can be seen, these are the 3rd and 4th largest genera of hard ticks.

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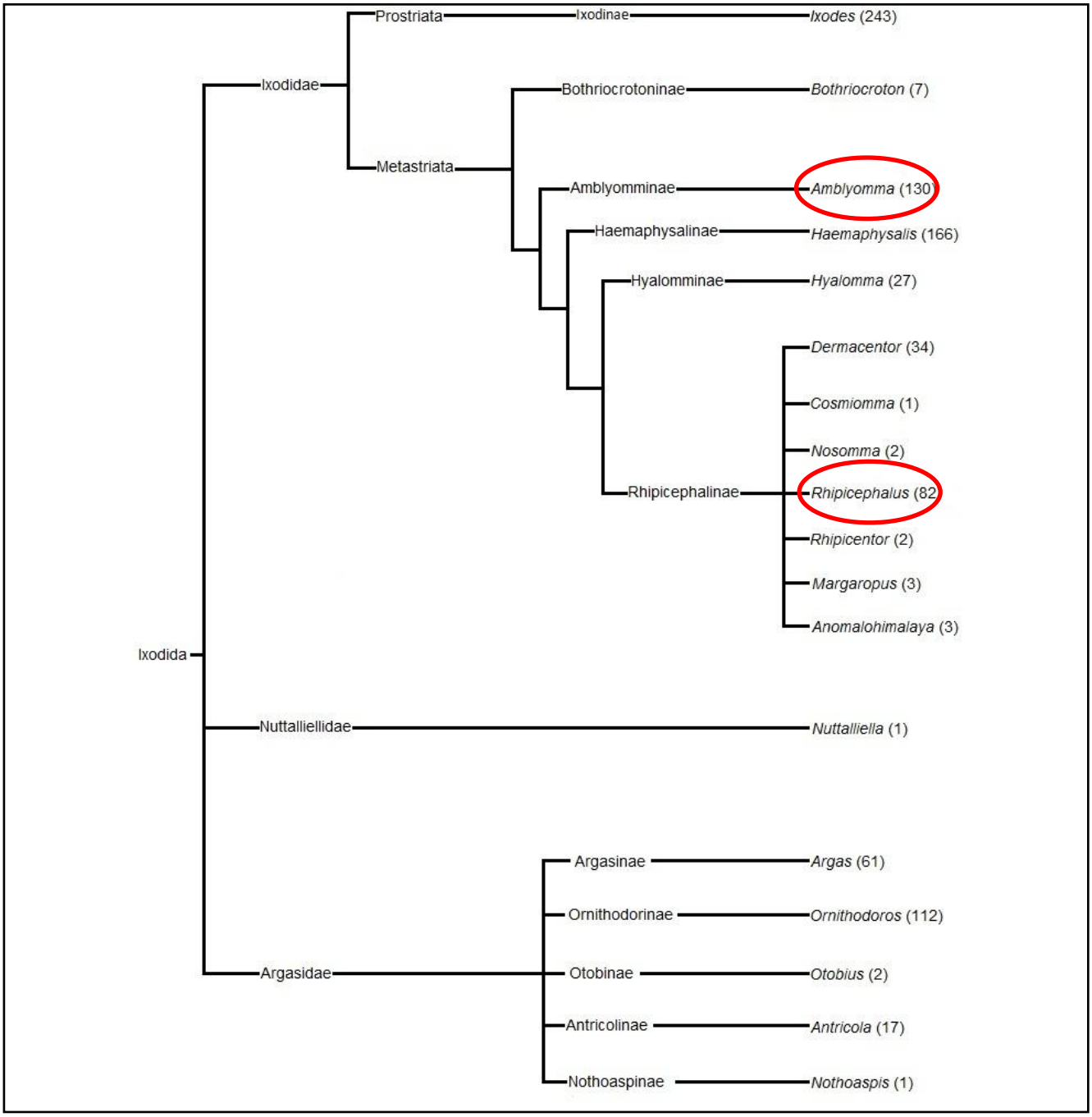

Figure 1.1. Phylogenetic relationship between ticks

Shown here is the phylogenetic relationship between ticks. Adapted from (Sonenshine, 1991). Numbers in brackets indicate the number of species in each genus (Barker and Murrell, 2002; Guglielmone et al., 2010; Klompen et al., 2002).

1.1.2 *Rhipicephalus* ticks in South Africa

Despite the large number of tick species, those of primary veterinary importance in South Africa are *Amblyomma hebraeum*, *Rhipicephalus microplus*, *R. decoloratus*, *R. appendiculatus* and *R. evertsi evertsi* (Horak et al., 2002; Jongejan and Uilenberg, 2004;

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Nyangiwe and Horak, 2007). In this study, emphasis was placed on *R. microplus* and *R. decoloratus* because they most adversely affect the livestock industry in South Africa.

I. *R. microplus* and *R. decoloratus*

Rhipicephalus decoloratus is indigenous to Africa, but *R. microplus* has been introduced to the continent during the nineteenth century, when cattle were imported from Madagascar after the rinderpest epidemic in 1896 (Tønnesen et al., 2004; Zeman and Lynen, 2010). *Rhipicephalus decoloratus* is more resistant to a dry and cold environment, and can parasitize a wider variety of hosts than *R. microplus*. This includes sheep and wild African ungulates (Jongejan and Uilenberg, 2004; Lynen et al., 2008; Solomon and Kaaya, 1998). Furthermore, recent reports have indicated that *R. microplus* is displacing endemic *R. decoloratus* in South Africa (Tønnesen et al., 2004), spreading into areas that were previously unoccupied (Lynen et al., 2008). This is due to the fact that *R. microplus* has a higher reproductive rate than *R. decoloratus* and is less susceptible to host resistance (Lynen et al., 2008; Zeman and Lynen, 2010). This is a concern, as *R. decoloratus* ticks transmit *B. bigemina*, whereas *R. microplus* transmits both *B. bigemina* and *B. bovis* (Homer et al., 2000; Jongejan and Uilenberg, 2004). Compared to *B. bigemina,* serious infections with *B. bovis* lead to cerebral babesiosis, resulting in paralysis as well as fever, which results in premature abortions (Bock et al., 2004; Suarez and Noh, 2011). In South Africa, co-infestation of cattle with both *R. microplus* and *R. decoloratus* occurs (Figure 1.2).

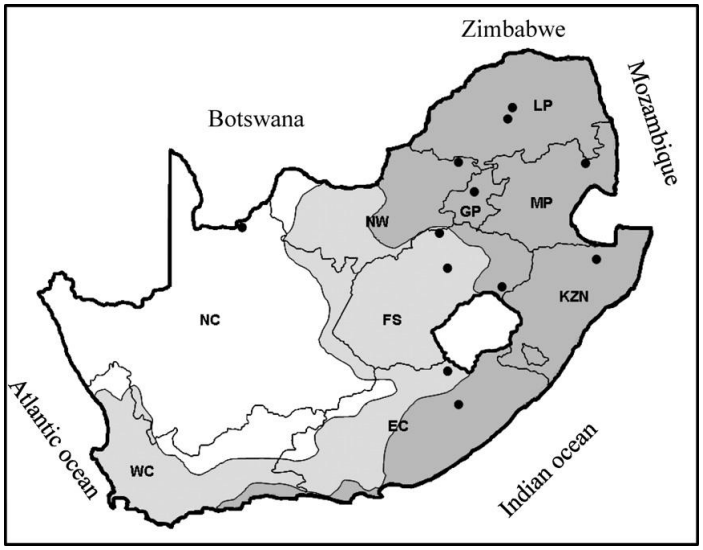

Figure 1.2. Distribution of *R. microplus* and *R. decoloratus* in South Africa

Gray indicates the distribution of *R. decoloratus*, while dark gray indicates areas where both *R. decoloratus* and *R. microplus* occur (Terkawi et al., 2011).

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II. Morphological and genetic differences

Macroscopically, *R. microplus* and *R. decoloratus* appear virtually identical but the adults can be distinguished based on some key morphological differences. The teeth on the hypostome (feeding apparatus) of *R. decoloratus* adult females are arranged in 3+3 columns (Figure 1.3A), whereas in *R. microplus*, they are arranged in 4+4 columns (Figure 1.3B).

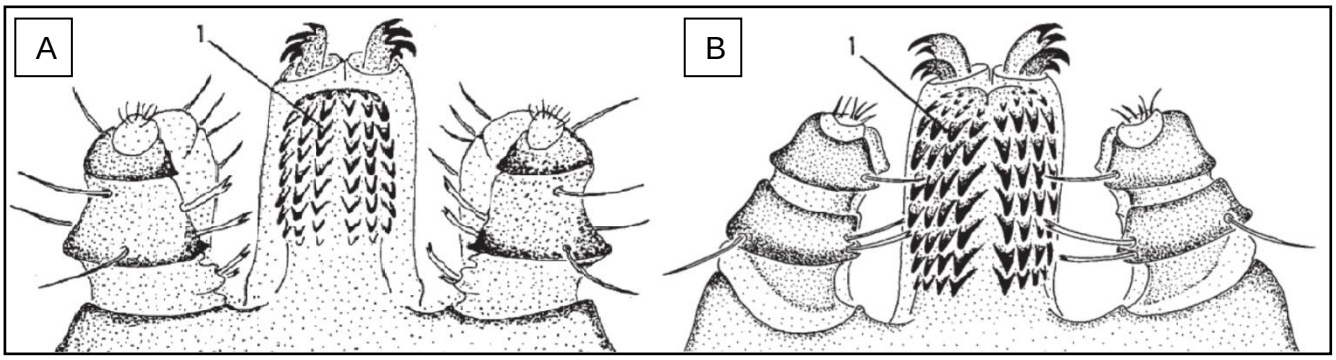

Figure 1.3. Mouthparts of adult *R. decoloratus* and *R. microplus*

Shown here is the feeding apparatus of adult *R. decoloratus* (*A*) and *R. microplus* (*B*) ticks, shown with 1, to indicate their morphological differences (Walker et al., 2003).

Differences between adult males are more distinct, as the spurs on the adanal plates of *R. decoloratus* (Figure 1.4A) can easily be distinguished from *R. microplus*, where no distinct spurs are found (Figure 1.4B).

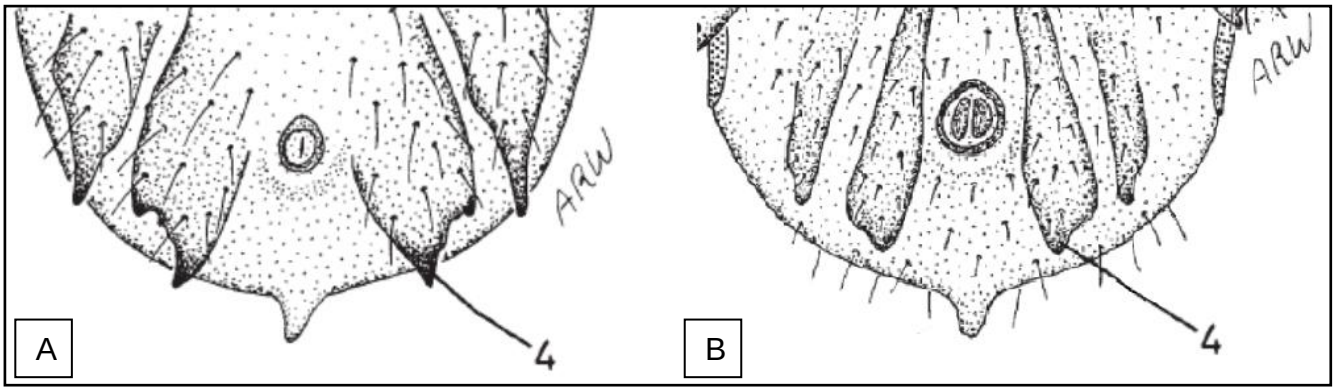

Figure 1.4. Ventral aspect of adult male *R. decoloratus* and *R. microplus* ticks

Shown here are the ventral aspects of adult male *R. decoloratus* (*A*) and *R. microplus* (*B*) ticks. Note the distinct adanal spurs in (*A*) compared to (*B*), indicated with a 4 (Walker et al., 2003).

Apart from their morphological similarities, molecular techniques have also shown that *R. microplus* and *R. decoloratus* are related phylogenetically. Alignments with their mitochondrial 12S and cytochrome c oxidase I sequences (Murrell et al., 2001b) showed 93% and 88% sequence identity, respectively. Sequence analysis of internal transcribed spacer 2 of rhipicephaline ticks indicates that *R. annulatus* is more closely related to *R. microplus*, followed by *R. decoloratus* (Murrell et al., 2001a). PCR-based techniques have been developed to differentiate between these ticks (Lempereur et al., 2010). The species

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barrier between *R. microplus* and *R. decoloratus* is further indicated by the observation that interbreeding between these ticks leads to sterile progeny, creating a barrier zone that is difficult for either tick species to cross (Bock et al., 2004).

III. Life cycle

Most ixodid ticks have a three-host life cycle, which means that three different host species are required for completion of the life cycle. In comparison, both *R. microplus* and *R. decoloratus* are one-host ticks, requiring only one host to complete their life cycles (Sonenshine, 1991).

The one-host ixodid life cycle (Figure 1.5) starts with the hatching of eggs, after which larvae quest to find a suitable host. The larvae attach to the host and feed to engorgement and moult into nymphs, which, after successful feeding, moult into adults. Adults feed to engorgement and mate, after which females drop from the host before oviposition takes place (Sonenshine, 1991).

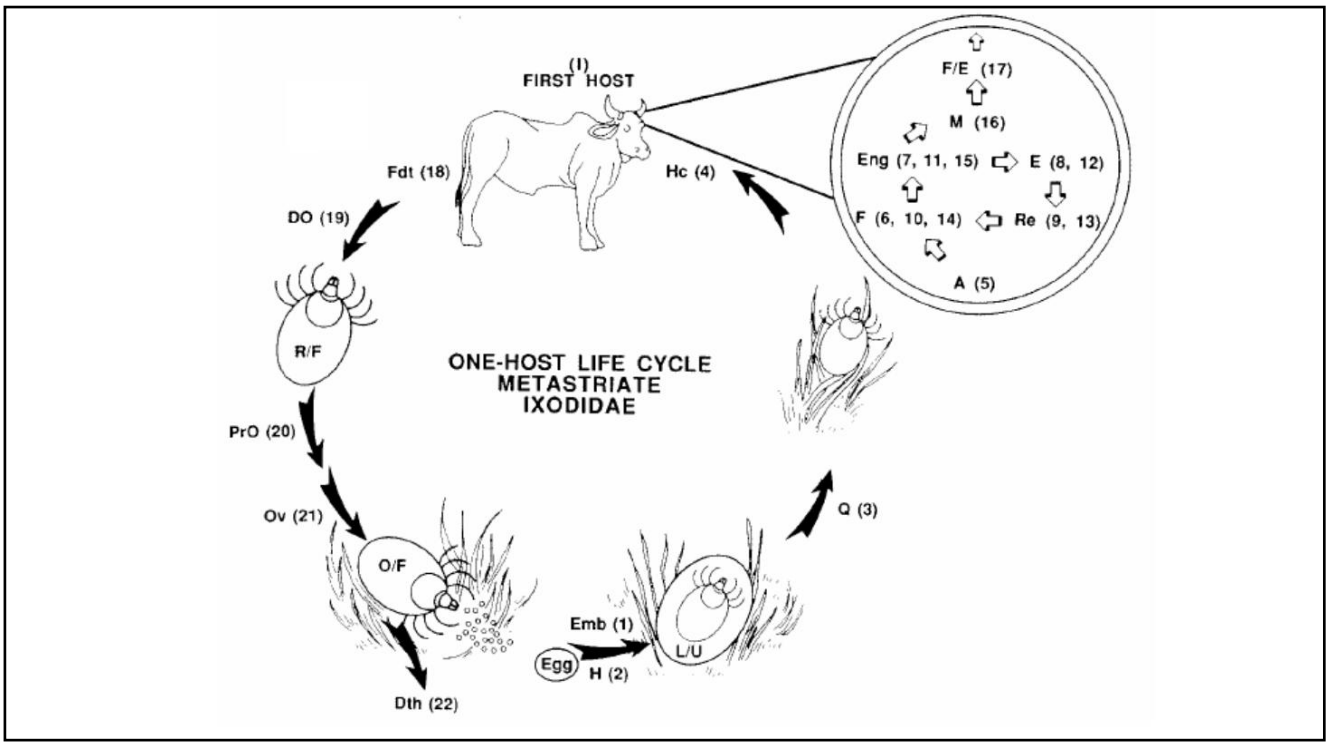

Figure 1.5. Life cycle of a one-host ixodid tick

The one-host life cycle starts when eggs hatch (*2*) after successful embryogenesis (*1*) and larvae start their questing behaviour (*3*). After host contact (*4*), larvae attach (*5*) and feed (*6*) until engorgement (*7*). The larvae ecdyse (*8*) and moult into nymphs, which reattach (*9*) to feed (*10*) until engorgement (*11*). The nymphs undergo ecdysis (*12*) and moult into adult ticks, which reattach (*13*) to feed (*14*) and engorge (*15*). Adults mate (*16*) and females feed until fully engorged (*17*), before detaching from the host (*18*) and dropping off (*19*). The life cycle concludes with preovipositional development (*20*), oviposition (*21*) and death of the female (*22*) (Sonenshine, 1991).

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1.1.3 Effects of tick infestation

I. Tick infestation alone

Tick infestation has direct effects such as a decreased appetite, anaemia and weight loss (Jonsson, 2006; Rajput et al., 2006) and indirect effects due to the various pathogens that ticks transmit. The first is due to the secreted biologically active molecules from the salivary glands that have profound effects on the host's physiology. This is necessary for successful feeding, as insertion of the hypostome causes damage to blood vessels and the dermis, activating haemostatic and inflammatory pathways in the host. If these host immune responses are not sufficiently suppressed, the tick will not be able to feed successfully. Tick saliva contains, amongst others, various enzymes, enzyme inhibitors, host protein homologues and immunoglobulin and amine binding proteins, to ensure a successful blood meal (Steen et al., 2006). As a result, the host's immune system becomes compromised. For example, *R. microplus* infestations lead to a decline in the number of circulating T lymphocytes, reducing antibody titres after vaccination with ovalbumin (Inokuma et al., 1993). Moreover, proteins in the salivary glands from *R. microplus* lead to a decrease in Th1 promoting cytokines, potentially exacerbating the pathogenicity of tick-borne diseases (Brake and Pérez de León, 2012).

II. Tick-borne diseases

However, tick-borne diseases are far more detrimental to the health of cattle, as they can be lethal (Bock et al., 2004). The four most prominent diseases affecting the livestock industry are anaplasmosis, babesiosis (redwater), cowdriosis (heartwater) and theileriosis (Marcelino et al., 2012). It is estimated that 80% of the world's cattle population is at risk of ticks and tick-borne diseases (de Castro, 1997). This would therefore mean that 11.28 million cattle in South Africa are at risk, as there are 14.1 million heads of cattle in total (DAFF, 2011).

Anaplasma marginale, an obligate rickettsial parasite, is the most prevalent tick-borne pathogen of cattle, where it causes anaplasmosis (Kuttler, 1984; Suarez and Noh, 2011). This mild to severe haemolytic disease is transmitted primarily by ixodid ticks, including *R. microplus* and *R. decoloratus* (Table 1.1)(Kocan et al., 2010). In cattle, *Babesia bovis* and *B. bigemina* are the primary causative agents of babesiosis. *Babesia bovis* is transmitted by *Rhipicephalus microplus* and *Rhipicephalus annulatus* ticks, while *Babesia bigemina* is transmitted by *R. microplus*, *R. decoloratus* and *R. annulatus* (Bock et al., 2004). Historically, these two tick-borne diseases were collectively known as cattle tick fever, as they had similarities, such as both being caused by intraerythrocytic parasites that are transmitted by

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Rhipicephalus ticks. Control of these diseases is based on the combination of vector control (acaricides) and parasite control (attenuated vaccines) (Suarez and Noh, 2011). The development of acaricide resistance, the variability in the efficacy of the available vaccines and these pathogens' ability to escape the host's immune response via antigenic variation exacerbate the effect of these pathogens on the livestock industry (Suarez and Noh, 2011).

Another disease of veterinary importance is heartwater, caused by the bacterium *Ehrlichia ruminantium*. It is transmitted by ticks belonging to the *Amblyomma* genus and is limited to sub-Saharan Africa and certain Caribbean islands (Bekker et al., 2002). Finally, Corridor disease (theileriosis) is transmitted by ticks belonging to the genera of *Rhipicephalus*, *Hyalomma* and *Amblyomma* (Minjauw and McLeod, 2003).

Diagnosis and treatment of diseases transmitted by R. microplus

The severity of tick-borne diseases is dependent on how easily they can be diagnosed and treated. Pharmacological intervention of tick-borne diseases is possible but the problem lies with their diagnosis. For example, one-way cross-reactivity of antibodies to antigens from *B. bovis* with those from *B. bigemina* can lead to false positive results. Serological tests for the detection of *B. bovis* are also hampered when animals start clearing the infection, leading to false positive results. As an alternative, PCR-based assays can be used to demonstrate the presence of tick-borne diseases but they are expensive and can be of little use to determine the severity of the infection (Bock et al., 2004).

Babesiosis can be treated with drugs such as diminazene aceturate and imidocarb dipropionate. However, these drugs can interfere with the development of immunity following vaccination using live vaccines. To circumvent this problem, long-acting oxytetracyclines can be used, significantly reducing parasitaemia without inhibiting the development of immunity. Unfortunately, they are unable to control infections with virulent strains (Bock et al., 2004). For the treatment of anaplasmosis in cattle, tetracyclines can be used to reduce parasitaemia but are ineffective at reliably eliminating infection (Aubry and Geale, 2011). Finally, pharmacological intervention of tick-borne diseases is costly and may select for drug-resistant pathogens. The importance of tick control therefore lies therein that ticks serve as vectors of potentially lethal pathogens that are both difficult to diagnose and treat.

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1.1.4 Effect of ticks on the South African economy

In South Africa, the agricultural sector gross domestic product (GDP) is estimated to be 7.174 million US$, representing 3.9% of the country's total GDP. The livestock industry, however, is responsible for 46% of this amount, indicating its importance in the South African economy, where trade in hides and skins, milk and beef represents 70% of exports (FAO, 2005). The 14.1 million heads of cattle are responsible for providing jobs to some 500,000 people, along with an additional 2,125,000 people whose livelihood depends on the livestock industry. Of these, 8.46 million heads of cattle are owned by commercial farmers and 5.64 million by emerging and communal farmers (DAFF, 2011).

For many farmers, the only viable form of agriculture is livestock farming. As a result, approximately 69% of agricultural land in South Africa is used for pastures (DAFF, 2011). This heavy reliance on the livestock industry renders farmers vulnerable to disease outbreaks that could have devastating effects. In the 1890s, 80-90% of cattle contracted the fatal rinderpest, resulting in some 2.5 million deaths. Fortunately, in 1897, Robert Koch reported that bile from an ox that had died from rinderpest could be used to immunise other cattle. This led to the eradication of rinderpest in South Africa in 1905 (Mack, 1970).

Taken together, this highlights the importance of veterinary research in South Africa. Tick-borne diseases such as corridor disease, where untreated cases can have fatality rates as high as 90% (Minjauw and McLeod, 2003), could have similar disastrous consequences for the livestock industry unless the control of ticks and tick-borne diseases becomes a priority.

1.1.5 Tick control

As the geographical distribution of tick-borne diseases coincides with that of the tick vectors (Bock et al., 2004), anti-tick control measures would help alleviate pathogen loads in herds. Such control measures would have to be effective against a wide variety of ticks, as the elimination of one tick species would lead to an increase in the population size of another. This is especially true for *Rhipicephalus* ticks, where *R. microplus*, *R. decoloratus* and *R. annulatus* ticks occupy the same geographical areas and may even infest the same host (Fragoso et al., 1998).

One of the factors contributing to the severity of tick-borne diseases in Africa is the susceptibility of exotic cattle breeds to ticks, despite their higher productivity (George, 2000).

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Various methods for controlling ticks have been attempted but with variable results. These include the use of tick-resistant cattle (de Castro, 1997), fungal species that target ticks (Kaaya and Hassan, 2001), plant extracts (Elango and Rahuman, 2011; Sousa et al., 2011) and acaricides, which will be discussed below.

I. Chemical control

Acaricides have been the main form of tick control since the introduction of arsenic-based dips in South Africa in 1893 (George, 2000). Resistance to arsenic-based compounds developed within 40 years, after which organochlorine and organophosphate-based chemicals were developed. This was followed by amidines, insect growth regulators and macrocyclic lactones (Graf et al., 2004). Unfortunately, resistance to virtually all classes of acaricides is becoming a global problem. It is said that resistance can be expected to develop within 10 years after the introduction of an acaricide (Rajput et al., 2006). Despite the increasing incidence of resistance to acaricides, these chemicals still remain the preferred method of tick control. The use of acaricides, especially organophosphates, has been associated with environmental pollution and chemical residues in food products. In South Africa, more than 20% of expenses relating to veterinary products are acaricides, amounting to 17.5 million US$ in 2003 (based on an exchange rate of 1ZAR = 10US$) (Peter et al., 2005). Moreover, the development of a novel acaricide has been estimated to cost around 100 million US$ (Graf et al., 2004). A study by the University of the Free State found that during 1998-2001, 36.1% of tick populations from KwaZulu Natal and the Eastern Cape were resistant to one major group of acaricides and 19.5% against two. During 2005-2008, however, this increased to 43.5% and 37.9%, respectively. In addition, the development of resistance to all three major groups of acaricides (amidines, synthetic pyrethroids and organophosphates) was noted in 2.25% of tick populations (Pesticide Resistance Testing Facility, University of the Free State, personal communication). These results show how wide spectrum acaricide resistance is becoming a reality in South Africa, necessitating the evaluation of alternative forms of tick control.

II. Cattle breeds

When cattle are infested with ticks, a form of naturally acquired immunity develops. This form of immunity starts with an immediate hypersensitivity reaction, which is insufficient to protect susceptible *B. taurus* cattle against ticks such as *R. microplus* (Willadsen and Kemp, 1988). Resistance to ticks is a heritable trait but the underlying molecular mechanisms remain to be elucidated (Jensen et al., 2007). It is well known that *Bos indicus* cattle are more resistant to

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tick infestations than *Bos taurus*. Exposed to the same number of ticks, purebred Brahmans' tick burdens are one tenth of that of Herefords. As can be expected, crossbreeds have intermediate resistance (Jonsson, 2006). A lot of inter-species variation also occurs, although highly resistant *B. taurus* cattle are very rare (Utech and Wharton, 1982). As ticks are increasingly becoming resistant to acaricides, using cattle that are resistant to tick infestation may form part of more sustainable environmentally-friendly practices (Donald, 1994; Frisch, 1999). Commercial farmers therefore need to make a compromise between using more productive and tick-susceptible *B. taurus* cattle and more resilient but less productive *B. indicus* cattle (Jensen et al., 2007).

Over the last 50 years, a more resistant line of cattle has been bred by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) from Australia. This *B. taurus* breed is a hybrid between Shorthorn and Hereford cattle, referred to as the Adaptaur (de Castro, 1997). Use of this breed might become an attractive alternative to chemical acaricides (de Castro, 1997). A microarray study was conducted to analyse gene expression differences between Adaptaur animals with high and low resistance to ticks. Collagen, which provides structural rigidity to the skin, was upregulated in highly resistant cattle. This was coupled with the downregulation of various immune responsive genes such as chemokine receptor 1 and *Bos taurus* MHC I antigen, showing that cattle might acquire resistance to ticks by altering the physical nature of their skins, along with being less responsive to the immunomodulatory components of tick saliva (Wang et al., 2007). This form of resistance is different to that previously noted in *B. taurus* cattle, where acquired resistance to ticks was shown to be effected via a T cell-mediated / hypersensitivity reaction (Schleger et al., 1981; Willadsen and Kemp, 1988). The T cell response has also been shown to be upregulated during tick infestation in *Bos indicus*, compared to *Bos taurus*, where inflammatory responses were more highly upregulated (Piper et al., 2009). This suggests that *B. taurus* cattle develop tick resistance via cellular immunity. They are simply less effective in doing so than *B. indicus* cattle, which are more resistant to the immunomodulatory effects of ticks (Piper et al., 2010). It is believed that due to co-evolution with *R. microplus*, the *B. indicus* host has developed the ability to suppress its response to infestation with this tick. Conversely, *B. taurus* cattle have only recently been exposed to *R. microplus*. As a result, their immune systems overreact, with the ensuing inflammatory responses leading to an environment rich in bodily fluids, which in fact might aid the tick in successfully acquiring a blood meal (Piper et al., 2010).

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III. Immunological control of ticks

Ground-breaking work was performed by Trager and colleagues (1939), who showed that extracts from various tissues of *Dermacentor variabilis* could be used to immunise guinea pigs against ticks (Labarthe, 1994). This was the first example of experimentally immunising animals against ticks. In 1986, it was demonstrated that immunisation of cattle with a crude extract from adult female *R. microplus* ticks protected cattle against subsequent tick challenge (Johnston et al., 1986). Compared to that acquired during natural infestation, vaccine-induced immunity to ticks is antibody-mediated (Willadsen and Kemp, 1988). Carbohydrate moieties are essential for the induction of sufficient immunity against *R. microplus* ticks, as treatment of gut membrane proteins with the oxidative agent periodate abolishes their protective capacity (Lee et al., 1991; Pazur et al., 1970). One of the effects on female ticks was a 70% reduction in fecundity, and damage in the gut was observed in 60% of female ticks. Female ticks with a damaged gut appeared red, as host erythrocytes had leaked into the haemolymph. Unique about this observation was that gut damage had never been reported in the rejection of ticks by cattle with naturally acquired resistance (Agbede and Kemp, 1986). This response was antibody-mediated, supporting the hypothesis that a vaccine against the cattle tick could be developed (Willadsen and Kemp, 1988). In order to identify the protein responsible for this response, approximately 50,000 adult female ticks were dissected and proteins from their guts were isolated and sub-fractionated. Cattle were then immunised and the proteins that conferred protection were identified.

A low-abundance glycoprotein, with an apparent molecular weight of 89 kDa, was identified and is known as Bm86 (Willadsen, 2006; Willadsen et al., 1989). It is bound to the membrane via a glycosylphosphatidylinositol anchor (Rand et al., 1989; Willadsen et al., 1989). When expressed recombinantly using *Pichia pastoris*, Bm86 is highly immunogenic and glycosylated, as opposed to when using *Escherichia coli* (García-García et al., 1998a) but the carbohydrate components of Bm86 alone do not confer protection against *R. microplus* (Willadsen and McKenna, 1991). Bm86 expressed in *P. pastoris* can even act as an immunostimulatory adjuvant (García-García et al., 1998b). It has also been determined that the effect of IgG alone, or with the aid of complement, is what causes damage to the tick gut. Despite this, protection due to cellular immunity cannot be excluded, especially since host lymphocytes were found in the midgut of female ticks feeding on vaccinated cattle (Kemp et al., 1989). Since cytotoxic T cells eliminate infected host cells (Larregina and Falo, 2005), this matter remains controversial.

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Initial reports on the Bm86 vaccine were highly favourable, indicating that three doses of purified antigen were enough to lead to a 92% reduction in the subsequent larval progeny (Willadsen et al., 1989). As part of an integrated pest management programme, the vaccine led to a two-third reduction in the number of acaricide treatments necessary to maintain acceptable levels of tick infestation (de la Fuente et al., 1998). The vaccine also confers protection against various other hard tick species, such as *Rhipicephalus appendiculatus* (Canales et al., 2009), *Hyalomma anatolicum anatolicum*, *H. Dromedarii*, *Rhipicephalus decoloratus* (de Vos et al., 2001) and *R. annulatus* (de la Fuente et al., 1999). Moreover, vaccination with Bm86 also led to a decline in the transmission of tick-borne pathogens (de la Fuente et al., 2007).

The Bm86-based vaccine suffers from many drawbacks, however. A high variability in the efficacy of this vaccine against *R. microplus* has been reported to range from 51% to 91% (de la Fuente et al., 1999). Moreover, against the *R. microplus* strain A from Argentina, the vaccine confers no protection. This has been linked to inter-strain sequence variation in the Bm86 gene (Freeman et al., 2010; García-García et al., 1999). The relationship between inter-strain sequence variation in the Bm86 gene (from the Yeerongpilly strain) and the efficacy of Bm86-based vaccines is not clear, as the vaccine's efficacy is below 60% when used against certain Mexican strains sharing sequence homology above 90% (de la Fuente et al., 2000). Furthermore, regular vaccinations are necessary, as the host does not come into contact with Bm86 during natural infestations (Nuttall et al., 2006). Due to the reported variability in its efficacy and other industrial considerations, TickGARD is no longer commercially available. GAVAC, however, is still marketed in North and South America, despite its reported shortcomings and a lack of widespread acceptance of this vaccine (Guerrero et al., 2012).

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1.1.6 Anti-tick vaccines – a reality?

To date, some 13 proteins have been evaluated as vaccine candidates against *R. microplus* (Table 1.2), with their reported efficacies ranging from 0-81%. However, as the genome of *R. microplus* contains some 13,500 putative open reading frames (Maritz-Olivier et al., 2012), this represents but a fraction of the total number of genes that could be evaluated.

Table 1.2. *R. microplus* antigens tested to date in cattle vaccine trials

Vaccination efficacy of antigens from *R. microplus*, indicated as follows: (-), no significant effect; (+), >25% efficacy; (++), 25-50% efficacy; (+++), 50-75% efficacy; (++++), 75-100% efficacy. Adapted from M.E. Botha, M.Sc thesis in preparation.

Antigen	Biological role	Vaccine efficacy	Reference
BmTI	Trypsin inhibitor. Putative role in larval attachment and feeding	+++	(Andreotti et al., 2002)
Bm95	Gut protein of unknown function. Bm86-like protein from the Argentinean strain of *R. microplus*	++++	(Canales et al., 2009) (Kumar et al., 2009) (Willadsen, 2004)
Bm86	Gut glycoprotein of unknown function. Putative role in digestion	+ to +++	(Cunha et al., 2012) (Willadsen, 2004) (Willadsen et al., 1989)
BYC	Aspartic proteinase that plays a role in embryogenesis	++	(da Silva Vaz Jr. et al., 1998)
Bm91	Sequence similarity with mammalian angiotensin-converting enzymes	++	(Riding et al., 1994)
Vitellin	Most abundant protein in *R. microplus* eggs	++	(Tellam et al., 2002)
VTDCE	Cysteine endopeptidase that plays a role in embryogenesis	+	(Seixas et al., 2008)
5'-Nucleotidase	5'-Nucleotidase that degrades nucleotide mono, di- and triphosphates, possibly for purine salvage.	-	(Hope et al., 2010)
Ferritin 2	Maintenance of iron homeostasis	+++	(Hajdusek et al., 2010)
Various mimotopes	Various putative biological functions	-	(Prudencio et al., 2010)
BMA7	Mucin-like membrane protein of unknown function	+	(McKenna et al., 1998)
Subolesin-MSP	Plays a role in tick fertility	++++	(Almazán et al., 2012)
Elongation factor-1α	Possible transcription factor	++	(Almazán et al., 2012; de la Fuente et al., 2008)
Ubiquitin-MSP	Tagging of proteins for proteasomal degradation	ND	(Almazán et al., 2012)

The numerous factors that need to be considered for a successful vaccine are shown (Figure 1.6) and discussed below:

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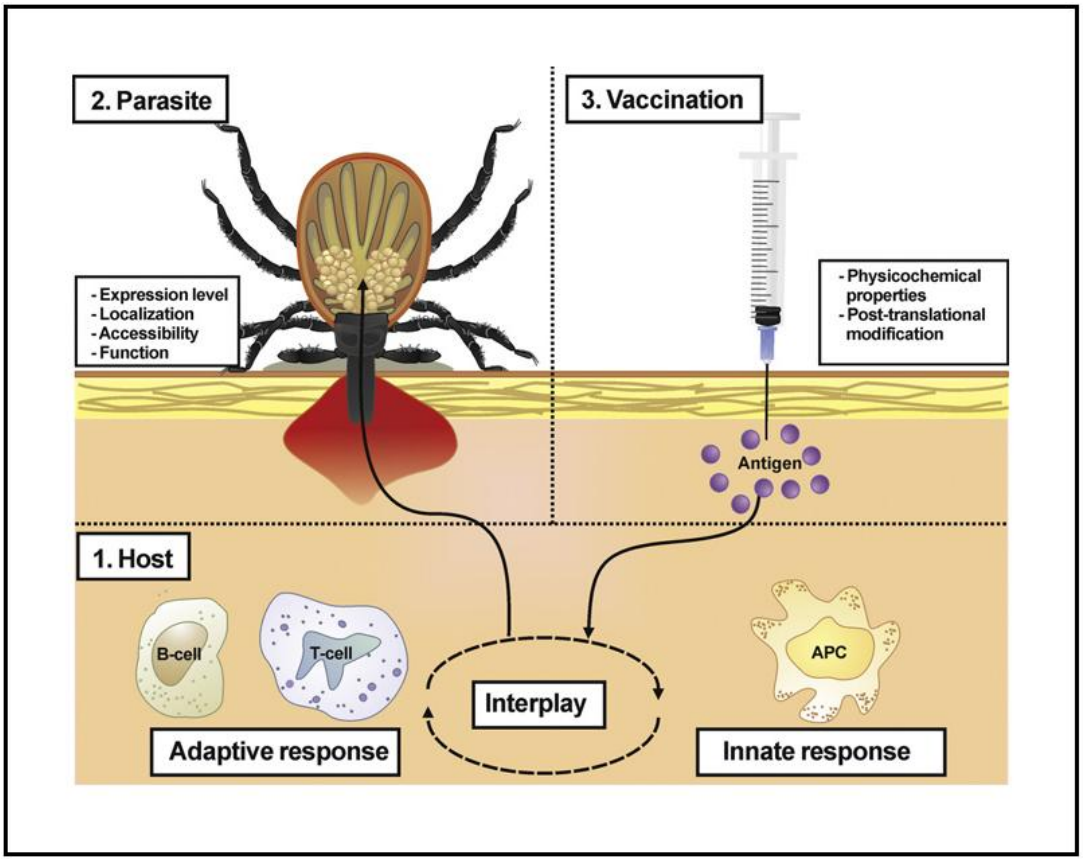

Figure 1.6. Vaccine-host-parasite interactions

Shown are the various factors that need to be taken into consideration when designing an anti-tick vaccine. A vaccine's formulation and preparation (*3*) need to be such that a sufficient immune response is elicited in the host (*1*) in order for vital proteins in the parasite (*2*) to be exposed to the host's immune factors, resulting in parasite death (Maritz-Olivier et al., 2012).

I. Host factors

The type of immune response elicited depends on the way in which exogenous antigens are presented. After phagocytosis of exogenous proteins by antigen-presenting cells, the proteins are degraded to peptide fragments that are displayed on MHC II. T helper cells that recognise this antigen-MHC complex become activated. Most microbes cause the T helper cells to favour a T helper 1 response, whereas the presence of helminths leads to a T helper 2 polarised response (Romagnani, 2000). Protection against different diseases can be conferred via various mechanisms. For example, an IgA-mediated response is sufficient for protection against cholera, while the involvement of T cells, macrophages and IFNγ is essential for immunity against tuberculosis (Zepp, 2010). Stimulation of the appropriate type of immune response is therefore necessary to confer immunity to the relevant organism. As mentioned earlier, *B. taurus* cattle develop natural resistance to *R. microplus* via cellular immunity. In comparison, vaccination using Bm86 confers protection via antibody-mediated immunity. It is likely that an antigen that elicits both types of immune responses would be of

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great benefit. The evaluation and prediction of the host's immune response are discussed in more detail in Chapter 3.

II. Parasite factors

Numerous factors are addressed in this section and will be expanded on in Chapter 3.

Expression level and function

The protein against which an immune response is launched needs to be present in sufficient amounts, otherwise the immune response, however successful it may be, would be of little use. Estimating protein expression levels using DNA microarray technology is discussed in Chapter 2. The contribution of a protein's function, in the case of membrane-bound proteins, is discussed in Chapter 3.

Localisation

When deciding on a protein-based vaccine candidate, the protein's localisation in the host-pathogen (or parasite) interface is important. Regarding immunity to *R. microplus* and other parasites, three classical localisations have been described.

a. *Exposed antigens*

During natural infestations with ticks, antigen-presenting cells residing in the host's skin take up and process exogenous antigens, including those from the tick's saliva. These are then presented to various immune cells, leading to the stimulation of a cellular or a humoral response (Larregina and Falo, 2005). The peptides and proteins that are found in a tick's saliva and cement are therefore exposed to the host's immune system and are termed '*exposed antigens*' (Nuttall et al., 2006). As mentioned earlier, many of these salivary proteins have potent immunomodulatory effects, which could make them inappropriate vaccine candidates. For example, guinea pigs developed undesirable cutaneous reactions at the site of feeding, following immunisation with a histamine-binding protein (Nuttall et al., 2006). During a natural infestation, the host is exposed to the tick's saliva and anti-tick resistance only develops to a certain extent, indicating that these proteins may not be highly immunostimulatory (Nuttall et al., 2006). It might also be argued that their immunomodulatory effects prevent the host from developing a protective immune response. Taken together, these findings suggest that immunomodulatory exposed antigens from tick saliva are not good vaccine candidates, as co-evolution with the host has led to the tick being able to

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circumvent and/or prevent the development of an immune response to these antigens (Guerrero et al., 2012).

b. Concealed antigens

Compared to exposed antigens, concealed antigens are those that are hidden from the host's immune system during natural infestation, for example antigens from the gut. The concealed antigens that are of interest with regard to vaccine design are those that have a vital function in the tick and interact with the host's immune factors (Nuttall et al., 2006). This class of antigens is well-represented by Bm86 (Willadsen et al., 1989). Due to the fact that concealed antigens are hidden from the host's immune system, natural infestations don't maintain immunity to these antigens, as is the case with Bm86, where regular vaccinations are necessary to maintain tick-resistance (de la Fuente et al., 1998; Nuttall et al., 2006).

c. Dual-vaccines

The third group combines the properties and advantages of exposed and concealed antigens. Host immunity to exposed antigens would be maintained by natural infestations, while concealed antigens have the advantage of not being subjected to the immunomodulatory effects of tick saliva. One example of such a protein is 64P, a cement protein from *Rhipicephalus annulatus*. When it was used to immunise guinea pigs, their antiserum also reacted with tick midgut proteins (Trimnell et al., 2002). Given the poor results of exposed antigens as vaccine candidates and the limitations of concealed antigens, some argue that these dual-action antigens are the most promising anti-tick vaccine candidates (Guerrero et al., 2012).

Whether the ideal vaccine is based on concealed or exposed antigens remains controversial. Concealed antigens, like Bm86, require regular vaccinations for long-lasting protection. Exposed antigens do not suffer from this drawback but it is argued that their usability is limited due to co-evolution with the host. Dual-vaccines are supposed to circumvent this problem by combining the advantages of both types of antigens. Regardless, the ideal vaccine remains elusive. Apart from finding the ideal vaccine candidate, the immunomodulatory effects of tick infestation also need to be taken into consideration. These include the downregulation of Th1-mediated immunity (Brake and Pérez de León, 2012) as well as immunoglobulin-binding proteins (Ferreira et al., 2002).

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d. Intracellular antigens

These belong to a subgroup of concealed antigens. Not only are they hidden from the host during natural infestations, they also do not come into direct contact with the ingested blood meal, unlike Bm86.

Expression library immunisation using cDNA from *Ixodes scapularis* was used to evaluate 4000 unique cDNAs. The cDNAs were subsequently used to immunise mice and their ability to confer anti-tick resistance was evaluated. Using this technique, three such cDNAs were identified (Almazán et al., 2003; Almazán et al., 2005). Their ability to confer anti-tick protection to sheep was subsequently evaluated and the protein with the highest efficacy was 4D8, which is now known as subolesin (Almazán et al., 2005). Results from gene knockdown experiments using RNAi suggest that subolesin plays a role in the modulation of blood ingestion and reproduction in ticks (de la Fuente et al., 2006). An orthologue to the Akirins found in insects and mammals, subolesin is believed to be a transcription factor involved in innate immunity signalling pathways (de la Fuente et al., 2008; Goto et al., 2008). Surprisingly, despite recommendations that membrane proteins are the best vaccine candidates (Rappuoli and Bagnoli, 2011), subolesin is an intracellular protein but still manages to confer anti-tick protection when used as a vaccine. Furthermore, vaccination using subolesin led to a 98% and 99% decrease in *A. marginale* and *B. bigemina* infection, respectively, of ticks feeding on vaccinated animals (Merino et al., 2011). The potential use of subolesin to curb pathogen transmission was also demonstrated by immunising mice with vaccinia virus expressing subolesin from *I. scapularis*. Apart from a 52% reduction in tick infestation, the transmission of *Borrelia burgdorferi* to uninfected mice was reduced by 40%, compared to controls (Bensaci et al., 2012).

III. Vaccine formulation factors

A lot of progress has been made in terms of large-scale vaccine production. This includes the expression of Bm86 as a secreted product in *P. pastoris* (Canales et al., 2008), or the expression of vaccine candidates as membrane protein fusions (Almazán et al., 2012). The Bm86 vaccine was first expressed recombinantly in *E. coli* (Rand et al., 1989). Subsequent reports have shown that recombinant Bm86 can be obtained in a more immunogenic form when using *P. pastoris*. This is due to the fact that, when expressed in *P. pastoris*, recombinant Bm86 is found in a particulate and highly glycosylated form (García-García et al., 1998a; Rodríguez et al., 1994). When expressed in *E. coli*, subolesin was found to have an efficacy of 51% against *R. microplus* (Almazán et al., 2010). However, when expressed as

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a fusion protein with MSP1, its efficacy increased to 81% (Almazán et al., 2012). This shows how the efficacy of an anti-tick vaccine can be improved by altering its formulation.

The type of adjuvant used can also determine the success of an anti-tick vaccine. When sheep were immunised with recombinant 5'-nucleotidase from *R. microplus,* significant protection was only conferred to experimental groups that received formulations containing the adjuvant ISA50. Antibody titres were more than fivefold lower when ISA773 was used as an adjuvant. By recruiting antigen presenting cells to the vaccination site, by improving the delivery of antigens to these cells, or by activating them to produce cytokines and provide activating signals to T cells, adjuvants enhance the immunogenicity of a vaccine (McKee et al., 2010). The adjuvant used therefore needs to be optimised for each vaccine.

Consumer expectations and industrial considerations

Despite some promising results, anti-tick vaccines are simply not efficacious enough. It has been noted that consumers expect vaccines to work as efficiently as chemical acaricides (Guerrero et al., 2012). This puts a lot of strain on the scientific community to come forth with a novel, efficacious vaccine against *R. microplus*. Moreover, overzealous marketing of substandard products exacerbates farmers' hesitance to adopt novel alternative forms of treatment. Anecdotal reports indicate that the demand for anti-tick products is very high, especially in South American countries. There, a crude protein extract from pulverised ticks was registered and marketed as an anti-tick vaccine. Not surprisingly, the product is under review due to poor performance (Guerrero et al., 2012).

Proof of principle is also but one of the first steps towards the ultimate commercialisation of a novel anti-tick vaccine. Regulatory requirements are constantly changing, which means that a vaccine that was registered 20 years ago might no longer be considered for registration today. Production methods need to comply with various quality control regulations and the final product needs to be safe for use (Heldens et al., 2008).

Given the rise in the development of acaricide resistance and the lack of promising alternative compounds, consumers may just be left with no choice other than to adopt vaccination strategies as part of their pest management.

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With the well-published shortcomings of Bm86 in mind, a global concerted effort for the development of an anti-*R. microplus* vaccine will definitely be necessary to eradicate this pest. Numerous genes still remain to be evaluated as anti-tick vaccines (Table 1.2), which presents a task both daunting and exciting for future *R. microplus* researchers.

1.2 Aims of this study

The overall aim of this project was to systematically evaluate all known *R. microplus* transcripts as possible vaccine candidates, based on the properties of an ideal vaccine. As expected, the only anti-*R. microplus* vaccine to ever get commercialised, Bm86, was used as a starting point. Existing knowledge of this protein in terms of localisation and its expression across the different life stages and adult tissues of *R. microplus* was used as a guide.

Chapter 2 aims to identify genes that are expressed in the midgut of *R. microplus* and *R. decoloratus* females, 20 days post infestation. This will provide information as to which transcripts are shared between or unique to either tick species in addition to information regarding their expression levels.

Chapter 3 aims to incorporate data generated in another study, where the transcriptome across the life cycle of *R. microplus* was analysed. Combined with *in silico* predictive tools, it is envisaged that novel vaccine candidates against both *R. microplus* and *R. decoloratus* can efficiently be identified. In addition, preliminary studies to evaluate the immunogenicity of identified antigens are included.

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Findings from this project have enabled the following contributions to anti-tick research:

Peer-reviewed publications

- Maritz-Olivier, C., van Zyl, W., and Stutzer, C. (2012). A systematic, functional genomics, and reverse vaccinology approach to the identification of vaccine candidates in the cattle tick, *Rhipicephalus microplus*. *Ticks Tick Borne Dis.* **3**, 179-187.
- Stutzer, C., van Zyl, W. A., Olivier, N. A., Richards, S., and Maritz-Olivier, C. (2013). Gene expression profiling of adult female tissues in feeding *Rhipicephalus microplus* cattle ticks. *Int. J. Parasitol.* **43**, 541-554.

Conference proceedings

- van Zyl, W. A., Stutzer, C., Olivier, N., Coetzer, N., Joubert, F., and Maritz-Olivier, C. (2010). A systematic approach towards anti-tick vaccines using an integrated immunoinformatics and functional genomics approach (poster). South African Society of Biochemistry and Molecular Biology (SASBMB), Bloemfontein, South Africa.
- van Zyl, W., Stutzer, C., and Maritz-Olivier, C. (2011). Comparative transcriptome analysis of the most prominent cattle ticks, *Rhipicephalus* (*B.*) *microplus* and *R. decoloratus* (oral presentation). Parasitology and Zoological societies of South Africa Conference, Stellenbosch, South Africa.
- van Zyl, W., Stutzer, C., and Maritz-Olivier, C. (2012). From prediction to protection: *In silico* identification and *in vitro* validation of promising anti-*Rhipicephalus* vaccine candidates (oral presentation). South African Genetics & Bioinformatics and Computational Biology Society Conference, Stellenbosch, South Africa. Best oral presentation award.

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Chapter 2

Comparative transcriptome analyses of the midgut of *R. microplus* and *R. decoloratus* female ticks

2.1 Introduction

2.1.1 Rationale of performing transcriptome analyses

Transcriptome analyses allow for the simultaneous evaluation of expression levels of individual or a large number of genes. As mentioned in the previous chapter, *R. microplus* and *R. decoloratus* ticks are typically found on the same cattle host in South Africa. The identification of genes that are expressed in the midgut of both ticks might provide candidate genes for further development of a vaccine against *R. microplus* and *R. decoloratus*.

2.1.2 Methods to perform comprehensive transcriptome analyses

To date, there are only two techniques available that can be used in order to analyse gene expression across the entire transcriptome of an organism. A recent advance has been the development of RNA-Seq, a technique that combines high-throughput sequencing of complementary DNA with mapping of sequencing reads to the target genome (Nagalakshmi et al., 2010). This technique allows for the identification of exons and intron boundaries, splice variants and the precise quantification of exon expression levels (Costa et al., 2010). Apart from certain disadvantages, such as its bias towards identifying more differential expression for longer transcripts (Oshlack and Wakefield, 2009), RNA-Seq is also computationally very demanding and expensive (Kumar et al., 2012; Nagalakshmi et al., 2008). At time of commencement of this project, facilities for performing an RNA-Seq experiment were not available. Furthermore, a custom *R. microplus*-specific microarray slide was being designed and was therefore the most convenient choice of experimental platform.

In comparison to RNA-Seq, DNA microarrays have been successfully applied for the study of gene expression levels in ticks (Table 2.1).

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Table 2.1. Microarray studies in ticks

Tick species	Aim	GEO accession number	Reference
R. microplus	Identification of genes that are associated with genes associated with *B. bovis* infection in larvae, ovary and midgut tissues	GSE10816, GSM273028-GSM273051	(Heekin et al., 2012)
R. microplus	Organ-specific transcriptional changes due to infection with A. marginale	GSE21690	(Mercado-Curiel et al., 2011)
R. microplus	Study off-target effects caused by RNAi	GSE27453, GSM678423-GSM678430	(Lew-Tabor et al., 2011)
R. microplus	Identification of genes that are differentially expressed in larvae and females before and after attachment to *Bos indicus* and *B. taurus* cattle	GSE20605	(Rodriguez-Valle et al., 2010)
R. microplus	Identification of acaricide-inducible genes in larvae	GSE10171	(Saldivar et al., 2008)
Ixodes scapularis	Analysis of salivary gland transcriptional responses due to feeding or infection with flaviviruses	Not available	(McNally et al., 2012)
Ixodes scapularis	Effect of infection with *Anaplasma* in cultured tick cells	GSE10222	(Zivkovic et al., 2009)
Amblyomma americanum	Analysis of gene expression changes during feeding	GPL3741	(Aljamali et al., 2009)

2.1.3 Functional genomics studies in ticks

The genome of *R. microplus* is 7.1 Gigabases long, consisting of 31% highly repetitive, 38% moderately repetitive and 30% unique DNA. These features make its genome distinct from most other arthropods (Ullmann et al., 2005). Sequencing of the *R. microplus* genome has been initiated (Guerrero et al., 2006) and will aid gene discovery, enabling an improved understanding of the tick-host-pathogen interface, and how it responds to environmental or chemical (acaricide-caused) perturbations (Bellgard et al., 2012)

Microarray technology is typically limited to model organisms, unless a substantial amount of sequence data is available (Naidoo et al., 2005). For this reason, studying the *R. microplus* transcriptome on a large scale has only recently been made possible by large-scale EST sequencing projects and freely available databases hosting such data. The BmiGI website is one such database and was launched in 2005 (Guerrero et al., 2005). It has subsequently been used for the design of custom microarrays to identify genes that are differentially expressed in larvae as a result of exposure to three classes of acaricides. Saldivar and colleagues found that glutathione S-transferase was upregulated upon exposure to organophosphates (Saldivar et al., 2008). *R. microplus* is a one-host tick, feeding predominantly on cattle. The exposure of cattle to larvae therefore represents the major determinant of the severity of infestation. Larvae that sensed a cattle host upregulated genes involved in tick cement proteins and/or antimicrobial activity, suggesting an important role for these genes to enable successful feeding on the host (Rodriguez-Valle et al., 2010). Abovementioned studies show that microarray technology is a viable tool for global

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transcriptome analyses of *R. microplus.* It is still relatively new to tick research because few microarray experiments have been performed on ticks, compared to other organisms, such as *Plasmodium*. On the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/gds/), there are 2000 datasets pertaining to *Plasmodium* but only 113 *Rhipicephalus* datasets (as of 2013/01/09).

2.1.4 Principles of DNA Microarray

DNA microarray experiments typically involve the use of two cDNA samples that are labelled with different fluorescent dyes. These samples are then competitively hybridised to a glass slide that contains DNA probes, which could be full-length cDNAs or chemically synthesised oligonucleotides (Hardiman, 2004). The extent of hybridisation is monitored by measuring the ratio of the fluorescence of each dye. Results from DNA microarray experiments yield information on the relative expression of each gene, compared between two samples (Yang and Speed, 2002).

2.1.5 DNA microarray platforms

Traditionally, DNA microarrays were produced by spotting cDNA onto glass slides, which was a very time-consuming process (Hardiman, 2004). The application of photolithography to synthesise custom oligonucleotide microarrays in the late 1990s has eliminated difficulties such as sample handling for the preparation of spotted microarrays. Hence, DNA microarrays can be designed *in silico* and synthesised chemically (Lipshutz et al., 1998; Schena et al., 1998).

DNA microarrays produced using photolithography can be obtained commercially from various companies, with probe length being the major difference between them. Longer probes generally work better but it is argued that the optimal probe length would be around 140 nucleotides (Ylstra et al., 2006). Affymetrix, the original pioneers of this technology, manufacture microarray slides with 25-mer probes (Hardiman, 2004; Ylstra et al., 2006). Hybridised biotinylated cDNA is then detected using phycoerythrin-labelled streptavidin (Welle et al., 2002). The short probes cannot discriminate properly between related sequences due to their poor hybridisation properties (Hardiman, 2004) and as a result, more

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probes (up to 250,000) are included per array. Furthermore, only one sample can be analysed at a time, necessitating the simultaneous evaluation of a series of normal reference samples (Ylstra et al., 2006). DNA microarrays from Agilent Technologies contain 60-mer probes and can be produced in different formats, ranging from 1x 244,000 to 8x 15,000 probes per slide. One major advantage of these DNA microarrays is that they are custom-made and designed *in silico* (Ylstra et al., 2006).

2.1.6 Microarray experimental design

When performing microarray experiments, expression level differences can be compared directly between two samples, or indirectly between two or more samples via a common reference sample (Yang and Speed, 2002). Different design types can be illustrated using the convention that the sample at the end of the arrow is labelled red (Cyanine 5 or Cy5) and the sample at the head of the arrow is labelled green (Cyanine 3 or Cy3). A simple direct comparison is shown in Figure 2.1A, where one sample is labelled with Cy5 and the other with Cy3. Performing a dye-swop reduces dye-bias and technical variation (Churchill, 2002). Microarray experiments are typically done using a reference design, where the reference sample is labelled with one dye and experimental samples are labelled with the other (Figure 2.1B). Moreover, dye-swops (Figure 2.1C) can be incorporated to reduce dye-biases (Churchill, 2002). Advantages of reference designs include the relative ease of data analysis and the fact that all comparisons are made with equal efficiency, since the path connecting two samples is never longer than two steps. This means that all the samples can be directly compared with one another. In addition, reference designs can be extended to multiple samples, provided that the reference sample RNA is not a limiting factor (Churchill, 2002). It is argued, however, that reference designs are not as efficient, as half of all hybridisations are done using the reference sample, which is of little interest. Results are also more variable due to the increase in technical variation, as compared to when performing direct comparisons (Churchill, 2002). When available arrays for experimentation are a limitation, the balanced block design can be used (Figure 2.1D). For simple comparisons, this design is more efficient than the reference design, as only n arrays are necessary for n samples, compared to $2n$ arrays when using a reference design (Simon and Dobbin, 2003). In all instances, comparing more samples would lead to higher degrees of freedom (Churchill, 2002). Using more biological samples is also more favourable than using more technical replicates, as biological variation is a bigger concern than technical variation (Klebanov and Yakovlev, 2007).

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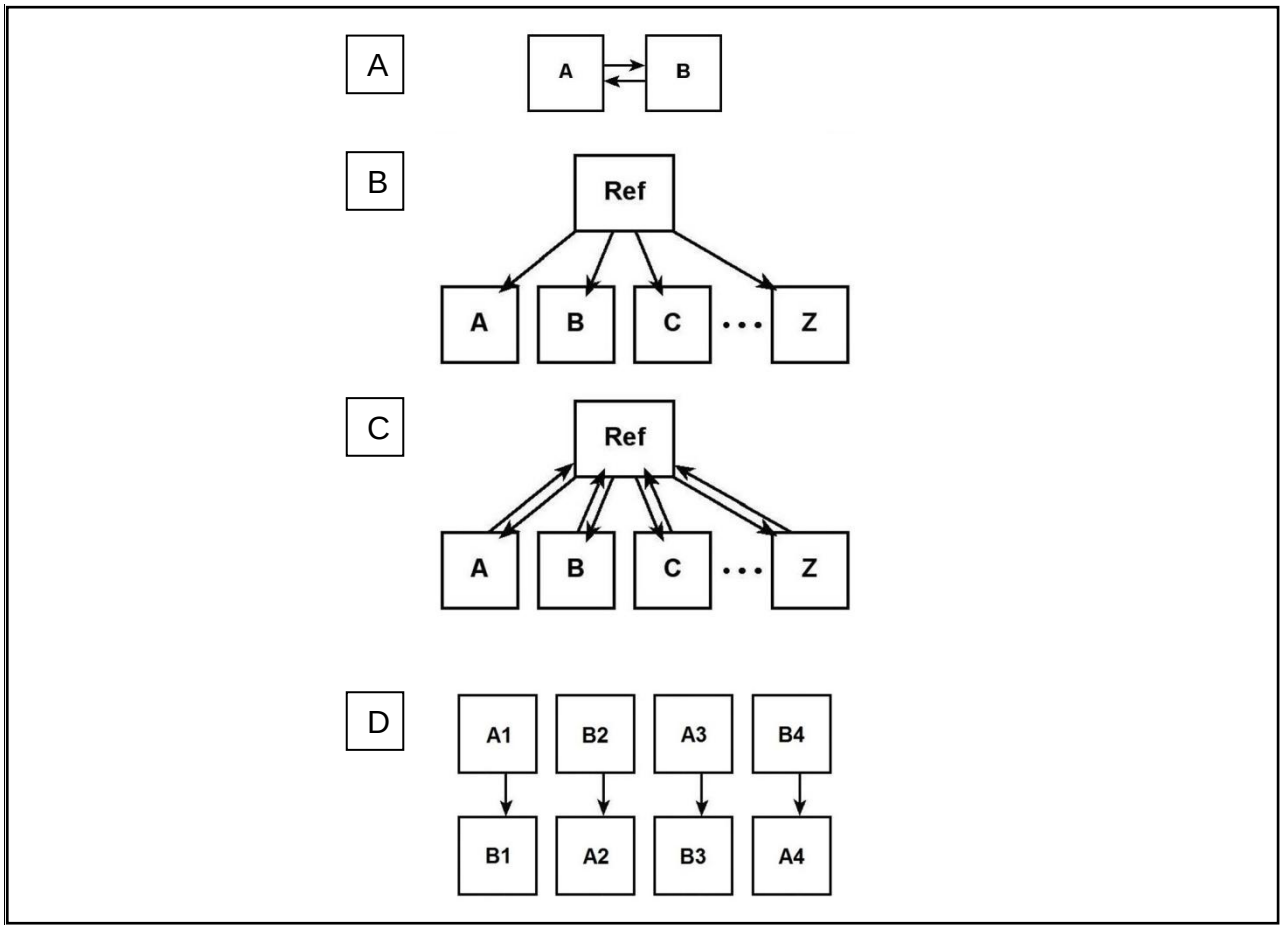

Figure 2.1. Microarray experimental design types

Figures show a direct comparison with dye-swop (*A*), a reference design without a dye-swop (*B*) and with dye-swop (*C*), and (*D*) a balanced block design without dye-swop. Redrawn as described in (Churchill, 2002; Simon and Dobbin, 2003).

2.1.7 Data normalisation and interpretation

Before data analysis is possible, the data need to be normalised first, in order to compensate for unequal amounts of starting material or biases due to differences in labelling or detection efficiencies between the fluorescent dyes used (Quackenbush, 2002). Prior to data normalisation, background correction has to be performed to remove effects due to non-specific binding (Ritchie et al., 2007). One approach includes subtracting the local background around each spot from the spot's intensity. This has been evaluated and has been found to cause high variability in the measured ratios of low-intensity spots, as well as a high number of spots with negative values (Ritchie et al., 2007; Yang et al., 2001). For two-colour arrays, the variance-stabilising effect of adding a positive offset value to each spot, combined with an adaptive background correction method, has been found to be optimal (Ritchie et al., 2007; Smyth et al., 2011). After background correction has been performed, the data first need to be normalised within arrays and then between arrays. Intensity-dependent dye biases are usually removed using global **lo**cally **we**ighted **s**catterplot

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smoothing, referred to as LOWESS or LOESS (Quackenbush, 2002; Smyth et al., 2011). Such biases can be visualised using a *MA* plot, where *M* refers to the $\log_2$ratio of the signal intensities and *A* refers to the average $\log_2$intensity (Smyth et al., 2011):

$$M = \log_2 R - \log_2 G \quad \text{Equation 2.1}$$

$$A = (\log_2 R + \log_2 G)/2 \quad \text{Equation 2.2}$$

Genes that are not differentially expressed will not have a large difference between the $\log_2$ratio of their respective dye intensities and the *M*-value will therefore be close to 0. Dye-specific biases will therefore be visible when the median *M*-value on an *MA* plot is far from zero. Intensity-dependent effects can be seen when the distribution of the *M*-values is more skewed at low or high intensities (Quackenbush, 2002). Other types of normalisation include print-tip LOESS, which normalises *M*-values within print-tip groups (not applicable to microarrays from Agilent Technologies) and robust spline, which is a compromise between global and print-tip LOESS normalisation methods (Smyth et al., 2011). LOESS normalisation adjusts the discrepancy in the distribution of *M*-values within arrays, but does not normalise the measured intensity levels between arrays. Aquantile normalisation is used to normalise intensity values across all arrays (Smyth et al., 2011). Finally, once all the data normalisation is complete, a linear model is fitted onto the data, after which an empirical Bayes method is used to moderate the estimated $\log_2$ratios (Smyth et al., 2011).

One of the concerns with microarray studies is that there is a low level of standardisation as to how the experiments are performed and analysed (Allison et al., 2005). As a consequence, the **m**inimum **i**nformation **a**bout a **m**icroarray **e**xperiment (MIAME) guidelines stipulate that the information regarding microarray experiments should be detailed enough to allow comparisons to similar experiments and structured such that data analysis is possible by other scientists (Brazma et al., 2001). Supplementary data can be uploaded to websites such as ArrayExpress of the European Bioinformatics Institute and the Gene Expression Omnibus of the National Center for Biotechnology Information at the National Institutes of Health (Stoeckert et al., 2002).

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2.1.8 Analysis of *R. microplus* and *R. decoloratus* using DNA microarray

The rate-limiting step in vaccine production in the past has been the identification of potential antigens (Mulenga et al., 2000; Willadsen, 2001). The use of DNA microarrays for the identification of transcripts that are expressed in the gut of feeding adult female *R. microplus* and *R. decoloratus* ticks is therefore an attractive option. As mentioned in Chapter 1, *R. microplus* and *R. decoloratus* are phylogenetically related, based on their similar mitochondrial 12S and cytochrome c oxidase I sequences. For this reason, it is hypothesised that an oligonucleotide microarray, based on *R. microplus* sequences, can also be used to assay gene expression levels in *R. decoloratus.*

However, it must be borne in mind that sequences that are unique to *R. decoloratus* will be missed with the current experimental approach but it is anticipated that vaccine candidates with high sequence identity and similar expression levels will be detected.

Results from this chapter will also be subjected to an *in silico* pipeline incorporating various predictive tools and a preliminary *in vivo* antigenicity assay in order to evaluate promising anti-*Rhipicephalus* vaccine candidates. This pipeline is the focus for Chapter 3.

Presented here is a comparative study on the transcriptional profiles of midgut tissues from *R. microplus* and *R. decoloratus* ticks, collected 20 days post-infestation, when adult ticks are rapidly engorging, using an in-house designed 60-mer oligonucleotide microarray. Along with the identification of transcripts that are expressed in both ticks, some preliminary insight into their biology is also provided.

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2.2 Hypothesis

An in-house designed 60-mer oligonucleotide microarray can be used to identify genes that are shared between and unique to *R. microplus* and *R. decoloratus*

2.3 Aims

- To identify transcripts that are shared between the midgut tissues from *R. microplus* and *R. decoloratus* using DNA microarray technology
- To study the differences in basic biology of abovementioned ticks using *in silico* tools
- To validate results from the microarray experiment using real-time PCR

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2.4 Materials

Bos taurus calves (~six months old) with limited previous exposure to ticks were housed at the University of Pretoria Biomedical Research Centre in Onderstepoort. Larvae from *R. microplus* and *R. decoloratus* were a gift from Josephus Fourie, ClinVet International (Bloemfontein, South Africa). Ethical clearance was granted by the Department of Agriculture, Forestry and Fisheries as well as the Animal Use and Care Committee from the University of Pretoria (EC022-10). TRI Reagent (Molecular Research Center, Inc., USA) and ethidium bromide were from Sigma-Aldrich. The RNeasy Mini and QIAquick PCR Purification kits and RNase-free DNase set (Qiagen, USA) were from Whitehead Scientific. Primers were synthesised by Inqaba Biotec (South Africa). RNasin® ribonuclease inhibitor, deoxynucleotides and O'GeneRuler™ 100 bp molecular marker were from Fermentas, USA. Superscript® III reverse transcriptase (Invitrogen) was from Life Technologies. The NucleoSpin Extract II kit was from Macherey-Nagel, Germany. KAPA SYBR® FAST and KAPA Taq 2X ReadyMix were from KAPA Biosystems, South Africa.

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2.5 Methods

2.5.1 Tick rearing and sample collection

Two *Bos taurus* calves were housed in separate stables at the University of Pretoria Biomedical Research Centre (UPBRC), at the Onderstepoort veterinary campus. One calf (Holstein-Friesian) was infested with three thousand *R. microplus* larvae and the other (Ayrshire) with *R. decoloratus*. Feeding adult female ticks were collected on 20 days post-infestation and dissected. For each species three samples (consisting of the midgut tissues from 10 female ticks) were collected. The tissues were placed in 500 µl TRI Reagent, snap-frozen in liquid nitrogen and stored at -70°C.

2.5.2 RNA isolation

Total RNA was isolated from the collected tissues following the acid guanidium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Tissues were homogenised in the presence of 1 ml TRI Reagent, a denaturing solution containing a proprietary mix of phenol and guanidium thiocyanate, and the homogenate was subsequently separated into an aqueous and organic phase upon addition of 200 µl chloroform. After incubation at room temperature for 15 minutes, the samples were centrifuged at 12,000*g* at 4°C for 15 minutes. The RNA-containing aqueous phase was transferred to a new polypropylene tube and 500 µl isopropanol was added to cause the RNA to precipitate. The samples were incubated at room temperature for 15 minutes after which they were centrifuged at 12,000*g* at 4°C. The supernatants were removed and the precipitated RNA washed with 1 ml 70% ethanol, followed by a 5 minute centrifugation step at 7,500*g*. Again, the supernatants were removed and 1 ml 70% ethanol was added before the samples were centrifuged at 7,500*g* at 4°C for 5 minutes. Excess ethanol was removed and the samples were air-dried, after which 100 µl nuclease-free water was added to solubilise the RNA. Further purification of RNA was performed using the RNeasy® kit. The kit works on the principle of nucleic acids (RNA in this case) binding to a silica membrane in the presence of chaotropic salts. The exact composition of each buffer is proprietary, but buffer RLT contains guanidinium thiocyanate, buffer RPE contains ethanol, buffer RW1 contains ethanol and guanidium thiocyanate and buffer RDD contains magnesium for optimal DNase I activity. To ensure binding to the silica membrane, 350 µl of buffer RLT and 250 µl of 100% ethanol were added to each sample, after which it was pipetted onto an RNeasy® spin column and placed in a 2 ml collection tube. Samples were centrifuged for 15 seconds at 8,000*g* and the flow-through was reloaded

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to repeat the centrifugation step, after which the flow-through was discarded. The silica membranes were washed by addition of 350 µl of buffer RW1 and centrifugation for 15 seconds at 8,000*g*. In order to remove any potentially contaminating DNA from the silica membrane, 10 µl of DNase I solution (27 Kunitz units) was mixed with 70 µl of buffer RDD and pipetted onto the membrane. Samples were incubated at room temperature for 15 minutes and the DNase I was removed by adding 350 µl of buffer RW1 and centrifuging the samples for 15 seconds at 8,000*g*. The membranes were washed by adding 500 µl buffer RPE and centrifuged at 8,000*g* for 15 seconds, followed by an additional 500 µl buffer RPE and a final centrifugation step for 3 minutes at 8,000*g* to remove any traces of ethanol. Finally, RNA was eluted by adding 30 µl of nuclease-free water and centrifuging the samples for 1 minute at 8,000*g*. To maximise yield, the flow-through was reloaded onto the column and samples were centrifuged for 1 minute at 8,000*g*.

2.5.3 Quantification of nucleic acids and assessment of RNA quality

RNA concentration was determined using the Nanodrop-1000 (Thermo Fisher Scientific, USA) spectrophotometer. The absorbance at 260 nm is measured and the concentration of RNA is calculated using the Beer-Lambert law

$$A = \varepsilon c l \quad \text{.....Equation 2.3}$$

Where A is the absorbance at 260 nm, ε is the molar extinction coefficient for RNA (40 µl/(ng.cm)), c is the concentration (ng/µl) and l is the path length (cm). RNA purity was indicated by the A_{260}/A_{280} ratio, where ratios from 1.8-2.0 represent pure RNA. Lower ratios indicate protein contamination, as the aromatic amino acids tryptophan, tyrosine and phenylalanine absorb light at 280 nm (Sambrook et al., 1989). Finally, in order to evaluate RNA integrity, analyses using the Experion® (Bio-RAD, USA) were performed at the ACGT Microarray facility (University of Pretoria). This highly sensitive system combines capillary electrophoresis and a fluorescent RNA-binding dye to determine RNA integrity, expressed as an RNA Quality Indicator (RQI) value, which ranges from 1.0 (fully degraded) to 10.0 (intact RNA). Relative expression levels can be determined using degraded RNA, but with significantly higher variability (Denisov et al., 2008). An RQI value >8 is therefore desirable whenever possible.

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2.5.4 cDNA synthesis

First strand cDNA synthesis for each sample was initiated by adding 250 pmol of an oligo-dT primer (5'-$(T)_{25}$ VN-3'; N=ACGT; V=ACG) and 775 pmol of a random nonamer to 3 µg of RNA. This was incubated at 70°C for 10 minutes, followed by 4°C for 10 minutes to allow primers to anneal. In order to inhibit potentially contaminating RNases, 40 units of RNasin® ribonuclease inhibitor and 10 mM dithiothreitol were added (Chen et al., 2004). This was followed by the addition of 6 µl of 5x SuperScript first-strand buffer, SuperScript® reverse transcriptase (340 units) and deoxynucleotides (dCTP, dGTP, dTTP and aminoallyl-dUTP, 0.3 mM each and dATP, 0.6 mM). Reactions were incubated at 42°C for 20 hours, after which the RNA was hydrolysed by adding 0.14 M NaOH and 0.07 M EDTA (pH 8) and incubation at 65°C for 15 minutes. Newly synthesised cDNA was purified using the NucleoSpin® Extract II kit. This kit works on the principle that nucleic acids (cDNA in this case) bind to a silica membrane in the presence of chaotropic salts. The exact composition is proprietary, but buffer NT contains chaotropic salts and buffer NT3 contains ethanol. To ensure binding to the silica membrane, 10 volumes of buffer NT were added to each cDNA sample, which was loaded onto a NucleoSpin® Extract II column and incubated at room temperature for 4 minutes. The columns were centrifuged at 13,000*g* for 1 minute and the flow-through was discarded. The silica membranes were washed three times by addition of 500 µl buffer NT3 and centrifugation at 13,000*g* for 1 minute. The membranes were dried to remove residual ethanol by centrifugation at 13,000*g* for 2 minutes. Finally, cDNA was eluted by adding 30 µl of nuclease-free water (pre-heated to 37°C), followed by incubation at 37°C for 4 minutes and centrifugation at 13,000*g* for 90 seconds. DNA purity and concentration were assessed as previously discussed, with the difference being that single-stranded DNA has a molar extinction coefficient of 33 µl/ (ng.cm).

2.5.5 cDNA labelling with cyanine dyes

Purified cDNA samples were dried *in vacuo* and solubilised in 2.5 µl nuclease-free water. To ensure a high pH for optimal cyanine dye coupling to the reactive amines of the aminoallyl-dUTP, 5 µl sodium bicarbonate buffer (pH 9, 0.1 M) was added to the cDNA before 2.5 µl of the appropriate cyanine dye was added to the samples (Table 2.2 and Figure 2.2).

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Table 2.2. Microarray hybridisation layout

R. microplus 1-3 and *R. decoloratus* 1-3 represent three biological repeats from samples isolated from the midgut of these ticks.

Array number	Cy3	Cy5
1	*R. microplus* 1	*R. decoloratus* 1
2	*R. decoloratus* 1	*R. microplus* 1
3	*R. decoloratus* 2	*R. microplus* 2
4	*R. microplus* 3	*R. decoloratus* 3

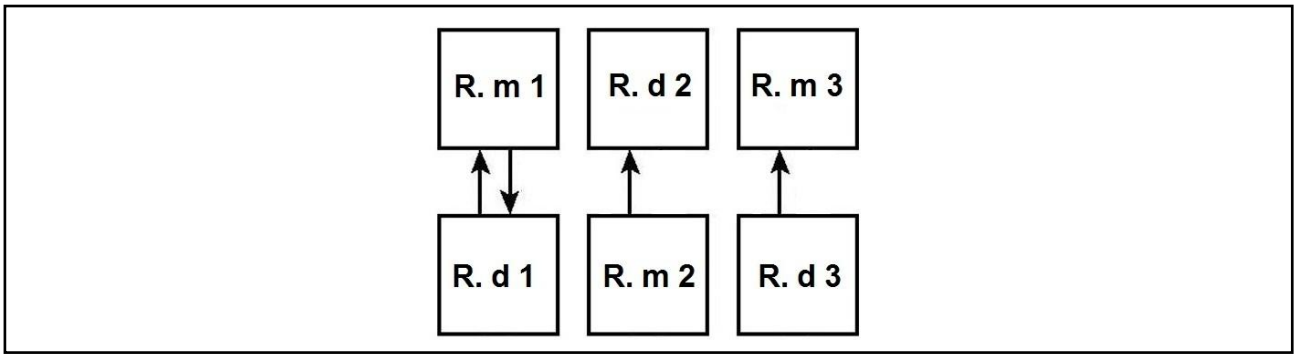

Figure 2.2. Microarray experimental layout employed in this study

A balanced block design incorporating one dye-swop was used. Abbreviations used indicate labelled cDNA from the midgut of: R. m (*R. microplus*) and R. d (*R. decoloratus*).The head of the arrow represents the sample labelled with Cy3 and the sample at the end of the arrow is labelled with Cy5.

The samples were incubated in a dark desiccator at room temperature for 2 hours. Excess dye was removed using the QIAquick® PCR Purification Kit. This kit works on the same principles as the NucleoSpin® Extract II kit. Again, the composition of the buffers is proprietary information, but buffer PB contains chaotropic salts and buffer PE contains ethanol. To ensure binding of labelled cDNA to the silica membrane, 10 volumes of buffer PB were added to each sample and mixed. These were transferred to a QIAquick® column and incubated for 4 minutes at room temperature, after which the samples were centrifuged at 13,000*g* for 1 minute. The flow-through was discarded and the silica membranes washed three times by addition of 500 µl of buffer PE and centrifugation at 13,000*g* for 1 minute. The membranes were dried to remove residual ethanol by centrifugation at 13,000*g* for 2 minutes. Pure labelled cDNA was eluted by adding 30 µl of nuclease-free water (pre-heated to 37°C) directly to the silica membranes, followed by incubation at 37°C for 4 minutes and centrifugation at 13,000*g* for 90 seconds.

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The extent of dye incorporation was determined using the Nanodrop-1000, by measuring the ratio of labelled nucleotides to unlabelled nucleotides. This ratio should be at least 1:100. Assuming that the average cDNA molecule is 1000 base pairs long and considering that the average dNTP has a molar mass of 324.5 pg/pmol, the following formula was used:

$$Labelling\ efficiency = \frac{\text{pmol dye /µl x } \frac{324.5\text{ pg}}{\text{pmol}}}{\text{ng cDNA /µl}} \quad \text{.....Equation 2.4}$$

2.5.6 Microarray slide

To ensure complete coverage of all available *R. microplus* sequence data available, an in-house designed custom oligonucleotide microarray slide was designed by Mr C Stutzer. Available ESTs from Genbank (http://www.ncbi.nlm.nih.gov/nucest) and BmiGI version 2.1 were downloaded (Wang et al., 2007). A script was written in Python (www.python.org) by Mr Philip Law from the Bioinformatics and Computational Biology Unit (University of Pretoria). It incorporated the following steps: Contaminating vector sequences were removed using VecScreen (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen_docs.html) and remaining sequences were clustered using CD-HIT (http://www.bioinformatics.org/cd-hit/) and CAP3 (http://genome.cs.mtu.edu/cap/cap3.html). These sequences were uploaded to the eArray microarray design website (https://earray.chem.agilent.com/earray/) and a set of unique 60-mer probes was designed. The slide was manufactured by Agilent Technologies, USA.

2.5.7 Hybridisation and scanning of oligonucleotide array

Equal molar amounts (20 pmol each) of both Cy3- and Cy5-labelled cDNA were mixed in a polypropylene tube. Various buffers of which the composition is proprietary information of Agilent Technologies were used. These include a 10x blocking buffer, a 25x fragmentation buffer, a 2x GE hybridisation buffer and wash buffers 1 and 2. Five microlitres of 10x blocking buffer and 2 µl of 25x fragmentation buffers were added to each sample. The final volume in each tube was adjusted to 25 µl using nuclease-free water. Reaction tubes were incubated at 60°C for 30 minutes to hydrolyse any remaining RNA in the samples before 25 µl of 2x GE hybridisation buffer was added to each tube. The gasket slide was loaded into the hybridisation assembly and 40 µl of each sample was pipetted onto the gasket slide in

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separate chambers. The microarray slide was loaded into the chamber, which was firmly closed and placed into a G2545A hybridisation oven (Agilent Technologies, USA) set at 65°C, 10 rpm. After 17 hours, the microarray slide was washed twice in wash buffer 1 (1 minute at a time) and once in wash buffer 2 (preheated to 37°C) for 1 minute. The slide was dried by centrifugation at maximum speed in a Spectrafuge™ Mini Centrifuge (Labnet International, Inc., USA). The microarray slide was scanned using an Axon GenePix 4000B scanner (Molecular Devices, USA) at the ACGT Microarray facility (University of Pretoria).

2.5.8 Data analysis

Scanned images of the slide were analysed using Axon GenePix Pro 6.0 (Molecular Devices, USA). The program's default settings were used to assess spot quality. Spots with irregular shapes, or where the background signal around the spot was higher than the spot itself, were flagged as such and not included in subsequent analyses. Spots were also visually inspected. In order to identify genes of which the expression levels were similar between *R. microplus* and *R. decoloratus*, the LIMMA package (www.bioconductor.org) was used. An adaptive background correction method incorporating the addition of a positive offset value to each spot was used (Ritchie et al., 2007), after which normalisation of the *M*-values within arrays was performed by making use of global LOESS. Aquantile normalisation was performed to minimise average signal intensity (the *A*-value) variation between arrays, leaving the *M*-values unchanged. In order to evaluate the fold change in expression values between the two *Rhipicephalus* species, the data were fitted onto a linear model ('lmfit' function in LIMMA). Agreement with the linear model was assessed using empirical Bayes statistics and expressed as a *p*-value, corrected for false discovery rate. Data pre-processing was kindly performed by Mr Nicky Olivier from the ACGT Microarray facility (University of Pretoria). Shared transcripts where the absolute $\log_2$fold change was not greater than 1.7 were identified. This relates to a fold change of not more than 3.25 between the species.

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2.5.9 Functional annotation of transcripts shared between *R. microplus* and *R. decoloratus*

A desktop cDNA annotation system (dCAS) (Guo et al., 2009) that incorporates BLAST (basic local alignment tool) functionality and consolidates data into a tab-delimited file was used for annotation. The latest versions (as of 30/9/2010) of the Gene Ontology (Ashburner et al., 2000) cluster of eukaryotic genes (Tatusov et al., 2003), Protein Family (Finn et al., 2010), non-redundant and SMART (Schultz et al., 2000) databases were downloaded (http://exon.niaid.nih.gov/cas/dbsupport.jsp) and loaded into dCAS. Transcripts of interest were put in FASTA format as input for dCAS. See Tab 1 on the electronic supplementary data.

2.5.10 Primer preparation for use in semi-quantitative real-time PCR (semi-qPCR)

In order to confirm the results of the microarray study, oligonucleotide PCR primers were designed using Oligo 7 (Molecular Biology Insights, Inc., USA). Primers (Table 2.3) had similar melting temperatures and were designed such that the amplicon would be around 150 base pairs in length. New cDNA was synthesised using 500 ng of RNA from each sample used in the microarray study. A cDNA pool, consisting of 500 ng in total from all six samples, was also prepared in order to construct standard curves. For cDNA synthesis, the iScript kit was used. The buffer components of the kit are proprietary information, but the reverse transcriptase is pre-blended with RNase inhibitor and the 5x reaction mix contains oligo-dT and random hexamer primers. For each reaction, 500 ng of RNA from each of the samples used in the microarray study were added to a polypropylene tube. This was followed by 4 µl of 5x reaction mix and 1 µl of reverse transcriptase (number of units is proprietary information). The final volume in each tube was adjusted to 20 µl using nuclease-free water. The reaction was incubated for 5 minutes at 25°C, followed by 30 minutes at 42°C and 5 minutes at 85°C. Newly synthesised cDNA was diluted 5 times in nuclease-free water and standard PCR was performed to test the primer pairs. This was done by adding 12.5 µl of KAPA Taq ReadyMix, 9.5 µl of nuclease-free water, 1 µl of both the forward and reverse primer (10 pmol/µl) to 1 µl of diluted cDNA. PCR was performed in a 2720 Thermal Cycler (Applied Biosystems, USA) and thermal cycling conditions consisted of an initial DNA denaturing step at 94°C for 2 minutes, followed by 35 cycles consisting of denaturation at 94°C for 30 seconds, primer annealing at 56°C for 30 seconds and primer extension at 72°C for 30 seconds. A final extension step was performed at 72°C for 5 minutes, followed by

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cooling the reaction tubes to 4°C. Agarose gels (2.5%) were prepared in TAE buffer (40 mM Tris-HCl, 20 mM acetic acid, 1 mM EDTA, pH 8) and 5 µl of each sample was mixed with 1 µl of loading dye (0.03% w/v bromophenol blue and 30% v/v glycerol). O'GeneRuler™ 100 bp DNA Ladder (Fermentas, USA) was used as a molecular marker. After agarose gel electrophoresis, gels were soaked in an ethidium bromide solution (10 µg/ml) for 30 minutes at room temperature, before PCR products were visualised using a UV-transilluminator.

2.5.11 Semi-qPCR

Standard curves were constructed for each transcript to be evaluated using semi-qPCR. The cDNA pool, consisting of three samples each from *R. microplus* and *R. decoloratus*, was used as a template (1 µl). The template for the standard curve was diluted 1/5, 1/10, 1/20, 1/50, and 1/100 in nuclease-free water. Each well contained 1 µl of template and 9 µl of reaction mix consisting of 2 µl of nuclease-free water, 1 µl of forward primer (10 pmol), 1 µl reverse primer (10 pmol) and 5 µl 2x KAPA SYBR® FAST Master Mix, with a final $MgCl_2$ concentration of 2.5 mM. To measure the relative expression levels in each sample, 1 µl of 5x diluted cDNA was added to 9 µl of abovementioned reaction mix. All semi-qPCR experiments were carried out in triplicate on a 384-well plate. Thermal cycling was carried out in the LightCycler® 480 (Roche Applied Science, Germany). PCR conditions consisted of an initial denaturation step at 95°C for 5 minutes, followed by 40 cycles consisting of template denaturation at 95°C for 3 seconds, primer annealing at 56°C for 7 seconds and primer extension at 72°C for 4 seconds. After each cycle, the fluorescence at 483-533 nm was measured and recorded. After completing the PCR, melt curve analysis was performed by raising the temperature to 95°C for 30 seconds, lowering it to 55°C for 30 seconds and raising it again to 95°C, while continually measuring the fluorescence. Measured quantification cycle values were exported to qBase (Hellemans et al., 2007) and normalised against three reference genes. Fold changes were calculated by dividing the average normalised relative quantities of each gene in the three *R. microplus* samples with those in *R. decoloratus*.

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Table 2.3. Primers used for semi-qPCR

Transcript	Classification	Primer	Sequence	Length	Product size	Tm (°C) (Rychlik et al., 1990)
Contig1269	Shared transcript	F	AAGGGAGAGGACTTCGTCAAGA	22	91	60.3
		R	ATCCACTGATGGGCACTTAACAC	23		60.6
Contig1362	Shared transcript	F	CGCTTATTGTTCCCACAGACTGA	23	97	60.6
		R	CGATGACAGATGCCACGACA	20		59.4
Contig1514	Shared transcript	F	TCAACACGCCCCGCAAGGAA	20	125	61.4
		R	GCCACGGAAAGGAATACCCTC	21		61.8
Contig3185	Shared transcript	F	AGCCATTGATTCTGCATCGTCG	22	93	60.3
		R	CGCTGCCCACAAGAGTAGGA	20		61.4
CV456291	Shared transcript	F	CCACCCAGAAGACCGTGTGC	20	103	63.5
		R	ACTACCGTATGCCGCTCCAC	20		61.4
Contig5295	Shared transcript	F	ACCACTGATGGACTCGTGCTC	21	113	61.8
		R	ACAATCTTGCCTTTCACGTCTGC	23		60.6
Contig8515	Shared transcript	F	CCCCTTTCTACCCCATAGCG	20	140	61.4
		R	GCACATTTACAAGCACTCTCCGA	23		60.6
EF1α	Reference gene (Nijhof et al., 2009)	F	CGTCTACAAGATTGGTGGCATT	22	90	58.4
		R	CTCAGTGGTCAGGTTGGCAG	20		61.4
PPIA	Reference gene (Nijhof et al., 2009)	F	CTGGGACGGATAGTAATTGAGC	22	133	60.3
		R	ATGAAGTTGGGGATGACGC	19		56.7
Contig8723	Reference gene (In-house validated)	F	ATGATCGGCAAGAAGCGTCT	20	100	57.3
		R	GGAAACCCTTTGTGACACCCTT	22		60.3

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2.6 Results and discussion

2.6.1 RNA isolation and analysis

RNA was isolated from the midgut of *R. microplus* and *R. decoloratus* females, collected on day 20 post-infestation. RNA yields ranged from 5-15 µg per tick, with average A_{260}/A_{280} ratios at 2.16, indicating pure RNA (Table 2.). RNA integrity was estimated by visual inspection of virtual gel images and electropherograms generated by the Experion® system's software after RNA analysis. Figure 2.3 shows defined bands and low smearing in the RNA samples, indicating that the RNA was of high quality. Results of RNA yield and quality assessment are summarised in Table 2.4. RNA yield was high and RNA quality was excellent in four out of six samples, where the RNA quality was found to be similar to that of 'intact RNA' (Denisov et al., 2008). The remainder of the samples were less intact but the RNA was considered to be usable for downstream applications, based on the electropherograms.

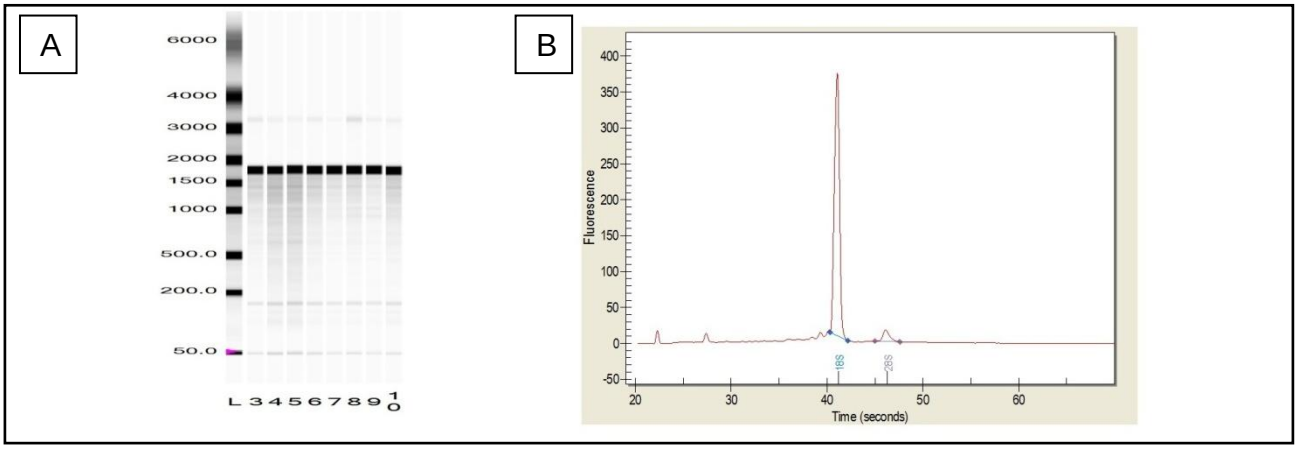

Figure 2.3. Analysis of RNA integrity using the Experion®

Virtual gel (*A*) and electropherogram (*B*) showing typical results for RNA quality assessment.

Table 2.4. RNA yield and quality assessment

Midgut RNA sample number	Concentration (ng/ µl)	RNA quality indicator value	RNA quality	A260/A280
R. decoloratus 1	2173.24	8.7	Intact	2.16
R. decoloratus 2	1995.44	6	Less intact	2.16
R. decoloratus 3	1869.76	5.6	Less intact	2.16
R. microplus 1	1537.84	9.2	Intact	2.16
R. microplus 2	1728.8	9.7	Intact	2.17
R. microplus 3	2956.88	8.4	Intact	2.13

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2.6.2 Sample preparation for microarray studies

A slide that allowed eight microarray hybridisation experiments per slide was used. For the comparison between *R. microplus* and *R. decoloratus*, only four of these microarrays were available. Trial experiments were performed to determine the efficiency of cDNA synthesis and subsequent labelling using cyanine dyes. Three micrograms of RNA starting material were used per sample to synthesise cDNA. On average, 2.1 µg of cDNA was recovered after purification, indicating a yield of 70%. This cDNA was subsequently labelled and re-purified, with an average yield of 47.8%. Results for cDNA synthesis and labelling are summarised in Table 2.5. The A_{260}/A_{280} ratios are low, but this could be due to the fact that these ratios are for labelled cDNA that contains aminoallyl-dUTP, which has an absorbance maximum at 289 nm (Shimkus et al., 1985). An increase in absorbance at 289 nm would therefore lead to a decrease in the A_{260}/A_{280} ratio. Labelling efficiencies ranged from 23-48 labelled nucleotides per 1000 nucleotides. Although it has been reported that optimal labelling efficiency ranges from 40-50 ('t Hoen et al., 2003), others recommend a minimum labelling efficiency of at least 10 (Smit, 2010). For the hybridisation, twenty picomoles of Cy3- and Cy5-labelled sample were hybridised per array.

Table 2.5. cDNA synthesis and labelling efficiency

Labelled midgut cDNA sample number	cDNA (ng/µl)	A_{260}/A_{280}	%Yield (RNA converted to cDNA)	Cy dye	pmol dye/ µl	Labelled cDNA (ng/ µl)	A_{260}/A_{280}	Recovery of cDNA after labelling (%)	Labelling efficiency
R. decoloratus 1	69.5	1.42	67.2	Cy3	4.9	33.1	1.6	47.6	48.0
R. decoloratus 1	73.4	1.38	71.0	Cy5	4.2	34.6	1.67	47.1	39.4
R. decoloratus 2	85.3	1.46	82.5	Cy5	4.1	38.4	1.64	45.0	34.6
R. decoloratus 3	64.7	1.76	62.5	Cy3	3.6	34.7	1.55	53.6	33.7
R. microplus 1	70.2	1.82	67.9	Cy5	3.4	30.2	1.62	43.0	36.5
R. microplus 1	52.7	1.68	51.0	Cy3	3.8	35.0	1.66	66.4	35.2
R. microplus 2	90.9	1.68	87.9	Cy3	2.8	39.6	1.63	43.6	22.9
R. microplus 3	73.7	1.62	71.2	Cy5	2.9	26.3	1.55	35.7	35.8

2.6.3 Quality control checkpoints for microarray analysis

Having hybridised and scanned the microarray slide, some analyses had to be performed to assess the hybridisation quality. Oligonucleotide microarray slides manufactured by Agilent Technologies contain various control spots, such as bright and dark corners. Probes that form hairpin structures are printed on the dark corner control spots, preventing any cDNA from hybridising to them. Conversely, probes containing endogenous sequences are printed in the so-called bright corners, ensuring a bright signal (Giles et al., 2010).

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Figure 2.4 shows the scanned images of arrays 1 and 2. The image on the left is predominantly green, indicating preferential binding of the *R. microplus* sample, which was labelled with Cy3 (green). Conversely, the image on the right is predominantly red, as the *R. microplus* sample for this array was labelled with Cy5 (red). The corners of each array contain control spots, and these have been enlarged. The bright corners were saturated and the dark corners produced no signal as expected (Figure 2.4 A and B, respectively). Contig8501, representing Bm86, is also shown. Note that Figure 2.4 shows the raw scanned image of the arrays. As the data are not normalised at this point, it is only for illustration purposes.

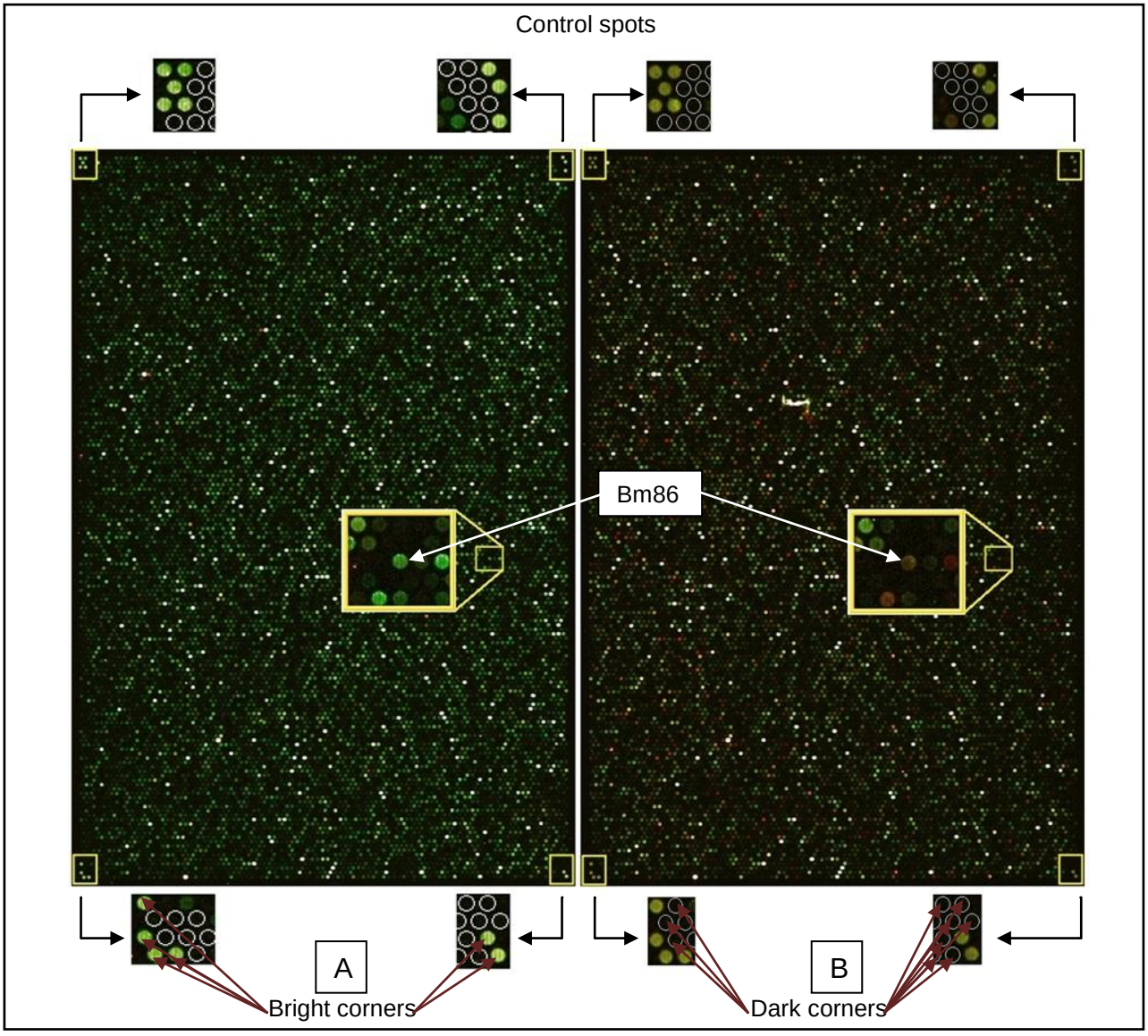

Figure 2.4. Example of scanned microarrays

Scanned microarrays hybridised using cDNA from *R. microplus* and *R. decoloratus* midgut tissues. Control spots are included on each array. The bright (*A*) and dark (*B*) corners have been enlarged. The spot representing Bm86 is indicated.

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In order to decide whether it might be necessary to normalise the data, box plots are constructed to show the distribution of *M*-values across all arrays. Pre-normalisation box plots are shown in Figure 2.5A. The median *M*-values for arrays 1 and 4 are centred at -2, while they are centred closer to 0 for arrays 2 and 3. This indicates that normalisation was required. After global LOESS normalisation, the *M*-values of all the arrays are centred at 0 (Figure 2.5B), indicating no differential expression for the median transcript, which is an assumption made when performing global LOESS normalisation (Smyth et al., 2011).

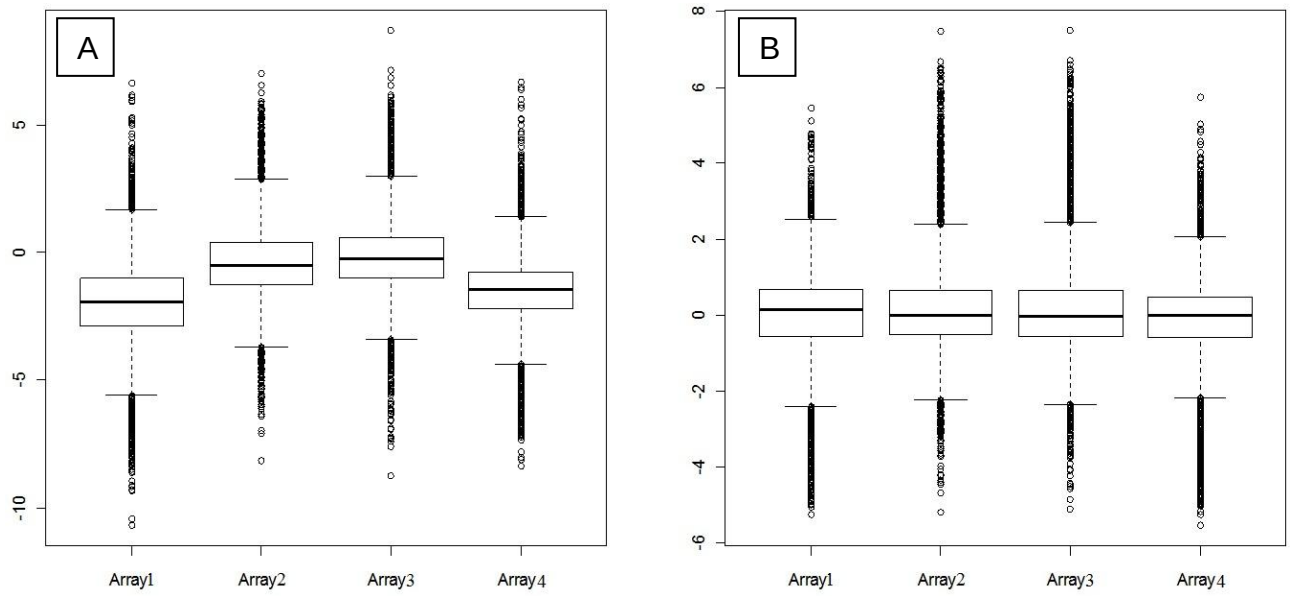

Figure 2.5. Box plots before and after normalisation

Graphs show the *M*-value distribution of each array before (*A*) and after (*B*) global LOESS normalisation.

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The need to normalise the *M*-values of the data can also be visualised by constructing red-green density plots. Prior to normalisation, it is evident that there is a large discrepancy between the red vs. green $\log_2$ratios, or *M*-values (Figure 2.5A and Figure 2.6A). After global LOESS normalisation, the discrepancy between these is much smaller; as the red and green graphs have similar shapes (Figure 2.6B).

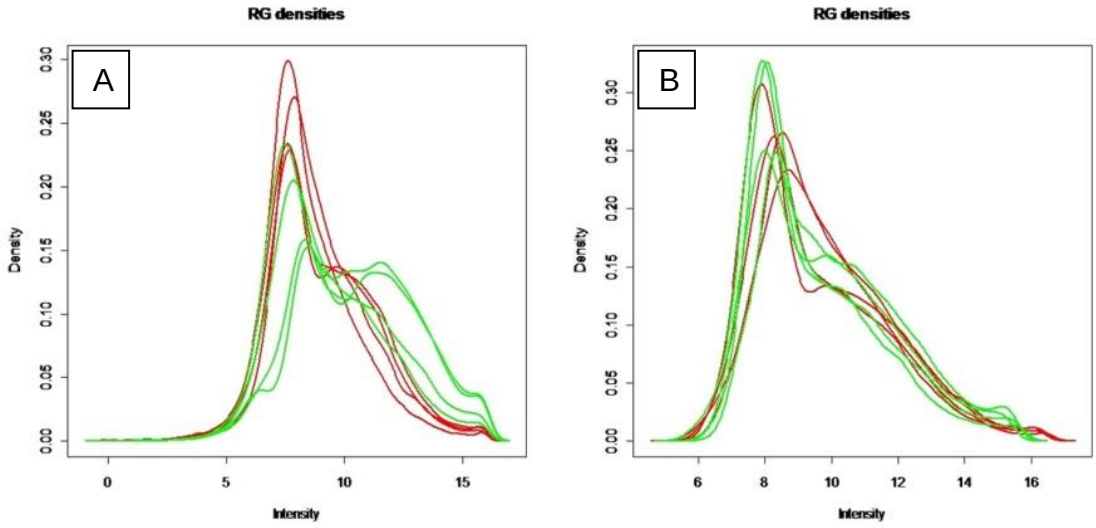

Figure 2.6. Red-Green density plots of all arrays before and after normalisation

Before (*A*) and after (*B*) global LOESS normalisation density plots of all four arrays.

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The quality of microarray data can also be assessed by looking at the *MA* plot. Assuming that the majority of genes is not differentially expressed, the majority of data points on an *MA* plot would have an *M*-value of close to 0, corresponding to no difference between the $\log_2$-transformed signal intensities of the two channels (Formula 2.1). Before global LOESS normalisation, the most data points from arrays 2 and 4 have negative *M*-values (Figure 2.7, A and C) and after normalisation, the *M*-values lie between 2 and -2 (Figure 2.7, B and D). When the majority of the *M*-values centre around 0, global LOESS normalisation is complete and the next normalisation step can be performed.

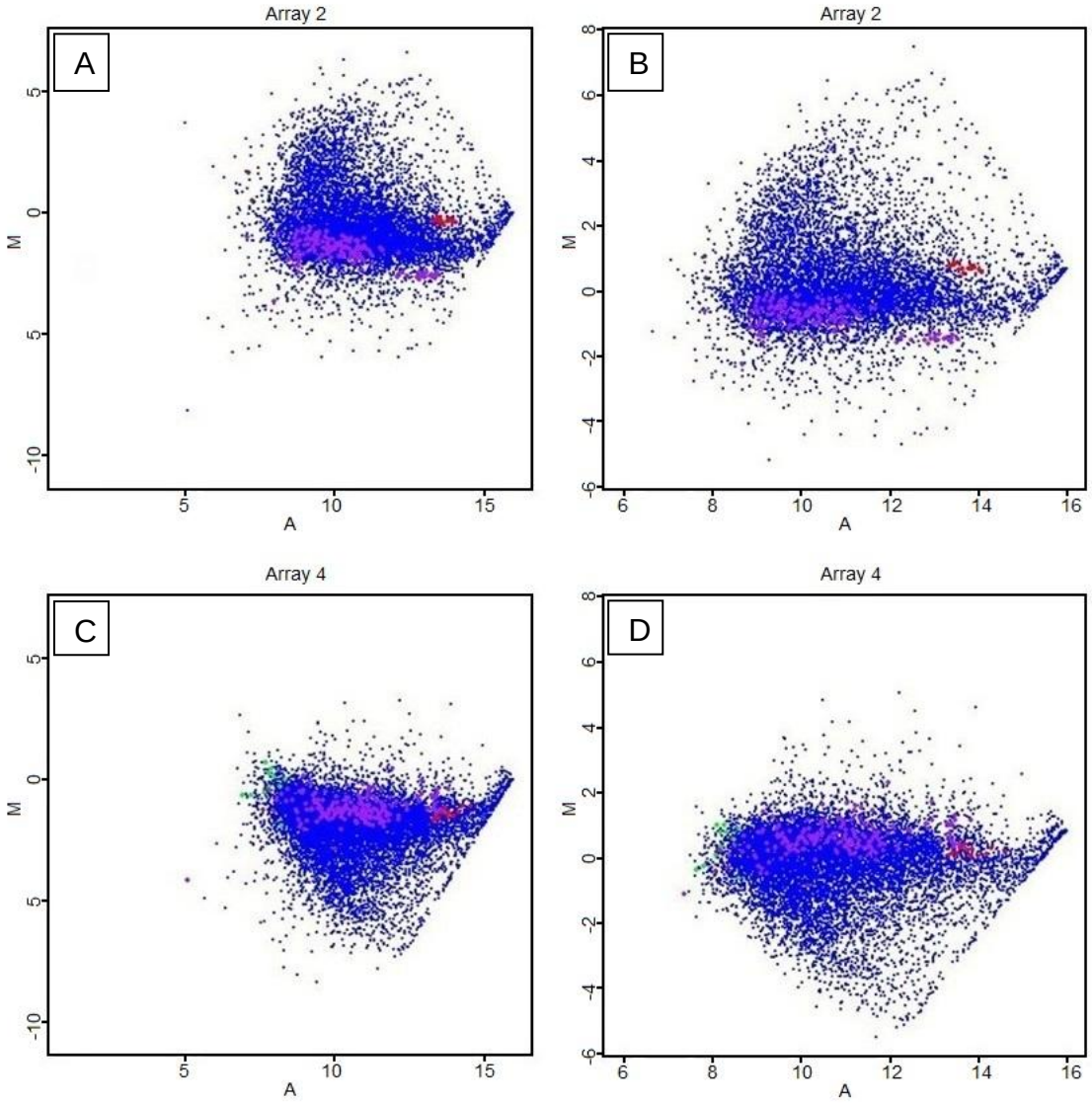

Figure 2.7. MA plots of arrays 2 and 4 before and after normalisation

Arrays 2 and 4 before (*A* and *C*) and after (*B* and *D*) global LOESS normalisation.

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Finally, having normalised the $\log_2$intensity ratios of the red and green channels within all the arrays, the average spot intensity values between arrays had to be normalised. This was done using Aquantile normalisation, where the *M*-values remain unchanged and only the distribution of *A*-values is normalised between arrays. A red-green density plot can be used to visualise the effect of Aquantile normalisation, shown in Figure 2.8. Here it can be seen that the average $\log_2$intensity as well as $\log_2$ratios of the red and green channels across all arrays are in a similar range, as all the graphs have both similar shapes and sizes.

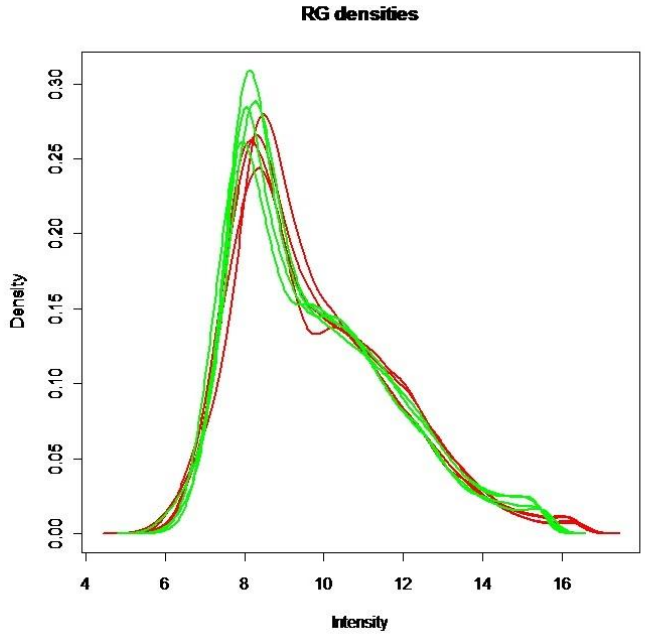

Figure 2.8. Red-Green density plots

Density plots show the intensity distributions of the red and green channels across all four arrays.

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2.6.4 Microarray data analysis

The log_2ratios of each gene's fold change between *R. microplus and R. decoloratus* were exported using LIMMA. These were then analysed to find genes of which the expression levels were similar between the two species. At a 95% confidence level, there were 2476 shared genes (-1.7 ≤ log_2fold change ≤ 1.7), 1084 with a higher expression level in *R. microplus* (log_2fold change > 1.7) and 136 with a higher expression level in *R. decoloratus* (log_2fold change < -1.7). This can be illustrated by using a graph that plots the adjusted *p*-value against the log_2fold change. Transcripts that are shared between *R. microplus* and *R. decoloratus*, as well as transcripts specific to either tick species, are shown in Figure 2.9. Validation of these findings using proteomics is currently under way. This is necessary to determine the relationship between mRNA and protein levels in *R. microplus* and *R. decoloratus*.

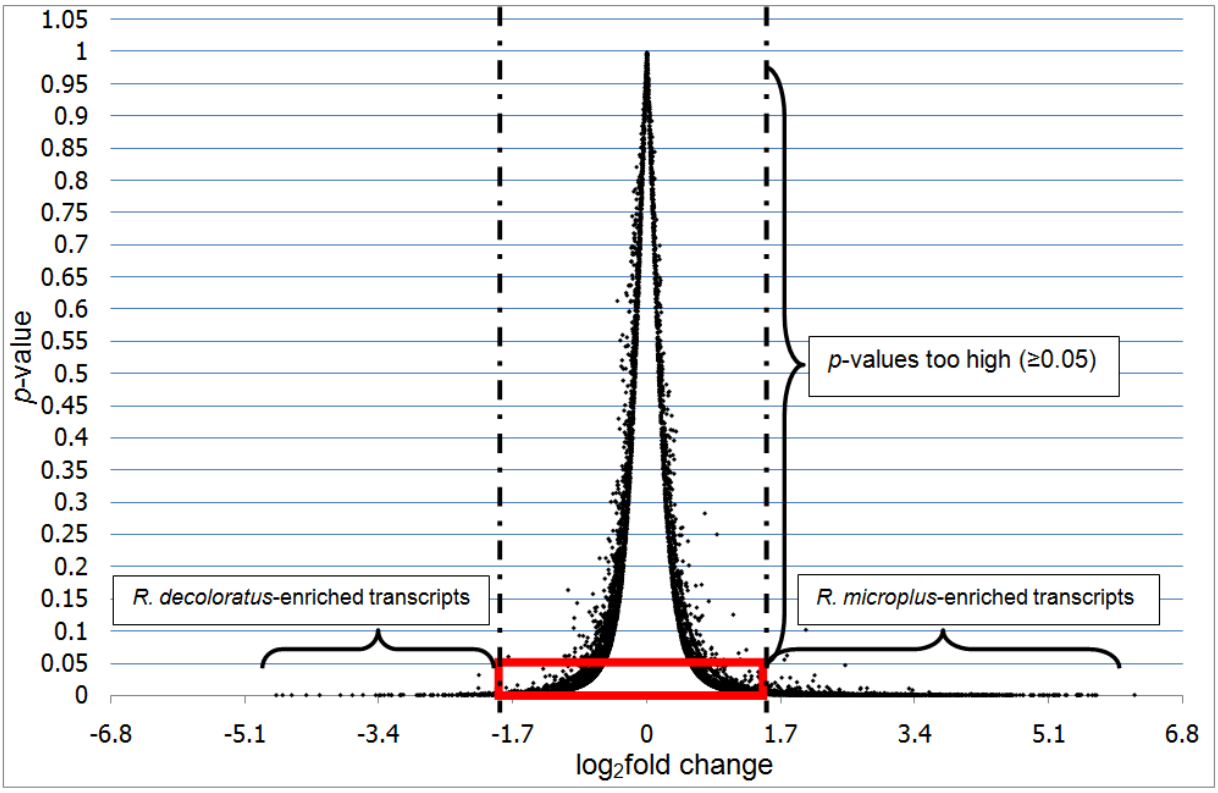

Figure 2.9. Identification of transcripts shared between *R. microplus* and *R. decoloratus*

Plotting the adjusted *p*-value versus the log_2fold change for each transcript shows which transcripts are shared between the two ticks and which are unique to either. Note the large number of transcripts where the *p*-values were greater than 0.05, indicating a large number of genes where no conclusion could be drawn. Shared transcripts are outlined by the red box.

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To further visualise the results, the distribution of log_2fold changes is indicated as a bar chart in Figure 2.10. Negative values indicate that expression levels in *R. decoloratus* were higher and positive values indicate that expression levels in *R. microplus* were higher. Transcripts where the absolute log_2fold change between the two tick species was smaller than or equal to 1.7, are shown in Figure 2.9 in the red box. These transcripts, having a relatively small fold change in expression levels between the two tick species, were considered to be shared between them. In total, 2476 putatively shared transcripts were identified.

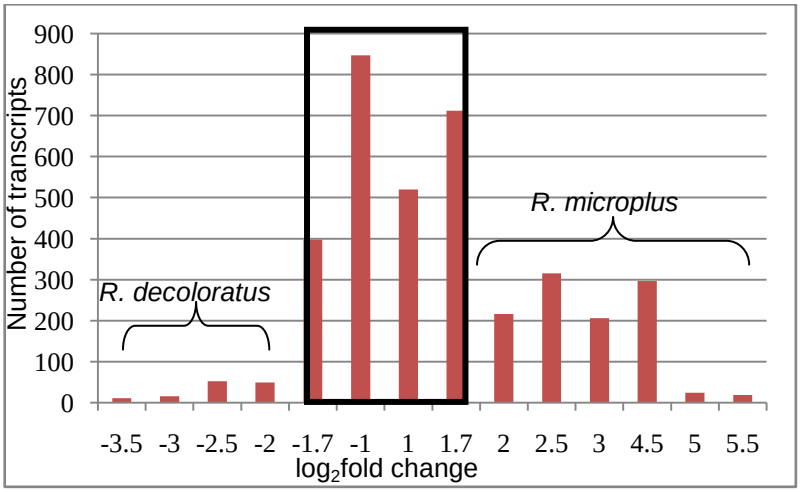

Figure 2.10. Log_2ratio distribution

Log_2fold changes of *R. microplus* vs. *R. decoloratus* transcripts with *p*-value < 0.05. Transcripts shared between the species are indicated (-1.7≤log_2fold change≤1.7) in the black box.

2.6.5 Functional classification of transcripts

Using the automated cDNA annotation system, transcripts were classified according to which eukaryotic cluster of orthologous genes they belong to. See Tab 1 on the electronic supplementary data. An *E*-value cut-off of $1x10^{-10}$ was chosen to functionally annotate transcripts. Out of 136 transcripts found to be upregulated in *R. decoloratus*, only 32 (24%) were annotatable using KOG (Figure 2.11). The three largest clusters were posttranslational modification, protein turnover and chaperones (25.0%), lipid transport and metabolism (15.6%) and signal transduction mechanisms (12.5%). This leaves 104 (76%) unannotatable transcripts. At an *E*-value cut-off of $1x10^{-25}$, 44% of *R. microplus* sequences had hits against other databases (Guerrero et al., 2005), which is higher than the reported 24% of transcripts that had hits against the KOG database. Out of the 136 transcripts upregulated in *R. decoloratus*, 40 (30%) transcripts had hits against the non-redundant database, however.

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This shows that the transcripts that are upregulated in *R. decoloratus* are even less annotatable.

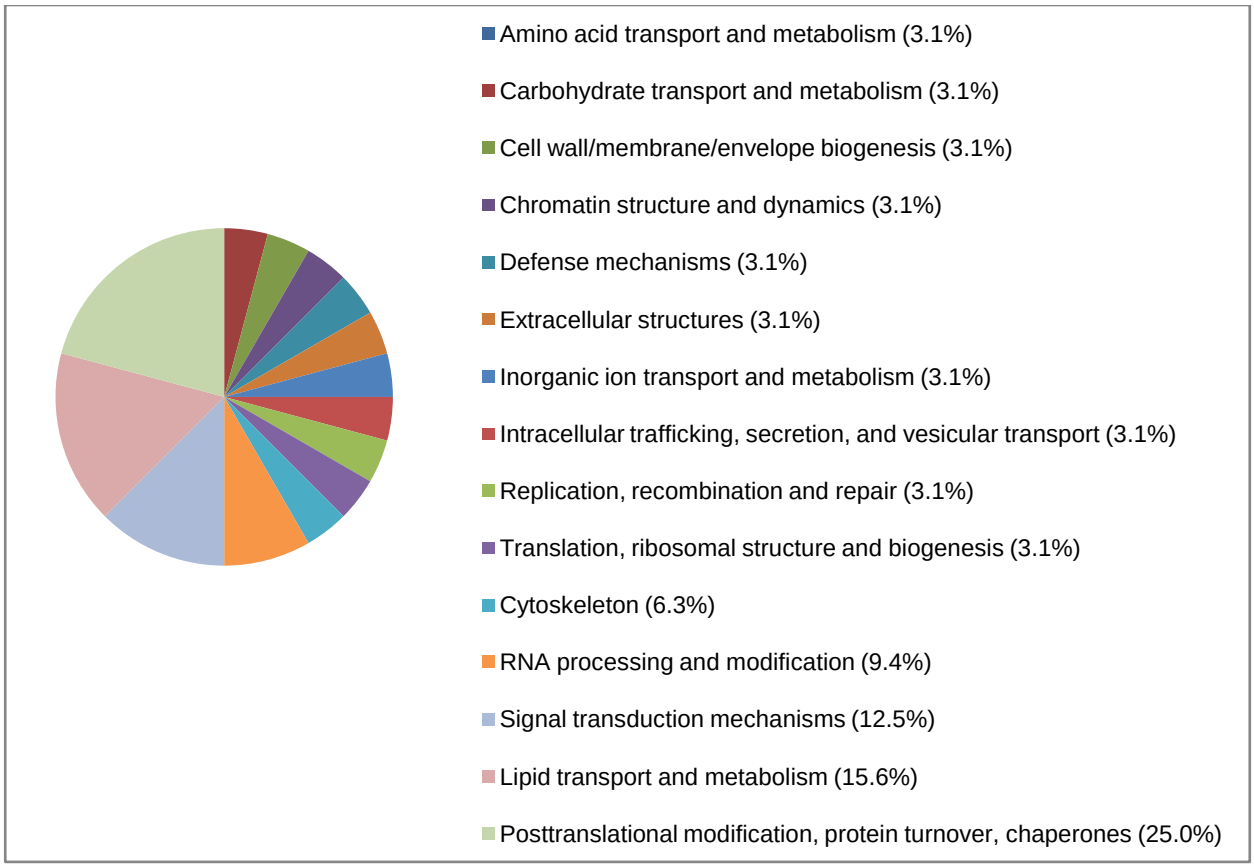

Figure 2.11. Annotation of transcripts upregulated in *R. decoloratus* using KOG

Only annotations with an *E*-value greater than or equal to $1x10^{-10}$ were considered.

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Figure 2.12 shows the biological classification of the 323 (30%) annotatable genes that were upregulated in *R. microplus*. Interestingly, the top three clusters are the same as for the genes upregulated in *R. decoloratus*: lipid transport and metabolism (7.7%), signal transduction mechanisms (10.8%) and posttranslational modification, protein turnover and chaperones (17.0%). Most genes were unannotatable using KOG (761 or 70%).

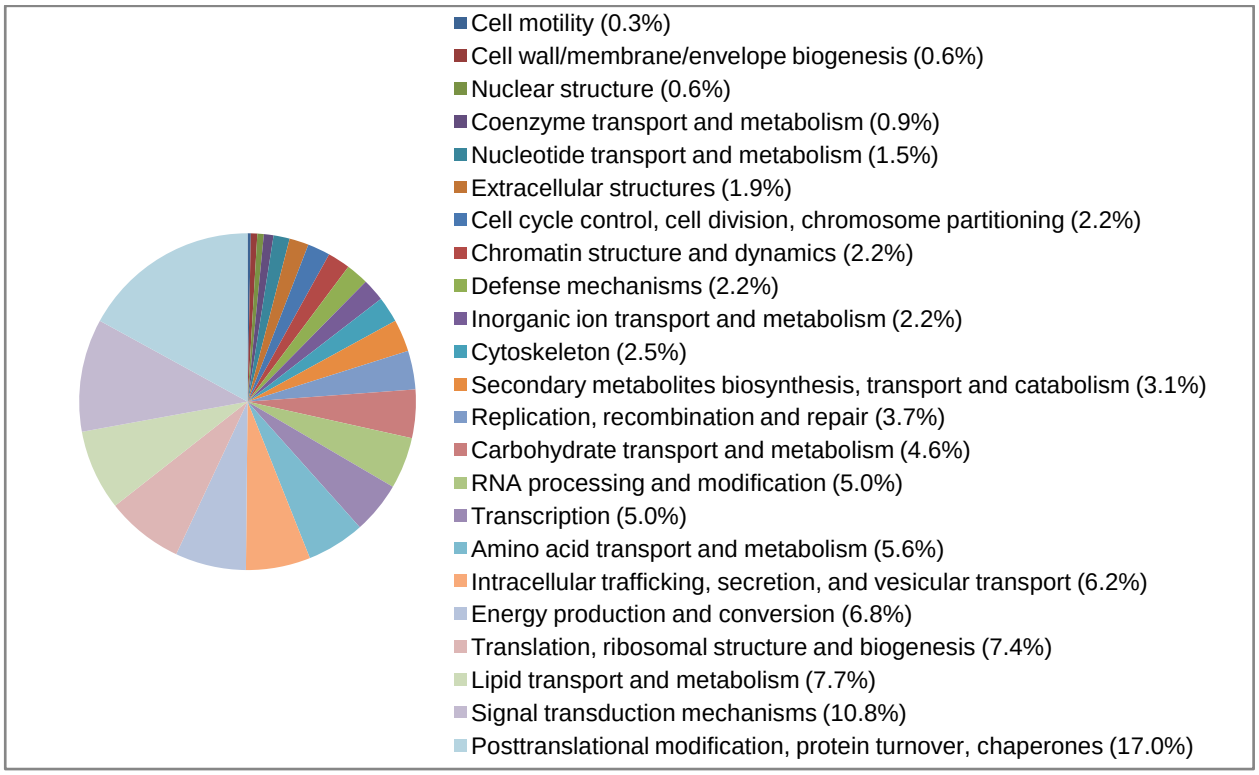

Figure 2.12. Annotation of transcripts upregulated in *R. microplus* using KOG

Only annotations with an *E*-value greater than or equal to $1x10^{-10}$ were considered.

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Lastly, Figure 2.13 shows the KOG classification of the 718 (29%) annotatable genes that were found to be shared between *R. decoloratus* and *R. microplus*. Again, signal transduction mechanisms (9.7%) and posttranslational modification, protein turnover and chaperones (13.8%) are found in the three largest clusters. Transcription-related genes (10.2%) now occupy the 2nd largest cluster in this group of genes. Lipid transport and metabolism (5.0%) however, is now only the 9th largest cluster. As with the *R. microplus*-enriched genes, 1758 (71%) of the genes are unannotatable.

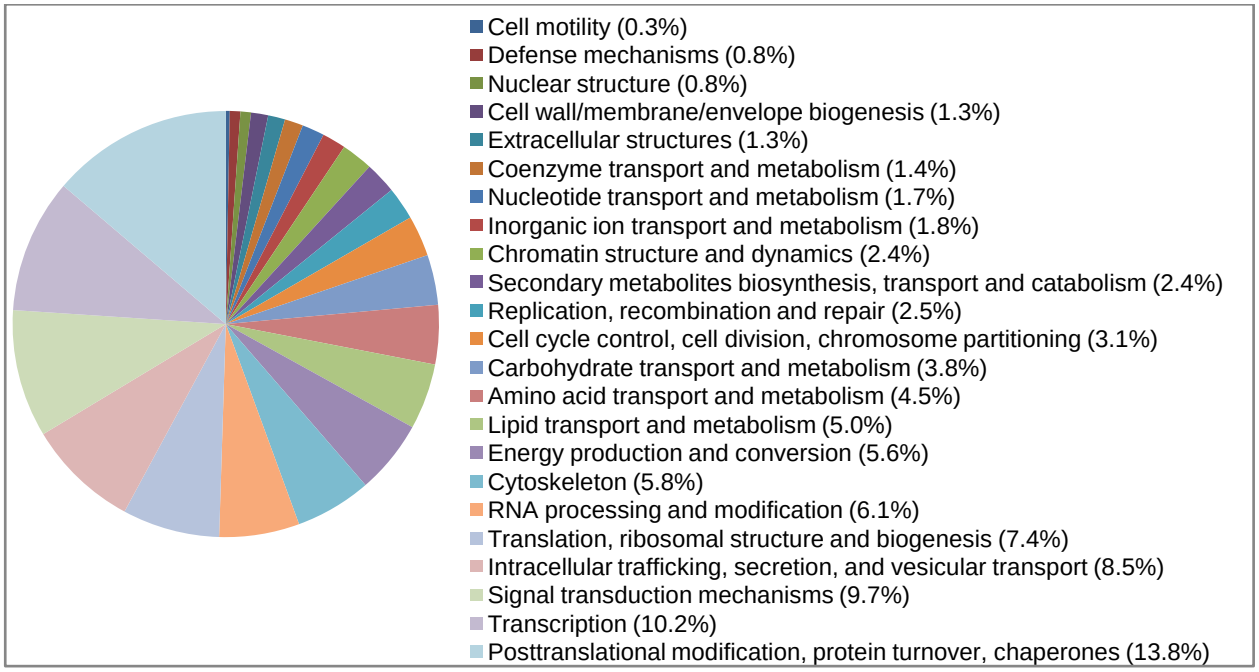

Figure 2.13. Annotation of transcripts shared between *R. microplus* and *R. decoloratus* using KOG

Annotations were based on the different eukaryotic clusters of orthologous genes. An *E*-value cut-off $1x10^{-10}$ was selected to assign transcripts to functional classes.

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When looking at the 20 annotatable genes that are most highly expressed in *R. microplus* and in *R. decoloratus* (Table 2.6), again it is evident that genes involved in posttranslational modification, protein turnover and chaperones represent a major group of upregulated genes in both species. Curiously, six of the twenty most upregulated annotatable genes in *R. decoloratus* are involved in lipid transport and metabolism, whereas only two such genes were identified in the corresponding list of *R. microplus* genes.

Table 2.6. Annotation of the twenty most highly upregulated transcripts in *R. microplus* and *R. decoloratus*

Name	log_2fold change	*E*-value	General biological process (KOG)	Best match to databases (NR, KOG and GO)
Upregulated in *R. microplus*				
Contig6100	5.690532	1E-70	Posttranslational modification, protein turnover, chaperones	Cathepsin L-like proteinase, Peptidase C1 family
Contig6280	5.659406	1E-14	Signal transduction mechanisms	Membrane glycoprotein LIG-1
Contig1826	5.52198	3E-37	Posttranslational modification, protein turnover, chaperones	Glutathione transferase (GSTE3), GST superfamily
Contig2900	5.50104	1E-85	Translation, ribosomal structure and biogenesis	Ribosomal protein S7
Contig250	5.489688	4E-25	Posttranslational modification, protein turnover, chaperones	Cathepsin D, Peptidase A1 family
Contig9019	5.464995	2E-11	Energy production and conversion	NADH-ubiquinone oxidoreductase chain 5
Contig809	5.335701	3E-28	Nucleotide transport and metabolism	GMP synthase
Contig6473	5.194716	2E-61	Translation, ribosomal structure and biogenesis	60S ribosomal protein L26 (RPL26), Ribosomal protein L24P family
Contig6406	5.179166	5E-29	Amino acid transport and metabolism	Serine protease elastase 2 like (ela2l)
Contig5592	5.158764	1E-21	Signal transduction mechanisms	Calcium-activated neutral proteinase 3
Contig9025	5.083948	9E-72	Translation, ribosomal structure and biogenesis	Elongation factor 1-gamma
Contig2302	4.782331	1E-19	Lipid transport and metabolism	Phosphoethanolamine N-methyltransferase (NMT), Methyltransferase superfamily
Contig3850	4.778745	2E-39	Amino acid transport and metabolism	Serine protease chymotrypsinogen B (CTRB1), secreted peptidase
Contig953	4.625647	1E-63	Posttranslational modification, protein turnover, chaperones	Cathepsin B, Peptidase C1 family
Contig2797	4.580061	4E-40	Intracellular trafficking, secretion, and vesicular transport	Receptor expression-enhancing protein 5-like (REEP), DP1 family
Contig1729	4.579455	3E-26	Lipid transport and metabolism	Proactivator polypeptide (PSAP), Prosaposin
Contig1863	4.573586	1E-131	Posttranslational modification, protein turnover, chaperones	Legumain (LGMN), Peptidase C13 family
Contig4841	4.549125	2E-60	Inorganic ion transport and metabolism	Superoxide dismutase [Cu-Zn]
Contig1506	4.512081	4E-53	Posttranslational modification, protein turnover, chaperones	Dipeptidyl-peptidase I (CTSC), Cathepsin C, Peptidase C1 family
Contig4369	4.457761	1E-14	Signal transduction mechanisms	Membrane glycoprotein LIG-1
Upregulated in *R. decoloratus*				
Contig7457	-4.32966	3E-21	Posttranslational modification, protein turnover, chaperones	Papilin; involved in modulation of metalloproteinases during organogenesis
CK174578	-3.24583	8E-32	Lipid transport and metabolism	N-acetylglucosaminyltransferase complex, subunit PIG-A/SPT14
CK187607	-3.13244	2E-16	Posttranslational modification, protein turnover, chaperones	Chaperone protein Zgc:153268 from *Danio rerio* involved in protein folding
CV438503	-3.11833	7E-13	RNA processing and modification	RNA binding motif protein 34
Contig7499	-2.92757	1E-17	Lipid transport and metabolism	Phosphatidylinositol transfer protein SEC14
Contig42	-2.75135	4E-63	Defence mechanisms	Protein Serpinb3c
Contig4499	-2.72137	9E-21	Lipid transport and metabolism	Phosphatidylinositol transfer protein SEC14
CK184995	-2.58648	6E-54	Posttranslational modification, protein turnover, chaperones	Ubiquitin-like modifier-activating enzyme 1
CK179409	-2.56264	1E-133	Posttranslational modification, protein turnover, chaperones	Heat shock protein 68
Contig4008	-2.40069	7E-16	Posttranslational modification, protein turnover, chaperones	FKBP-type peptidyl-prolyl cis-trans isomerase
CV436151	-2.27744	7E-24	Signal transduction mechanisms	Sodium-neurotransmitter symporter
Contig1229	-2.27625	2E-75	Intracellular trafficking, secretion, and vesicular transport	Translocon-associated complex TRAP, alpha subunit
Contig3281	-2.11637	3E-54	Chromatin structure and dynamics	Histone H4

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Name	log_2fold change	*E*-value	General biological process (KOG)	Best match to databases (NR, KOG and GO)
CK191207	-2.08936	4E-23	Inorganic ion transport and metabolism	Sodium-coupled monocarboxylate transporter 1
Contig6920	-2.08243	2E-41	Lipid transport and metabolism	Acetyl-coenzyme A synthetase
Contig2063	-2.07151	2E-75	Posttranslational modification, protein turnover, chaperones	Endoplasmin (HSP90B1), Heat shock protein 90 family
Contig3808	-2.05657	1E-27	Lipid transport and metabolism	Peroxisomal NUDIX hydrolase
Contig717	-2.01761	1E-13	Lipid transport and metabolism	Acyl-CoA synthetase
CV444468	-2.01457	1E-51	Posttranslational modification, protein turnover, chaperones	Protein geranylgeranyltransferase type II, alpha subunit
Contig4745	-2.00528	6E-39	Carbohydrate transport and metabolism	Beta-galactosidase

2.6.6 Link to biology

When looking at Figures 2.11-2.13, it is evident that not all the biological processes are equally represented. In order to determine whether some of these were significantly enriched when comparing the shared, *R. microplus*-favoured or the *R. decoloratus*-favoured gene lists, χ^2 analysis was performed (Rivals et al., 2007). Before starting such an analysis, normal levels first need to be established. The entire in-house assembled *R. microplus* database with each transcript's KOG classification was used for this purpose.

Chi-square analysis is performed using the formula:

$$\chi^2 = \sum \frac{(Observed - Expected)^2}{Expected} \quad \text{Equation 2.5}$$

Results for χ^2 analysis are summarised in Table 2.7. Shown are the percentages of each biological term comprised by the respective dataset. The percentage in brackets, as per χ^2 analysis, indicates the chance that a similar sample would have to find the same observed occurrence for the respective biological term. This therefore means that smaller percentages are more significant.

Only 2 biological terms were found to be significantly enriched among all comparisons evaluated. Intracellular trafficking, secretion, and vesicular transport was significantly higher (p=0.031) in the list of genes shared between *R. microplus* and *R. decoloratus*. This is not surprising, as the RNA samples had been collected from ticks on 20 days post infestation, where ticks are engorging rapidly.

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Lipid transport and metabolism was significantly enriched in both the *R. microplus* and *R. decoloratus* gene lists (p=0.035 and p=0.007, respectively). This suggests a possible preference for lipids as a nutrient source during feeding, 20 days post infestation.

Table 2.7. Identification of significantly enriched biological terms

Numbers indicate the percentage occurrence of the biological term in the respective gene list. The percentages in brackets indicate the χ2 probability that there is no difference in the occurrence of the biological term in the gene list compared to its occurrence in the in-house assembled *R. microplus* database. Smaller percentages are therefore more significant. Green boxes indicate enriched biological terms and red boxes indicate depleted ones.

Biological process	**% Occurrence in *R. microplus* database**	**% Occurrence in shared genes (χ^2 probability)**	**% Occurrence in *R. microplus*-upregulated genes (χ^2 probability)**	**% Occurrence in *R. decoloratus*-upregulated genes (χ^2 probability)**
Amino acid transport and metabolism	5.6	4.5 (23.50%)	5.6 (98.9%)	3.1 (55.0%)
Carbohydrate transport and metabolism	4.0	3.8 (72.3%)	4.6 (60.3%)	3.1 (79.2%)
Cell cycle control, cell division, chromosome partitioning	2.5	3.1 (39.2%)	2.2 (70.7%)	0 (36.4%)
Cell motility	0.1	0.3 (41.4%)	0.3 (46.7%)	0 (83.1%)
Cell wall/membrane/envelope biogenesis	0.9	1.3 (44.0%)	0.6 (56.2%)	3.1 (20.7%)
Chromatin structure and dynamics	2.5	2.4 (82.8%)	2.2 (70.7%)	3.1 (82.4%)
Coenzyme transport and metabolism	1.2	1.4 (61.5%)	0.9 (70.0%)	N/A (53.9%)
Cytoskeleton	4.3	5.9 (7.60%)	2.5 (11.1%)	6.3 (59.6%)
Defence mechanisms	1.3	0.8 (29.30%)	2.2 (20.7%)	3.1 (37.2%)
Energy production and conversion	5.3	5.6 (74.30%)	6.8 (24.1%)	0 (18.2%)
Extracellular structures	1.0	1.3 (58.70%)	1.9 (16.9%)	3.1 (24.5%)
Inorganic ion transport and metabolism	2.4	1.8 (30.20%)	2.2 (75.2%)	3.1 (80.6%)
Intracellular trafficking, secretion, and vesicular transport	6.3	**8.6 (3.10%) ↑**	6.2 (94.2%)	3.1 (46.1%)
Lipid transport and metabolism	5.0	5.1 (99.9%)	**7.7 (3.5%) ↑**	**15.6 (0.7%) ↑**
Nuclear structure	0.8	0.8 (85.3%)	0.6 (76.6%)	N/A (61.9%)
Nucleotide transport and metabolism	2.2	1.7 (39.9%)	1.5 (46.1%)	N/A (40.0%)
Posttranslational modification, protein turnover, chaperones	14.2	13.9 (76.6%)	17.0 (16.8%)	25 (8.30%)
Replication, recombination and repair	2.8	2.5 (67.1%)	3.7 (34.1%)	3.1 (90.9%)
RNA processing and modification	7.3	6.2 (26.9%)	5.0 (11.7%)	9.4 (65.2%)
Secondary metabolites biosynthesis, transport and catabolism	2.5	2.4 (86.2%)	3.1 (49.8%)	N/A (36.7%)
Signal transduction mechanisms	11.3	9.8 (21.7%)	10.8 (78.6%)	12.5 (83.6%)
Transcription	8.4	10.3 (13.3%)	**5.0 (2.90%) ↓**	N/A (28.1%)
Translation, ribosomal structure and biogenesis	8.0	7.4 (59.10%)	7.4 (72.9%)	3.1 (9.6%)

The transcripts that are upregulated in either *R. microplus* or *R. decoloratus* are shown in Table 2.8. Most of these transcripts (9) play a role in β-oxidation. This suggests an important role of lipids for the production of energy in *R. microplus*. During the slow feeding phase, lipid inclusions of varying size are found in digestive cells in the midgut (Sonenshine, 1991). With tick control in mind, the transcripts that are involved in lipid metabolism might be used as targets for tick control strategies.

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Table 2.8. Transcripts involved in lipid transport and metabolism

Biological role inferred using UniProt (http://www.uniprot.org/).

Transcript	*E*-value	KOG annotation	Biological role
Contig8380	7E-89	Sterol O-acyltransferase/Diacylglycerol O-acyltransferase	Anabolism of cholesterol ester / triglycerides
Contig2916	2E-62	Acetyl-CoA acetyltransferase	Cholesterol synthesis
Contig74	6E-49	Cholesterol transport protein	Cholesterol transport
Contig7583	1E-77	Lipid exporter ABCA1	Lipid transport
Contig2020	1E-105	Ethanolamine kinase	Phospholipid biosynthesis
Contig3708	3E-21	Long chain fatty acid acyl-CoA ligase	Phospholipid biosynthesis
Contig7499	1E-17	Phosphatidylinositol transfer protein SEC14	Transfer of phosphatidylinositol/ phosphatidylcholine
Contig4499	9E-21	Phosphatidylinositol transfer protein SEC14	Transfer of phosphatidylinositol/ phosphatidylcholine
Contig8127	7E-11	Predicted lipoprotein	Unknown
Contig2302	1E-19	SAM-dependent methyltransferases	Unknown – general prediction only
Contig7995	1E-104	Acid sphingomyelinase	Apoptosis
Contig6561	2E-30	Mitochondrial/plastidial beta-ketoacyl-ACP reductase	Fatty acid biosynthesis
Contig3801	8E-29	Mitochondrial/plastidial beta-ketoacyl-ACP reductase	Fatty acid biosynthesis
Contig2638	2E-36	beta-ketoacyl-ACP reductase	Fatty acid biosynthesis
Contig6832	2E-41	15-hydroxyprostaglandin dehydrogenase	Prostaglandin inactivation
Contig6062	8E-56	Gamma-butyrobetaine,2-oxoglutarate dioxygenase	L-carnitine synthesis
TC22152	6E-24	Lipid phosphate phosphatase	Stress response signalling
Contig3710	2E-51	Lysophospholipase	Breakdown of lipids
Contig2988	8E-65	Lysophospholipase	Breakdown of lipids
Contig6686	1E-41	Triglyceride lipase-cholesterol esterase	Catabolism of cholesterol ester / triglycerides
Contig6920	2E-41	Acyl-CoA synthetase	β-oxidation
TC15677	4E-89	Acyl-CoA synthetase	β-oxidation
Contig717	1E-13	Acyl-CoA synthetase	β-oxidation
Contig4388	3E-26	Acyl-CoA synthetase	β-oxidation
Contig4293	2E-80	Acyl-CoA synthetase	β-oxidation
Contig890	5E-28	2-enoyl-CoA hydratase / 3-hydroxyacyl-CoA dehydrogenase / Peroxisomal 3-ketoacyl-CoA-thiolase	β-oxidation
CV447153	7E-20	2-enoyl-CoA hydratase / 3-hydroxyacyl-CoA dehydrogenase / Peroxisomal 3-ketoacyl-CoA-thiolase	β-oxidation
Contig2857	1E-134	Medium-chain acyl-CoA dehydrogenase	β-oxidation
Contig984	4E-95	Enoyl-CoA hydratase	β-oxidation

2.6.7 Microarray validation using semi-qPCR

Validation of transcripts expressed in both tick species was done using semi-qPCR. Seven putatively shared transcripts were chosen at random and their relative abundance was normalised using three reference genes: previously evaluated cyclophilin and elongation factor 1α from *R. microplus* (Nijhof et al., 2009) and an in-house identified reference gene, Contig8723. Results are summarised in Table 2.9. The $\log_2$fold changes obtained for seven transcripts using semi-qPCR and microarray are shown, as well as whether they are in the same direction i.e. whether the techniques agree that the transcript was more highly expressed in the same tick species. For elongation factor 1α, the $\log_2$fold change was 0.005 from the semi-qPCR results and -0.13 from the microarray data. This means that its expression was $2^{0.005}$(=1.003) times as much in *R. microplus* than in *R. decoloratus*. From the microarray data, this value is $2^{-0.13}$ (=0.914). Both techniques therefore gave highly similar results for elongation factor 1α. Similarly, Contig8723 was found to be $2^{-0.18}$ (=0.88) times as abundant in *R. microplus* compared to *R. decoloratus*, using semi-qPCR. According to the microarray data, this value was found to be $2^{0.13}$ (1.09). Therefore, despite the fact that there

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was a disagreement between the trends observed by the two techniques when evaluating expression levels of two reference genes, the measured differences were small. The third gene where the results from the two techniques indicated opposite trends was CV456291. Here, semi-qPCR results indicated its expression to be $2^{0.85}$ (=1.8) times higher in *R. microplus* than in *R. decoloratus*, whereas this ratio was found to be $2^{-0.75}$ (=0.6) according to the microarray data. These results show the problems that may arise when validating a large dataset of microarray data by performing semi-qPCR on a very small subset. Others also argue the relevance of this form of validating microarray results (Allison et al., 2005). The genome of *R. microplus* is said to be rich in repetitive sequences (Ullmann et al., 2005) but no mention is made of the complexity of sequences obtained from a large-scale EST sequencing project (Guerrero et al., 2005). It is therefore argued that the observed disparity in the results from the two different techniques can be ascribed to differences in their experimental methodology and method for processing results. Clearly, there is a large difference in the kinetics of hybridisation of 60-mer probes, compared to the kinetics of real-time PCR. Moreover, hybridisation of the labelled cDNA onto the oligonucleotide microarray took place at 65°C. The smallest Tm of all the probes was 67.76°C, and the highest Tm was 99.34°C. Suboptimal hybridisation conditions could therefore also affect the integrity of results obtained for some of the probes, stressing the importance of validating results with an additional technique, such as semi-qPCR.

Table 2.9. Validation of microarray results using semi-qPCR

Transcript	qPCR log_2fold change	Microarray log_2fold change	Same direction?
Contig1269	0.27	0.81	Yes
Contig1362	-1.64	-0.76	Yes
Contig1514	7.96	0.56	Yes
Contig3185	2.17	0.58	Yes
CV456291	0.85	-0.75	No
Contig5295	0.95	0.82	Yes
Contig8515	1.28	0.56	Yes
EF1α	0.005	-0.13	No
PPIA	0.12	0.26	Yes
Contig8723	-0.18	0.13	No

2.6.8 Additional data mining

In order to perform reliable gene expression analysis using quantitative or semi-quantitative real-time PCR, normalisation of data using reference genes is essential. A survey of 20 peer-reviewed publications on tick research showed that β-actin was the most popular reference gene chosen (Nijhof et al., 2009). Due to the diversity in the expression stability of putative

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reference genes, such as β-actin, it is suggested that reference genes are validated for every particular experimental setup (Zyżyńska-Granica and Koziak, 2012).

A reference design microarray was performed by Mr. C. Stutzer to study the transcriptome of *R. microplus* across its entire life cycle. This was done by employing a reference pool experimental design, where the reference pool consisted of RNA from all the life stages and organs of *R. microplus*. Expression levels of some 13,500 genes were evaluated in larvae, nymphs and salivary glands, ovaries and the midgut from feeding adult ticks. The reference pool was labelled with Cy3 (green) and cDNA from larvae, nymphs, salivary glands, ovaries and the midgut was labelled with Cy5 (red). In order to identify transcripts that represent putative reference genes for use in real-time PCR, the absolute intensity values corresponding to the Cy5 (red) channel were calculated. After normalisation of the microarray slides, the *M*- and *A*-values were exported. Recall that

$$M = \log_2 R - \log_2 G \quad \text{Equation 2.1}$$

$$A = (\log_2 R + \log_2 G)/2 \quad \text{Equation 2.2}$$

From this it follows that:

$$R = \sqrt{2^{2A}.2^{M}} \quad \text{Equation 2.6}$$

The non-parametric one-way analysis of variance Kruskal-Wallis method was performed by Ms Nanette Coetzer (Bioinformatics and Computational Biology Unit, University of Pretoria) in order to identify transcripts where expression levels among the three tissues and two life stages were relatively constant. This would be useful for future real-time PCR studies where reference genes in *R. microplus* are lacking. Results from the Kruskal-Wallis analysis of variance revealed 2348 transcripts that were putative reference genes. Contig8723, which

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was used as a reference gene, had previously been validated as a potential reference gene in *R. microplus* using results and materials from this study (Sabine Richards, Hons report 2010). A manuscript detailing the validation of these putative reference genes is currently in preparation. Table 2.10 shows the median signal intensities for all the tested tissues and life stages along with their respective *p*-values from Kruskal-Wallis analysis. See Tab 2 on the electronic supplementary data for the rest of the putative reference genes.

Table 2.10. Identification of putative reference genes

Shown are the median intensity values for the respective life stage or tissue from feeding female ticks, along with the Kruskal-Wallis *p*-value that would indicate significant differences between the groups evaluated. Large *p*-values indicate that no significant differences were observed and that the genes are therefore regarded as putative reference genes.

Name	Median Cy5 intensity					Kruskal-Wallis *p*-value
	Larvae	Nymphs	Salivary glands	Midgut	Ovaries	
Contig2088	4254.11	4970.706	4317.484	5108.336	5613.802	0.072561
CV442512	5126.607	5728.206	4896.531	5113.337	5651.732	0.382896
Contig2276	5917.751	4444.722	5812.835	4534.389	5319.282	0.085777
Contig2927	4116.173	4243.587	4688.981	5317.303	4481.805	0.093471
Contig8394	5635.563	4143.676	4825.408	4705.103	4449.522	0.10297
Contig8723	33980.89	34748.09	25450.22	27855.49	22182.85	0.155709
Contig4643	5678.391	5996.601	5508.119	5754.239	8586.075	0.664886
CV456066	30717.14	27449.04	33137.77	29960.28	26637.24	0.078229

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2.7 Conclusion

The development of acaricide resistance in ticks has been published extensively. As an alternative approach to tick control, the possibility of anti-tick vaccines has been demonstrated with the Bm86-based vaccine. To date, no alternative vaccines have been discovered and there are various limitations to the existing Bm86-based vaccine. To test the hypothesis that conserved proteins are present in *R. microplus* and *R. decoloratus*, a DNA microarray-based strategy was followed. Finding genes that are conserved between these two tick species would be advantageous as the primary limiting step in the identification of possible cross-protective anti-tick vaccines is the identification of suitable vaccine candidates.

Microarray technology is suitable for the identification of genes with similar expression levels across these tick species, as it allows the simultaneous analysis of expression levels of thousands of genes. Technological advances have allowed the production of custom oligonucleotide microarrays, enabling scientists to study any organism with sufficient available sequence information. As such, an in-house designed *R. microplus*-specific oligonucleotide microarray slide was designed and used. One logistical limitation was the number of available microarrays to perform experiments. Taking this and the research question into consideration, microarray experiments were performed based on a balanced block design, incorporating one dye swop to minimise technical variation and three biological replicates to account for biological variation.

The choice of starting material was the midgut tissues from *R. microplus* and *R. decoloratus* female ticks 20 days post infestation. When designing the oligonucleotide microarray, all available EST sequence data from *R. microplus* were downloaded and clustered into unique contiguous sequences. When two sequences were clustered, their original names became lost and a new name was assigned to the newly assembled cluster. This necessitated BLAST searches in order to find the contiguous sequence that corresponds to a particular gene of interest. Bd86 is the homologue of Bm86, arguably the most well-known *R. microplus* protein. It was therefore anticipated that the contiguous sequence encoding Bm86 would be found in the list of transcripts with expression levels that are similar between the two *Rhipicephalus* species. Not surprisingly, it was, with a $\log_2$fold change of 1.54, favouring *R. microplus*.

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Data analysis revealed that, at a 95% confidence level, 2476 transcripts were shared between *R. microplus* and *R. decoloratus* ($-1.7 \leq \log_2$fold change ≤ 1.7), 1084 were more highly expressed in *R. microplus* ($\log_2$fold change > 1.7) and 136 were more highly expressed in *R. decoloratus* ($\log_2$fold change < -1.7). The microarray slide was designed using *R. microplus* sequences, so it was expected that the majority of differentially expressed transcripts would be found in *R. microplus*. However, only 63% (2316) of the $\log_2$fold change values were positive (*R. microplus*), while 37% (1380) were negative (*R. decoloratus*), indicating that species-bias contributed to a deviation of only 13 percentage points from the expected 50%, had there been no differences in expression levels between the two species. Considering that gene expression levels in *R. decoloratus* were evaluated using an oligonucleotide microarray that had been designed using *R. microplus* sequences, it was expected that numerous sequences would not get detected in *R. decoloratus*. Based on alignments using their mitochondrial 12S and cytochrome c oxidase I sequences, *R. annulatus* is in fact more closely related to *R. microplus* than *R. decoloratus* is (Murrell et al., 2001). It is therefore expected that more *R. annulatus*-favoured transcripts would have been detected, had this comparative analysis been done on the midgut of *R. microplus* and *R. annulatus* female ticks 20 days post infestation.

In order to functionally annotate transcripts, large-scale BLAST searches against a series of databases, including the eukaryotic cluster of orthologous genes and the non-redundant protein database, were performed using dCAS. Figure 2.13 shows that the largest number of transcripts with similar expression levels between the two species played a role in signal transduction mechanisms (9.7%) and intracellular trafficking, secretion and vesicular transport (8.5%) and the maintenance of protein levels and state: posttranslational modification, protein turnover and chaperones (13.8%), transcription (10.2%) as well as translation, ribosomal structure and biogenesis (7.4%). Most of the shared transcripts remain unannotated, however. It has been reported before that only 44% of *R. microplus* sequences have hits against other databases at an *E*-value cut-off of $1x10^{-25}$ (Guerrero et al., 2005). Of the 3696 transcripts identified to be upregulated in *R. microplus*, *R. decoloratus* or shared between these ticks, 42% had good hits against the non-redundant database with the same *E*-value cut-off. This presents a problem with vaccine design in mind, as it would be impossible to reliably predict their function or cellular localisation, making these unannotatable genes undesirable as potential vaccine candidates, until the databases get expanded significantly. A potential solution to this problem might be integrative analyses,

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whereby results from BLAST searches are combined with predictive software and scientific judgement. Membrane-bound proteins are hypothesised to make the best vaccine targets (Rappuoli and Bagnoli, 2011), but until thorough and integrative software gets developed, manual curation and annotation of sequences will be necessary for the identification of such proteins as novel vaccine candidates. Software for the prediction of the correct reading frame and for predicting whether a protein is cytosolic or not, is discussed in Chapter 3.

Gene Ontology (GO) has become one of the most widely-accepted standards for gene annotation. An ontology is a controlled vocabulary, whereby terms are organised into a hierarchy based on their relationships (Brazma et al., 2006). GO classifies genes according to their biological process, molecular function or cellular component (Ashburner et al., 2000). In comparison, the cluster of orthologous genes (KOG) assigns a gene to one of 21 biological processes found in eukaryotes (Tatusov et al., 2003). In this study, sequences were analysed using KOG and GO and the results were stored in a tab-delimited file. The limitation observed with GO analysis was that various potential GO terms were assigned to each transcript. These GO terms are subsequently harder to compare to one another, compared with results from KOG, where each transcript was only assigned 1 biological process. The lack of tools for GO analysis for non-model organisms presents a major limitation to tick research and KOG annotations were therefore provided in this study.

In order to comply with the MIAME standards, real-time PCR was performed to validate the microarray results and seven transcripts that were found to be shared between the two species were selected for further analysis. Except for three transcripts, microarray and real-time PCR results were consistently in the same direction, i.e. if a transcript was found to be *R. microplus*-favoured during real-time PCR analysis, the same classification was made based on the microarray results. Although the direction of the results from the two techniques differed in the case of three transcripts, the differences were not very big. CV456291 was found to be *R. microplus*-favoured according to real-time PCR and found to be *R. decoloratus*-favoured according to the microarray analysis. The difference, however, is only 1.6, meaning that the difference between the *R. microplus: R. decoloratus* $\log_2$fold change from microarray and that of real-time PCR is only 3-fold. The aim was to identify transcripts where there was a small difference in expression levels between the two ticks and a list of such transcripts was produced. Real-time PCR analysis was used to corroborate the

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microarray results and again, the difference between the expression levels of these transcripts was small, except for Contig1514, which, according to real-time PCR results, was favoured in *R. microplus* (nearly 250 fold), compared to being only 1.4 times more highly expressed in *R. microplus*, according to microarray results.

These results show that numerous transcripts are in fact shared between *R. microplus* and *R. decoloratus*, indicating that it is possible to identify transcripts that are shared between two species using a microarray slide that is specific to only one of the species. Results also indicate that there is a very large pool of potential anti-tick vaccine candidates that remains to be tested in trials. Proteomic validation of their cellular localisation and expression levels would be the next logical step towards identifying promising vaccine candidates that could confer protection against *R. microplus* and *R. decoloratus*.

Chi-square analysis showed that *R. microplus* and *R. decoloratus* ticks readily transport and metabolise lipids on 20 days post infestation. These transcripts might provide an additional target for tick control as they play an important role in tick biology while female ticks are feeding rapidly. This is a curious observation, because it has been reported previously that intracellular absorption of nutrients from the blood meal is primarily directed at haemoglobin (Sonenshine, 1991), which is a protein. One would therefore expect an upregulation in genes involved in the breakdown of protein instead. As mentioned earlier, however, one of the largest clusters of biological processes included protein turnover, which might be responsible for the breakdown of haemoglobin. Apart from the absorption of haemoglobin, lipid inclusions of varying size are found in digestive cells during the slow feeding phase (Sonenshine, 1991). Chi-square analysis of the microarray results obtained in this study therefore highlighted the importance of lipid metabolism in feeding ticks. This is a novel finding, as information on genes that are involved in lipid metabolism in ticks is limited.

In this chapter, genes that might play an important role in the midgut of both *R. microplus* and *R. decoloratus* female ticks, 20 days post infestation, have been identified. The next chapter discusses ways of evaluating numerous genes in order to decide which ones to analyse further for the development of an anti-tick vaccine.

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Future aims:

1. As an alternative to DNA microarrays, RNA-seq analysis is planned for future studies on *R. decoloratus*. The advantages would be two-fold. Apart from validating results from this study, valuable sequence information will be provided for *R. decoloratus*.
2. Furthermore, structural predictions of *R. microplus* sequences with limited homology to sequences in existing databases might enable their annotation, further increasing the number of genes to be evaluated as anti-tick vaccine candidates.
3. The upregulation of genes involved in lipid transport and metabolism is a novel finding which warrants additional studies. RNAi studies might indicate which of these genes are vital for tick survival, after which they could be used as targets for rationally designed acaricides.

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Chapter 3
In silico identification and *in vitro* evaluation of novel putative anti-*Rhipicephalus* vaccine candidates

3.1 Introduction

3.1.1 Classical methodologies for vaccine development

The medical practice of immunisation using serous fluids from infected individuals was mentioned by explorers in Africa in the 16th century. However, it is likely that in China, human variolation (where the inoculum was of human source) dates back even further (Lombard et al., 2007). It was formally introduced into Western medicine by Edward Jenner, who isolated serous fluids from cows that were infected with cowpox in order to treat smallpox. Since the inoculum was derived from cows, he coined the term *vaccination*, as the Latin word for cow is *vacca* (Jenner, 1801). Nearly a century later, Louis Pasteur established the principles of vaccine development. These consisted of the isolation, inactivation and injection of the causative microorganism (Serruto and Rappuoli, 2006). Pasteur's principles have been used to develop vaccines against poliomyelitis, measles, mumps, rubella and hepatitis B (Sette and Rappuoli, 2010). Unfortunately, this approach has been unsuccessful for microorganisms that lack easily identifiable immunodominant protective antigens or those that cannot be readily cultivated *in vitro* (Serruto and Rappuoli, 2006). The success rate of vaccine development is further hampered by the variability of surface antigens of a pathogen (Rappuoli, 2007).

3.1.2 Modern approaches towards vaccine development

Before the invention of PCR and other technological breakthroughs, the hepatitis B vaccine was produced by heat-inactivation of the virus' surface antigen, isolated from the plasma of infected people (Rappuoli, 2007). Since 1982, recombinant DNA technology has allowed the large-scale production of this antigen in yeast (Valenzuela et al., 1982). Another breakthrough was represented by advances made in DNA sequencing. The first completed genome sequencing project was published in 1995, where the entire genome of *Haemophilus influenzae* was sequenced (Fleischmann et al., 1995). These techniques have been used in combination in order to identify vaccine candidates against *Neisseria meningitidis* serogroup

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B. The process of starting vaccine discovery *in silico*, using genomic information, as opposed to starting with the pathogenic organism itself, is called reverse vaccinology (Rappuoli, 2000).

The entire *N. meningitidis* genome was sequenced and scanned for open reading frames that might encode surface-exposed or exported proteins. These were then cloned for expression in *E. coli,* which was successful in 350 out of 570 selected open reading frames. Mice were subsequently immunised with the recombinant proteins and the bactericidal activity of their antiserum was evaluated (Pizza et al., 2000). This was done by adding sterile baby rabbit serum to heat-inactivated antiserum from the immunised mice, and determining the dilution needed to destroy 50% of bacterial colonies within 18 hours (Peeters, 1999). Ultimately this resulted in the discovery of a candidate vaccine that was tested in phase III clinical trials. Traditional methodologies for vaccine development against *N. meningitidis* have been unsuccessful, as the outer polysaccharide on the bacterial capsule is identical to a human self-antigen (Sette and Rappuoli, 2010). The example of *N. meningitidis* shows that reverse vaccinology is a feasible option for the design of novel vaccines against organisms where traditional methods have been unsuccessful.

More than a decade later, substantial advances have been made that allow a more systematic approach for the implementation of reverse vaccinology in order to develop novel vaccines. Technologies such as DNA microarray allow for the identification of genes that are actively transcribed under a variety of conditions. This represents an improvement on the work by Pizza and colleagues, who expressed open reading frames without prior knowledge of their expression profiles *in vitro* and *in vivo.* One drawback of reverse vaccinology, however, is the inability to identify carbohydrate antigens (Sette and Rappuoli, 2010).

A comprehensive reverse vaccinology approach consists of two principal steps. The first is to identify and predict vaccine candidates, after which their efficacy as vaccines is evaluated (Rappuoli and Bagnoli, 2011). As applicable to tick research, reverse vaccinology will be discussed below.

I. Choice of antigen

The traditional method to develop vaccines has been to cultivate pathogens, after which they are inactivated and tested as vaccines (Oberg et al., 2011). The discovery of Bm86 followed a similar approach, where numerous protein fractions from the midgut of *R. microplus* females were tested for their ability to confer protection against tick infestation (Willadsen et

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al., 1989). For the development of alternative anti-tick vaccines, however, the rate-limiting step has been the identification of suitable antigenic targets (Nuttall et al., 2006). The work on *N. meningitidis* was facilitated by the availability of a fully sequenced genome, which could be scanned for open reading frames. For this reason, the development of vaccines against ticks is heavily impeded, as no fully sequenced genomes are available for ticks belonging to the *Rhipicephalus* genus. Fortunately, expressed sequence tag (EST) sequencing projects have made large-scale transcriptomic analyses possible for *R. microplus* ticks (Mercado-Curiel et al., 2011; Wang et al., 2007).

A significant shortcoming of an *in silico* approach for the identification of vaccine candidates is the fact that open reading frames might be predicted incorrectly (Rappuoli and Bagnoli, 2011). In the case of *R. microplus*, where a fully sequenced genome is lacking, incorrect assembly of ESTs into contiguous sequences (contigs) could lead to errors. These include the clustering of sequences from the same gene into different contigs or the clustering of sequences from different genes into the same contig (Nagaraj et al., 2007). Truncated sequences would also adversely affect downstream analyses. For this reason, proteomic studies coupled with sequence-providing techniques such as mass spectrometry can validate protein sequences as being expressed under the given experimental conditions, thereby greatly aiding in vaccine discovery. Proteomic studies still suffer from the same drawbacks as the traditional methods for vaccine discovery, however, as the organism in question needs to be cultivated or reared on host animals. Studies such as those performed by Kongsuwan et al. represent a step in the right direction: 134 proteins from the midgut of feeding *R. microplus* female ticks were analysed using proteomic techniques. The deduced amino acid sequences could therefore subsequently be mapped to ESTs from this tick. Unfortunately, most of the identified proteins were cytosolic and therefore not ideal vaccine candidates (Kongsuwan et al., 2010).

Subcellular localisation

Once all available open reading frames are available, the subcellular localisation of the proteins needs to be predicted *in silico*. This is done using programs such as SignalP to identify signal peptidase I cleavage sites (Bendtsen et al., 2004) or TMHMM (transmembrane helices hidden Markov model) to identify membrane-spanning regions (Sonnhammer et al., 1998). In addition, programs such as Big-PI (Eisenhaber et al., 1999), PredGPI (Pierleoni et al., 2008) or GPI-SOM (Fankhauser and Mäser, 2005) can be used to predict potentially GPI-anchored proteins. The reason for determining the subcellular localisation for each protein is

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to identify those proteins that would be accessible to the host's antibodies (Rappuoli and Bagnoli, 2011). This project focuses on proteins that are predicted to be tethered to the membrane via a GPI anchor, whereas other members of the research group focus on integral membrane proteins. Figure 3.1 shows the types of membrane-bound or secreted proteins that could be relevant for anti-tick and/or anti-pathogen vaccines (Vivona et al., 2008). However, it must be borne in mind that subolesin, one of the most promising alternative anti-tick vaccine candidates (Almazán et al., 2012), is an intracellular protein. As such, cytosolic proteins cannot be disregarded as potential anti-tick vaccine candidates.

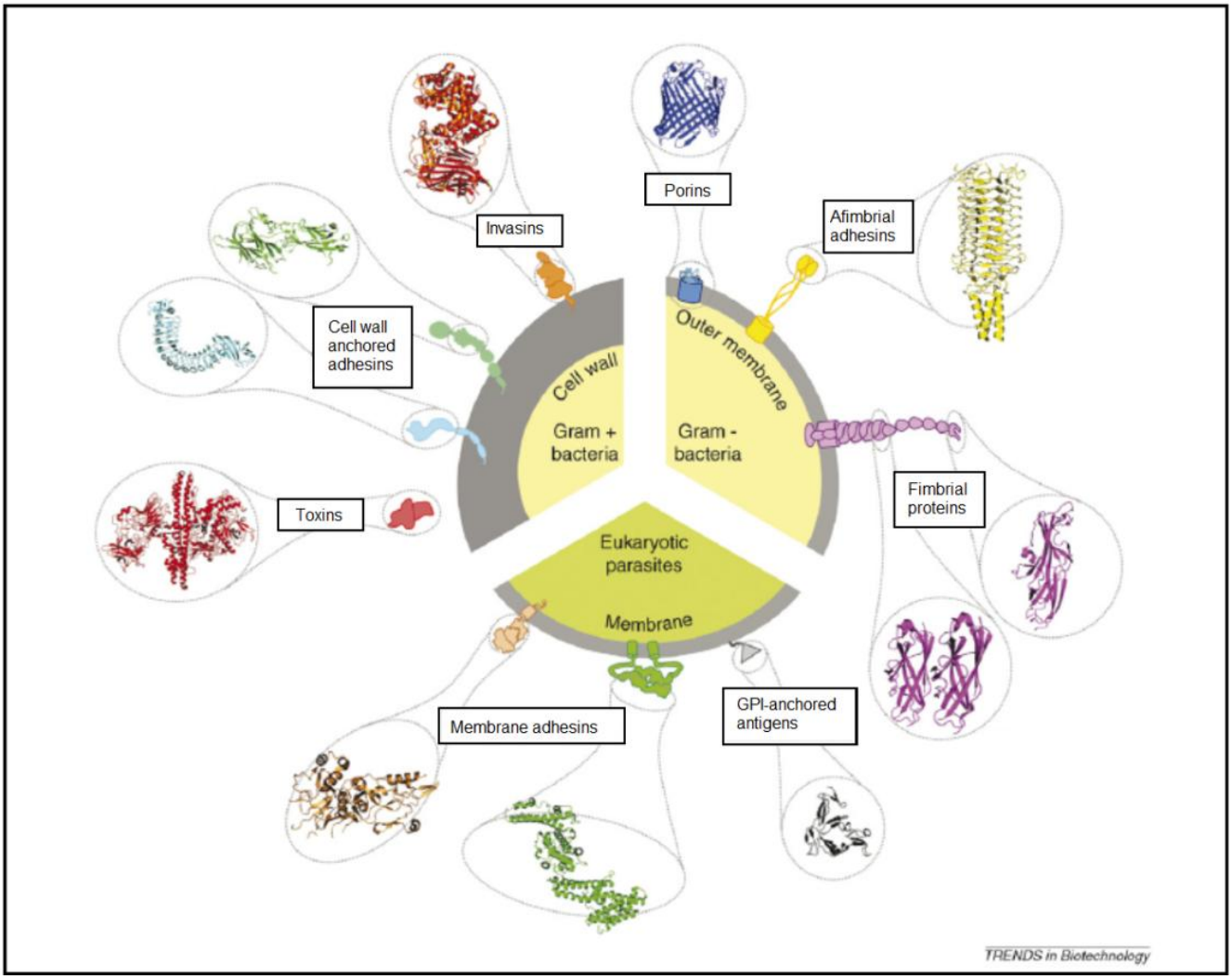

Figure 3.1. Surface-exposed and secreted proteins relevant to the pathogen- or parasite-host interface

Shown are the key role-players in the host-pathogen or host-parasite interface. These proteins participate in the pathogen- or parasite-host interface and are therefore accessible to the host immune system. They include proteins such as porins, adhesins, fimbrial proteins, GPI-anchored proteins, toxins and invasins (Vivona et al., 2008).

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Expression profile

Even if a highly immunogenic *R. microplus* protein were to be identified, it would be of little use as a vaccine candidate if it were only transiently expressed. Using global transcriptome analyses, one would be able to determine the expression profile of relevant genes across the entire life cycle (Rappuoli, 2000). Results from a previous study, where DNA microarray technology was used to study the expression levels of all known genes of *R. microplus*, were used in this chapter (Maritz-Olivier et al., 2012).

Biological function

Many researchers have attempted to identify anti-*R. microplus* vaccine candidates that function in physiologically important processes, such as tick embryogenesis (Seixas et al., 2008), tick attachment (Trimnell et al., 2002) or iron metabolism (Hajdusek et al., 2010), as it is believed that disruption of a critical biological process would be more deleterious to a parasite's survival than simply affecting a tissue surface layer (Willadsen, 1997). For pathogens, proteins involved in extracellular processes such as adhesion, invasion or avoidance of host responses are key players of the host-pathogen interface and therefore attractive vaccine targets (Vivona et al., 2008). This could be extended to the identification of anti-tick vaccines, where the focus would be on proteins that are involved in the host-parasite-pathogen interfaces

Using one- and two-dimensional gel electrophoresis, proteins that are upregulated in the midgut of *R. microplus* females in response to infection with *B. bovis* were isolated and analysed (Rachinsky et al., 2008). This led to the identification of Antigen2, which conferred 63% protection against *R. microplus* in cattle trials. This protein was isolated from midgut tissues, but analysis showed that it has significant similarity to putative salivary gland proteins found in *R. sanguineus* and *I. ricinus* (Guerrero et al., 2012). Vaccines that target such proteins that are upregulated in the midgut in response to infection with *B. bovis* might have a twofold advantage. First, they could reduce tick numbers by leading to direct damage caused to the tick's midgut. Second, by interfering with the midgut micro-environment, pathogen transmission might get curbed (Rachinsky et al., 2008).

The relevance of a protein's importance to a tick's survival as an indicator of its potential as a vaccine candidate remains controversial, however. This is best illustrated by the fact that gene silencing of Bm86 leads to a weak phenotype; although this is the only tick protein that ever became commercialised as an anti-tick vaccine (Nijhof et al., 2010).

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II. Type of immune response elicited

As mentioned in Chapter 1, vaccination using Bm86 primarily induces an antibody-mediated response against this midgut protein from *R. microplus*. Most vaccines targeted against pathogens of importance to human health elicit antibody-mediated responses, which are good enough to confer protection (Rappuoli, 2007). However, in some cases, induction of an antibody-mediated response is not enough. In such cases, it has been shown that T cell-mediated protection is superior (Belshe et al., 2007; Rappuoli, 2007). In the case of the human immunodeficiency virus (HIV), neutralizing antibodies are seldom formed, whereas T cell-mediated protection has been shown to decrease viral load, which might slow down disease progression (McMichael and Hanke, 2003). This might be the case for viruses or other intracellular pathogens, but it is believed that an anti-tick vaccine that induces a cytotoxic T cell response would be of little use, because these cells only affect host cells (Larregina and Falo, 2005). However, the involvement of T cells is essential for the development of anti-tick IgG. Taken together, this suggests that the ideal vaccine might be one that favours the development of a Th1-biased response. It would then be prudent to administer such a vaccine while tick burdens are low, before the ticks' immunomodulatory effects cause cattle to develop a Th2-biased immune response (Brake and Pérez de León, 2012). Maybe such a vaccine could even counteract the immunomodulatory effects of ticks.

When a successful vaccine confers protection against a pathogen, various biomarkers also indicate that the immune system has been stimulated. Immunity against *E. ruminantium*, for example, can be induced by vaccination with the major antigenic protein 1, which has been shown to induce T cells to produce IFN-γ. It is therefore hypothesised that a vaccine against *E. ruminantium* could be tested *in vitro* by its ability to induce IFN-γ, before proceeding with expensive vaccine trials (Liebenberg et al., 2012).

Another study evaluated the recognition of *R. microplus* serine protease inhibitor-3 by cattle that are resistant (*Bos indicus*) and susceptible (*Bos taurus*) to ticks. In this case, it was found that antiserum from resistant cattle recognises this protein, whereas antiserum from susceptible cattle does not (Rodriguez-Valle et al., 2012). Recognition of this protein after infestation with ticks might therefore be used as a biomarker for tick resistance.

When evaluating an anti-*R. microplus* vaccine, one could therefore first evaluate it using *in vitro* techniques. To test its effect on the humoral response, experimental animals could be immunised, and their antiserum evaluated for IgG1 production, using ELISA.

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III. Computational prediction of antigens

In order for an adaptive immune response against ticks to develop, dendritic cells need to take up exogenous antigen and present it to naive $CD4^+$ helper T cells. These helper T cells can then be induced to differentiate into Th1 cells to promote cellular immunity or Th2 cells to promote humoral immunity (Larregina and Falo, 2005). Humoral immunity is further mediated by the binding of an antigen to a B cell receptor, followed by internalisation, processing and finally presentation of antigen fragments to a helper T cell. This promotes isotype switching from IgM to IgG and the development of memory B cells (Zepp, 2010). This recognition of epitopes by B and T cells forms the core of the host's immune response to pathogens or ticks (Vivona et al., 2008).

The successful prediction of B and T cell epitopes therefore presents the major limitation to the rational *in silico* design of novel vaccines. B cell epitopes are notoriously difficult to predict accurately using single-scale propensity scales (Blythe and Flower, 2005). As the physicochemical properties of the amino acids in a protein determine which areas can be accessible to antibodies, these have been used by various researchers in order to identify putative B cell epitopes (Ansari et al., 2010). For this reason, various B cell epitope predictors were used in this study. As no single predictor performs significantly better than random predictions (Blythe and Flower, 2005), a consensus approach was followed. T cell epitopes, on the other hand, can be predicted with striking accuracy, as they are bound in a linear form to the major histocompatibility complex (MHC) (De Groot, 2006). Unfortunately for tick research, the prediction of binders to T cell epitopes is mostly based on experimental data with human alleles. For example, the immune epitope database analysis resource (IEDB-AR) hosts software that can predict only human MHC II binders (Zhang et al., 2008).

Fortunately, programs are also being developed for the prediction of binders to bovine MHC I molecules. NetMHCpan is a neural network trained on MHC-peptide binding data from human, mouse and other primates. The authors suggest that the model is retrained using more data from bovine MHC I peptide binding studies, after which it is proposed that cytotoxic T lymphocyte antigens could be predicted. This would be advantageous for development of vaccines against East Coast Fever, where the involvement of MHC I-restricted T cell antigens has been implicated in immunity to this disease (Nene et al., 2012).

Another method whereby additional antigens could be identified is by using sequence similarity searches. This means that a newly sequenced genome could be scanned for

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sequences that share similarity to known antigens (Flower et al., 2010). Such an approach would be of limited use if the sequence databases are insufficient, however. Moreover, over 60% of the genome of *R. microplus* shares no sequence similarity with other organisms (Wang et al., 2007). This would therefore render alignment-based approaches ineffective.

An alternative approach to performing sequence similarity searches is the use of statistical methods. As different proteins can have the same biological function or physiochemical properties without having any sequence similarity, alignment-based methods would fail to detect a link between them (Flower et al., 2010). The amino acids comprised by two immunogenic proteins might therefore have the same physiochemical profile without sharing significant identity. In order to perform alignment-independent comparisons of different proteins in terms of their immunogenicity, VaxiJen was developed. This program is based on autocross covariance (ACC) transformation of amino acid sequences into vectors that express their hydrophobicity, molecular size and polarity (Doytchinova and Flower, 2007). This therefore means that autocross covariance transformation is performed on a query sequence, comparing its hydrophobic, size and polarity profiles with those of experimentally validated immunogenic protein sequences in order to predict its potential as a vaccine target. As mentioned above, most *R. microplus* sequences don't share sequence similarity with any other sequences in public databases. It is therefore believed that an alignment-independent method, such as VaxiJen, would be the best alternative approach for the identification of novel putative vaccine targets. Validation of VaxiJen was done using a dataset of 26 previously validated parasitic antigens. Of these, 18 were predicted to be protective antigens, indicating a sensitivity of 69%. Predicted protective antigens included those from *Brugia malayi*, *Leishmania major* and *Schistosoma bovis* (Doytchinova and Flower, 2008).

3.1.3 Vaccines against other parasites

Worldwide, demand for safer alternatives to anti-parasitics is growing. This is understandable, as chemical residues can cause environmental contamination (Taylor, 2001). Various antigens have been tested as vaccines against other parasites of veterinary importance but with limited success (Willadsen, 2004). There are commercially available vaccines for diseases such as avian coccidiosis, toxoplasmosis or giardiosis (Dalton and Mulcahy, 2001). In the late 1980s, a vaccine against the cattle grub, *Hypoderma lineatum*, was developed and patented. Despite its efficacy, however, market-related factors prevented its successful commercialisation (Guerrero et al., 2012). Given the limited success of vaccines against ectoparasites, it seems that they are especially difficult to treat

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immunologically. Perhaps, owing to their adaptation to a blood-feeding behaviour, haematophagous parasites have evolved to circumvent the host's immune system. The immunomodulatory effects of ticks were discussed in Chapter 1. Despite these obstacles, however, the initial success of the Bm86 vaccine shows that immunological control of ticks is possible.

Vaccines against endoparasites

The most effective vaccine against a gastrointestinal worm is a 110 kDa integral membrane protein with aminopeptidase activity. This vaccine is very efficacious against *Haemonchus contortus*, conferring >90% protection to lambs. Moreover, egg output is reduced by 98%. Unfortunately, compared to antihelminthic drugs, purified antigens are not effective against a broad spectrum of organisms. Moreover, when expressed recombinantly in *E. coli*, antihelminthic vaccines do not work as efficiently (Dalton and Mulcahy, 2001).

3.1.4 Reverse vaccinology for tick-borne diseases

Recently, reverse vaccinology has been applied to one of the most important tick-borne diseases in southern Africa, heartwater, which is caused by *Ehrlichia ruminantium*. All the open reading frames from the annotated genome were used, except those that had been evaluated previously and those with more than four predicted transmembrane helices. Out of 272 selected open reading frames, 228 were successfully expressed recombinantly and 35 induced the production of IFN-γ in peripheral blood mononuclear cells from cattle and sheep. Of these, 11 proteins induced a cytokine profile similar to that when intact *E. ruminantium* is used. It remains to be seen whether these proteins confer protective immunity against *E. ruminantium*, which would then be the first vaccine against a tick-borne disease (Liebenberg et al., 2012).

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3.1.5 Vaccine candidate identification in ticks

Reverse vaccinology remains to lead to the identification of the next anti-tick vaccine but, apart from the identification of Bm86, techniques using classical methodologies have been unsuccessful. In contrast with these techniques, where only a few antigens are tested in an experiment, technological advances have allowed for the simultaneous evaluation of a large number of genes as possible vaccine targets in ticks. One such technique is expression library immunisation, which led to the discovery of subolesin. This was discussed in Chapter 1.

Other techniques that have been used to identify novel tick antigens include the use of antiserum generated by multiple infestations to identify antigens in crude protein extracts using immunoblotting (Mulenga et al., 2000). Abovementioned techniques aren't biased towards sequences with known function, affording each protein an equal chance, except for high abundance proteins that could mask antigenic low abundance ones. After immunising chickens with larval proteins from *R. microplus*, Prudencio and colleagues performed phage display using random peptide libraries of different lengths to identify potential B- cell epitope candidates. This led to the identification of novel immunogenic motifs, shown in Table 3.1 (Prudencio et al., 2011). These findings are significant because they show that B cell receptors had internalised the respective antigens, which led to B cell activation after antigen fragments were displayed to helper T cells.

Table 3.1. Novel motifs that occur in B cell-stimulating *R. microplus* proteins

From (Prudencio et al., 2011).

Immunogenic motif	***R. microplus* proteins containing this motif (http://www.ncbi.nlm.nih.gov/protein)**
NXXXKXXL	Salivary gland metalloprotease, G-protein-coupled receptor, calreticulin, glutathione S-transferase, glyceraldeyde-3-phosphate dehydrogenase
TPDKS	Egg cathepsin, Bm95, GP80 precursor, intracellular cystatin, Bm86 intestinal protein, phospholipids, salivary gland metalloprotease, cathepsin-type protein precursor, paramyosin, calreticulin, membrane B antigen, P450 CYP319A1, haeme-binding aspartic proteinase, reverse transcriptase-type protein, glyceraldehyde-3-phosphate dehydrogenase
PXXKXH	No alignments
LHS	Putative secreted protein, P450 CYP319A1
LHXXL	Acetylcholinesterase, GP80 precursor, Bm86 intestinal protein, paramyosin, P450 CYP319A1, esterase, angiotensin-converting enzyme
HTS	GP80 precursor, protective antigen 4D8
PXFF	Cytochrome oxidase subunit 1, NADH dehydrogenase subunit 2, cytochrome P450
LYGS	Acetylcholinesterase, egg cathepsin, GP80 precursor, glucose 6-phosphate dehydrogenase isoform, actin, salivary gland metalloprotease, ferritin, membrane B antigen, esterase, haeme-binding aspartic proteinase, cytochrome P450, carboxylesterase-related protein, angiotensin-converting enzyme

Despite these promising findings, however, no alternative vaccine exists to replace Bm86. The current situation of anti-tick vaccine research is comprehensively reviewed in an article by Guerrero and colleagues (Guerrero et al., 2012).

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As can be seen from the literature, the identification of novel vaccine candidates against *R. microplus* has been the primary limiting factor regarding the development of an effective anti-tick vaccine. Presented here is a reverse vaccinology approach for the identification of novel anti-*Rhipicephalus microplus* and *R. decoloratus* vaccine candidates. Due to the sheer number of proteins in this organism, protein expression and immunisation *en masse*, as performed by (Pizza et al., 2000), would be an extremely laborious undertaking, requiring a combined effort by a large number of researchers. Moreover, performing cattle trials is prohibitively expensive for most small research groups. Consequently, computer-based predictions were performed to evaluate only sequences that were predicted to be membrane-bound. In this study, emphasis was placed on those that are GPI-anchored. This was coupled with microarray studies to identify genes that are both shared between *R. microplus* and *R. decoloratus*, and expressed throughout the life cycle of *R. microplus* (larvae, nymphs and midgut tissues from adult females). Finally, an ELISA-based assay was used to evaluate predicted epitopes of selected proteins.

3.2 Hypothesis

Reverse vaccinology combined with immunoinformatics can be used to identify novel protective antigens from *R. microplus*.

3.3 Aims of this chapter

- To identify genes that are expressed throughout the life cycle of *R. microplus* using results from microarray studies performed by Mr C. Stutzer
- To consolidate these results with those from Chapter 2, where genes that are expressed in the midgut of both *R. microplus* and *R. decoloratus* were identified.
- To identify genes that are predicted to confer protective immunity upon vaccination using VaxiJen
- To identify genes that are predicted to be localised to the extracellular matrix via a glycosylphosphatidylinositol (GPI) anchor
- To predict epitopes for four antigens and to have the corresponding peptides synthesised
- To immunise mice immunised with a crude membrane protein fraction from the midgut of *R. microplus*
- To perform ELISA on the synthetic peptides using the mouse antiserum

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3.4 Materials

Microarray reagents were purchased as in Chapter 2. Synthetic peptides were purchased from GenScript. Ethical clearance for the use of BALB/c for the generation of antiserum was granted by the South African Department of Agriculture, Forestry and Fisheries as well as the University of Pretoria's Animal Use and Care Committee under project number EC022-10. All software used in this project is free for academic users. Goat anti-mouse IgG (heavy + light chain) conjugated to horseradish peroxidase was purchased from Cappel. *Ortho*-phenylene diamine substrate was purchased from Sigma-Aldrich. Sodium citrate and hydrogen peroxide were purchased from Merck.

3.5 Methods

3.5.1 Overview of the methodology followed in this study

In order to systematically reduce the number of proteins to be evaluated in further studies, a reverse vaccinology approach was followed. Microarray experiments were performed by Mr. C. Stutzer to identify transcripts that are expressed in *R. microplus* larvae, nymphs and the midgut of female adults. In Chapter 2, transcripts that are expressed in the midgut of feeding *R. microplus* and *R. decoloratus* females were identified. An alignment-independent approach for the identification of potential protective antigens further reduced the number of proteins. Finally, only proteins that were predicted to be GPI-anchored and devoid of transmembrane helices went into the last phase, where epitopes were predicted for four proteins. These predicted epitopes were then evaluated for their reactivity with antiserum from mice immunised with membrane proteins from the midgut of feeding *R. microplus* females.

The experimental methodology that was employed in this study is shown in Figure 3.2.

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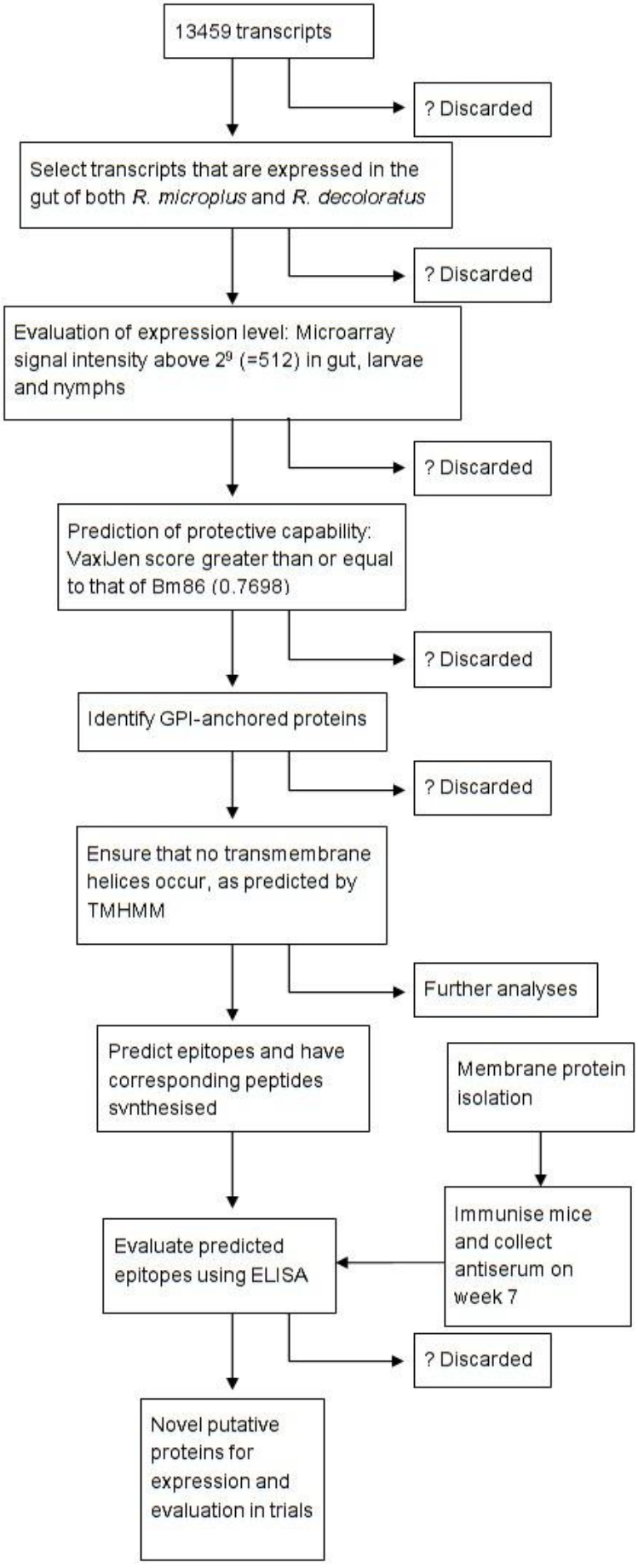

Figure 3.2. Experimental methodology employed in this study

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3.5.2 Identification of transcripts present in the midgut, larvae and nymphs of *R. microplus*

A reference design microarray was performed by Mr C. Stutzer to study the transcriptome from *R. microplus* (Maritz-Olivier et al., 2012). The reference pool was labelled with Cy3 (green) and cDNA from midgut, larvae and nymphs was labelled with Cy5 (red). In order to identify transcripts that are present in these three samples, the absolute intensity values corresponding to the Cy5 (red) channel were calculated. After normalisation of the microarray slides, as discussed in the previous chapter, the *M*- and *A*-values were exported. Recall that

$$M = \log_2 R - \log_2 G \quad \text{.....Equation 2.1}$$

$$A = (\log_2 R + \log_2 G)/2 \quad \text{.....Equation 2.2}$$

From this it follows that:

$$R = \sqrt{2^{2A}.2^{M}} \quad \text{.....Equation 2.6}$$

Upon visual inspection of scanned microarray images, spots with an average $\log_2$intensity of less than 9 were deemed too feint. It was therefore decided that the $\log_2$intensity of the red (sample) channel had to be more than 9. This meant that the red intensity had to be at least 512 (2^9=512) for the gene in question to be considered actively transcribed in the sample in question. Transcripts where the red intensity was not at least 512 in larvae, nymphs and the midgut were therefore not included.

3.5.3 Translation of sequences using Prot4EST

The most likely reading frame for each transcript was determined using the Prot4EST translation pipeline. Potentially ribosomal or mitochondrial genes are discarded, and similarity searches against SwissProt are performed to identify the most likely reading frame. Nucleotide sequences are translated into all six reading frames, which are used as query sequences for protein BLAST searches (BLASTP). High scoring segment pairs are identified and the protein sequence is assembled. The longest reading frame that is not interrupted by

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stop codons is used in the case of nucleotide sequences where none of the six reading frames yield a good result using BLASTP (Wasmuth and Blaxter, 2004). Prot4EST is free to academic users (http://www.nematodes.org/bioinformatics/PartiGene/) and was kindly run by Prof. F. Joubert at the Bioinformatics and Computational Biology Unit from the University of Pretoria

3.5.4 Prediction of protective antigens

All the *R. microplus* protein sequences, as determined by Prot4EST, were submitted to VaxiJen (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html). This program makes use of auto cross covariance transformations of input sequences, based on the physicochemical properties of the amino acids (Doytchinova and Flower, 2007). The parasite model was selected to base the predictions on. Contig8501, which represents Bm86, had a score of 0.7698, suggesting that it is a probable antigen. Only proteins where the VaxiJen score was greater than that of Bm86 were considered for further study.

3.5.5 Prediction of GPI-anchored antigens

GPI-anchoring signals in each protein were predicted using three web-based programs. Big-PI (http://mendel.imp.ac.at/gpi/gpi_server.html) makes use of position-specific independent counting of the physicochemical properties of amino acid residues in a protein sequence (Eisenhaber et al., 1999). PredGPI (http://gpcr.biocomp.unibo.it/predgpi/) makes use of hidden Markov models and a support vector machine to identify proteins that are GPI-anchored (Pierleoni et al., 2008). GPI-SOM (http://gpi.unibe.ch/) makes use of self organising maps for this purpose (Fankhauser and Mäser, 2005). Lastly, Memtype-2L (http://www.csbio.sjtu.edu.cn/bioinf/MemType/) combines pseudo position-specific (pse-PSSM) score matrix vectors with optimised evidence-theoretic K-nearest neighbour (OET-KNN) classifiers to predict membrane type (Chou and Shen, 2007). At least 2 of these programs had to predict a protein to be GPI-anchored for it to be considered for further analysis.

Due to the possibility of a false-positive prediction of a GPI-anchoring signal, potential **t**rans**m**embrane helices were identified using a **h**idden **M**arkov **m**odel-based program, TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) (Sonnhammer et al., 1998). Proteins with predicted transmembrane helices were avoided as it has been suggested that GPI-anchored proteins containing additional transmembrane segments exist that remain inside lysosomes,

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the ER or the Golgi apparatus (Eisenhaber et al., 1999). Nevertheless, these proteins can still be considered for further analysis.

3.5.6 Prediction of epitopes

For each GPI-anchored protein that was selected for further analysis, epitopes were predicted using a variety of programs. A propensity scale method that incorporates hydrophilicity, surface accessibility and flexibility of each amino acid in order to predict immunogenic regions was used (Kolaskar and Tongaonkar, 1990). Linear B-cell epitopes were predicted using an algorithm that combines a hidden Markov model with a propensity scale method based on the frequency of amino acids in secondary structures (Larsen et al., 2006; Levitt, 1978). The presence of β-turns was detected using a program that makes use of artificial neural networks (Kaur and Raghava, 2004). Surface accessibility across each protein was assessed using a method that incorporates the frequency of amino acid residues in surface-accessible areas (Emini et al., 1985; Janin and Wodak, 1978). A matrix-based prediction algorithm, employing the position and frequency of amino-acids in peptides binding to human leukocyte antigen (HLA) DRB1 alleles, was used to identify amino acids that occur in peptides that are predicted to be among the top 1% of binders to 51 HLA-DRB alleles (Singh and Raghava, 2001; Sturniolo et al., 1999). Finally, the top 10 binders to three HLA-DRB1 alleles were identified using MHCPred, a partial least squares-based approach (Guan et al., 2003). For each program, each amino acid that was part of a good predicted binder or β-turn received a point. Protein sequences were evaluated for areas that were recognised by the majority of the predictors, in order to select peptides to be purchased for subsequent analysis.

3.5.7 Preparation of a crude membrane protein isolate

Twenty female *R. microplus* ticks were collected on day 20 post-infestation of a Holstein-Friesian calf (~six months old). The ticks were dissected; their midgut tissues removed and stored in 10 mM PBS (phosphate-buffered saline, 150 mM NaCl, pH 7.4) and protease inhibitor cocktail (0.37 mg/ml). Midgut tissues were homogenised using 16G, 18G and 23G needles. This was followed by sonifying the samples 10 times using a VirSonic sonifier (VirTis, USA) at 3 W output (1 second pulse, 1 second rest) on ice. The samples were subsequently centrifuged at 100,000*g* for 1 hour, after which the supernatant was discarded and the pellet was washed with 10 ml PBS. Samples were centrifuged again at 100,000*g* for 1 hour, after which the supernatant was discarded and the pellet was resolubilised in PBS.

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Protein concentration was measured using the Nanodrop-1000 (Thermo Fisher Scientific, USA).

3.5.8 Visualisation of proteins

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (Laemmli, 1970) was performed using a 4% stacking gel and a 12% separating gel. Polymerisation was initiated by adding 0.05% (w/v) ammonium persulphate and 0.001% (v/v) N, N, N′,N′-tetramethylethylenediamine (TEMED). Gut membrane protein samples (2 µg) were diluted 1:4 in denaturing sample buffer (0.06 M Tris-HCl, pH 6.8, 2% sodium dodecyl sulphate, 0.1% glycerol, 0.05% β-mercaptoethanol and 0.025% bromophenol blue) and boiled for 5 minutes. PageRuler™ Unstained Protein Ladder (Fermentas, USA) was used as molecular marker. Electrophoresis was performed in running buffer (25 mM Tris-HCl, pH 8.3, 0.2 M glycine and 3.5 mM SDS) using a BG-verMINI gel system (BayGene Biotech Company, Hong Kong) at 80 V until the tracking dye reached the separating gel, after which the voltage was increased to 120 V until the tracking dye reached the bottom of the gel.

3.5.9 Silver staining

A silver staining technique (Merril et al., 1981) was performed to assess the integrity of the isolated membrane proteins. Proteins were fixed in the gel by immersing the gel in a solution containing 45% methanol and 5% acetic acid for 1 hour, after which the gel was sensitised for 2 minutes in 0.02% sodium thiosulphate. This was followed by rinsing the gel in distilled water twice, adding 0.1% silver nitrate for thirty minutes and finally in distilled water, and adding developing solution (2% sodium carbonate, 0.04% formaldehyde). Once visible protein bands appeared, further staining of proteins was stopped with the addition of 1% acetic acid.

3.5.10 Membrane protein quality check

A dot-blot was performed to confirm the presence of Bm86, known to be membrane-bound via a GPI-anchor. This was done by spotting the protein extract (70 ng) onto a nitrocellulose membrane and allowing the spot to dry. The membrane was immersed in 10 mM PBS (phosphate-buffered saline, 150 mM NaCl, pH 7.4) containing 5% (v/w) skim milk and incubated for 1 hour at 37°C. The membrane was washed three times with PBS for 5 minutes, after which bovine Bm86 antiserum was added at a dilution of 1:1000. The membrane was incubated for 1 hour at 37°C and washed three times with PBS for 5 minutes.

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The secondary antibody, horseradish peroxidase conjugated goat α-bovine IgG, was added at a dilution of 1:1000 and incubated for 1 hour at 37°C. This was followed by washing the membrane three times with PBS for 5 minutes. Finally, the substrate (0.06 g 4-chloronaphthol in PBS containing 0.015% H_2O_2 and 17% methanol) was added, after which the colorimetric reaction took place.

3.5.11 Antiserum production

Three six-week old female BALB/c mice were used for this study (ethical clearance number EC022-10). Naive serum was collected to serve as negative control, before the mice were immunised subcutaneously using 100 µg of the midgut membrane proteins mixed 1:1 with Montanide ISv50 adjuvant (SEPPIC, France) on weeks 1, 4 and 6. Antiserum was collected from the mice on week 7 and pooled.

3.5.12 Enzyme-linked immunosorbent assay (ELISA)

An enzyme-linked immunosorbent assay was performed in order to measure the reactivity of the pooled antiserum against the predicted antigenic synthetic peptides. Lyophilised peptides were dissolved in TBS (Tris-buffered saline, 25 mM Tris–HCl, 150 mM NaCl, pH 7.4) and in the case of acidic or basic peptides that did not dissolve readily, 100 mM NaOH or HCl was added respectively. Experiments were performed in quadruplicate and each well was loaded with 13 nmol of the appropriate peptide. The plate was dried to ensure complete adsorption of the peptides and subsequently blocked overnight at 4°C using TBSC (TBS with 0.5% w/v casein). The plate was washed four times using TBSC, after which 50 µl of 1:25 diluted antiserum was added to each well. The plate was incubated for 1 hour at room temperature, washed four times with TBSC and incubated for 45 minutes at room temperature with 1:1000 diluted horseradish peroxidase conjugated goat α-murine IgG (Sigma, USA). Following a final washing step, developing buffer (10 ml citrate, 10 mg *O*-phenylene diamine and 8 mg H_2O_2, pH 4.5) was added and the reaction was monitored at 450 nm using the Multiskan Plus reader (Thermo Fisher Scientific, USA). Due to the small amount of serum collected, ELISAs using naive serum were only performed in duplicate. A multiple comparison ANOVA (Holm-Sidak method) was performed to compare the results from the predicted immunodominant peptides with those from the peptides that correspond to antigenic regions in Bm86.

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3.6 Results and discussion

The results for this chapter can be summarised in Figure 3.3.

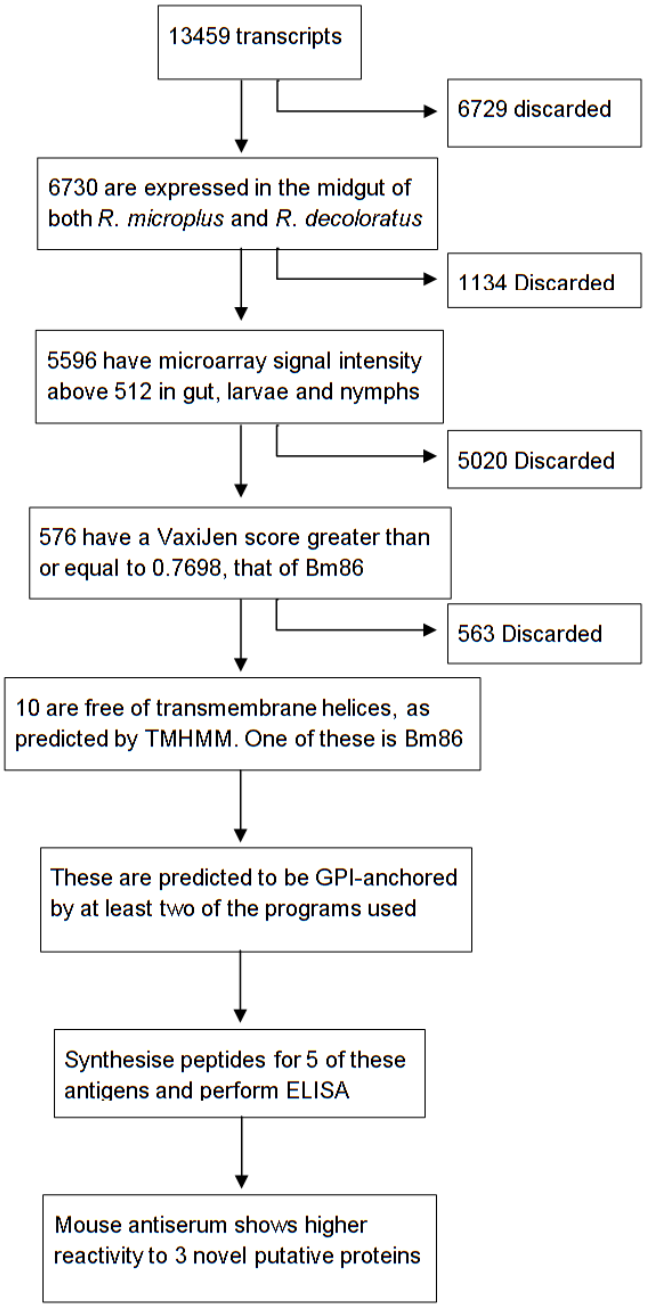

Figure 3.3. Summary of results obtained for Chapter 3

3.6.1 Identification of transcripts that are actively transcribed in the midgut of feeding *R. microplus* and *R. decoloratus* females

One of the primary aims of chapter 2 was to identify genes that are found in both tick species. Regardless of relative fold changes between the two species that were also determined and discussed in chapter 2, gene expression levels were also expressed as a function of median intensity across all four arrays. In order to classify a gene as being actively expressed in the

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midgut of a particular tick species, the median intensity of the respective Cy3 and Cy5 values was calculated for each gene for this species. This median intensity value had to be greater than the median intensity of the rest of the spots on the microarray for the gene to be considered to be actively expressed. Using this method, 6730 genes were identified to be expressed in both tick species and are therefore potential vaccine candidates. Note that this subset of genes does not necessarily exclude any of those that were found to be specific to *R. microplus* or *R. decoloratus* or shared between them. The genes considered to be of potential importance with vaccine design in mind are shown in Figure 3.4 in the red box and can be found on Tab 3 on the electronic supplementary data.

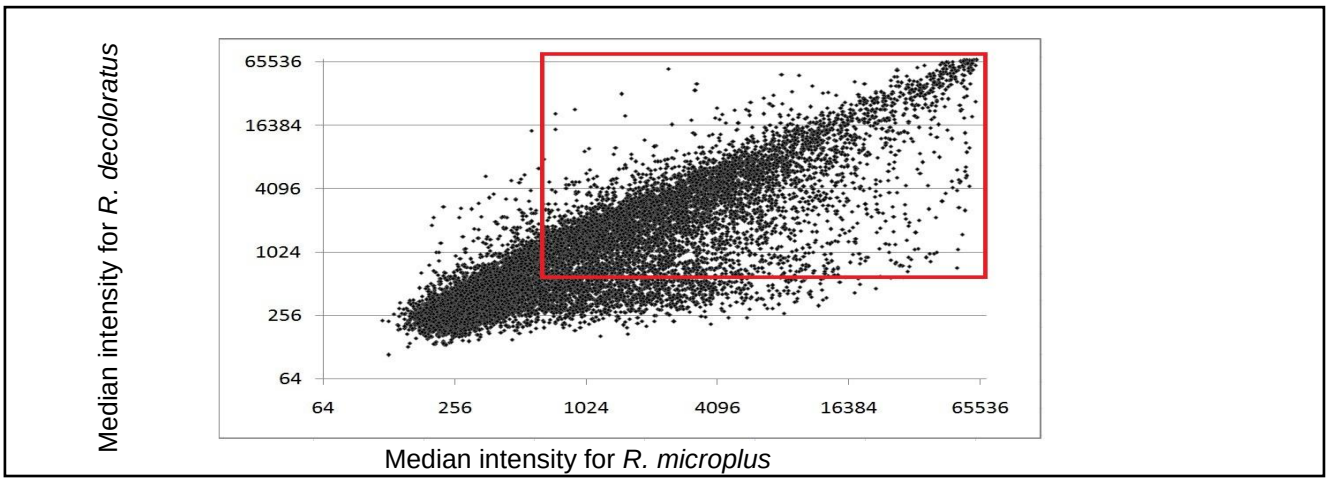

Figure 3.4. Identification of genes of potential interest for vaccine design

The red box indicates genes where the median intensity level for both ticks was above the median intensity for all spots on the array. Axis labels indicate median intensity level for Cy3 and Cy5 for each tick for a gene in question. Each spot represents a unique gene.

3.6.2 Identification of transcripts that are actively transcribed in the midgut, larvae and nymphs

The custom oligonucleotide microarray contained 13459 spots that represented unique *R. microplus* transcripts. Six thousand seven hundred and thirty transcripts were found in the midgut of both *R. decoloratus* and *R. microplus* females. Of these, 5596 were expressed in larvae, nymphs and the midgut of *R. microplus* females (defined as having a spot intensity of greater than or equal to 512). See Tab 4 on the electronic supplementary data.

3.6.3 Identification of predicted GPI-anchored protective antigens

Out of 5596 shared transcripts where the spot intensity was at least 512 in the midgut, larvae and nymphs of *R. microplus*, 576 had a VaxiJen score of greater than or equal to that of Contig8501, representing Bm86. These were then submitted to the various websites to

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predict GPI-anchoring signals and 15 protein sequences remained. Results from membrane topology prediction using TMHMM lead to the removal of five more proteins due to ambiguous predictions. Furthermore, two of the remaining sequences were highly repetitive and were therefore excluded. Finally, four candidates, chosen at random, along with Bm86 serving as a positive control, were selected for further analysis. Seven potentially novel vaccine candidates were therefore identified but financial constraints allowed only for further analyses of four of them (Table 3.3). Refer to Tab 5 on the electronic supplementary data.

3.6.4 Additional analyses of transcripts

Having identified proteins that could be evaluated as vaccine candidates, additional analyses were performed to determine the subcellular localisation of the transcripts that are actively transcribed across the life cycle of *R. microplus* as well as those found to be shared between or significantly up- or downregulated in the midgut of female *R. microplus* vs. *R. decoloratus* ticks. Results are summarised in Table 3.2 and show a similar distribution of subcellular localisations for all the different groups of transcripts.

Table 3.2. Membrane proteins identified in both *R. microplus* and *R. decoloratus*

Transcript classification	GPI-anchor	Cytosolic	Membrane spanning	Secreted
Active in the midgut, larvae and nymph of *R. microplus* (6198 total)	87 (1.4%)	4657 (75.1%)	895 (14.4%)	646 (10.4%)
Expressed in the midgut of both *R. microplus* and *R. decoloratus* females (5405 total)	74 (1.4%)	4038 (74.7%)	794 (14.7%)	573 (10.6%)
Upregulated in the midgut of *R. decoloratus* (136 total)	0 (0%)	99 (72.8%)	21 (15.4%)	16 (11.8%)
Upregulated in the midgut of *R. microplus* (1084 total)	13 (1.2%)	779 (71.9%)	165 (15.2%)	140 (12.9%)

3.6.5 Peptide design

Four predicted protective antigens were selected at random (Table 3.3) for further analysis using peptides corresponding to predicted epitopes. Ten peptides were purchased in total. Three of these represent the Mozambique strain variant of previously identified antigenic peptides in Bm86, serving as experimental controls (Freeman et al., 2010; Patarroyo et al., 2002; Patarroyo et al., 2009). Figures 3.5-9 show the peptides used for the study. Each peptide also received a linker consisting of three glycines and a cysteine for potential subsequent conjugation to keyhole limpet haemocyanin.

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Table 3.3. Expression levels and antigenicity prediction of the five chosen proteins, including Bm86 as positive control

Transcript	Expression level in *R. microplus*, expressed as median signal intensity			Expression level in the midgut, expressed as median signal intensity		VaxiJen score	Selected for peptide design?
	Midgut	Larvae	Nymphs	*R. microplus*	*R. decoloratus*		
Contig8501 (Bm86)	3108	835	1176	7235	2950	0.7698	Y
Contig4831	693	617	1322	2541	3512	0.7999	Y
Contig6380	565	1087	1288	837	1389	0.8357	Y
Contig6778	2693	3235	2339	4434	3734	0.9042	Y
Contig7603	8769	5681	10315	17857	18766	0.8461	Y
Contig157	3345	1297	2424	3626.481	4488.0821	0.7795	N
Contig2122	5790	6997	3014	6687.2337	3535.1471	0.8517	N
CK181624	746	921	1172	406.81765	271.16835	0.9919	N

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Figure 3.5. Predicted immunogenic peptides for Contig8501 (Bm86)

Red boxes indicate the chosen peptides. Predicted GPI-anchoring sites are indicated with a green circle (Pierleoni et al., 2008) and blue box (Eisenhaber et al., 1999). Numbers indicate the combined score of the six programs for each amino acid.

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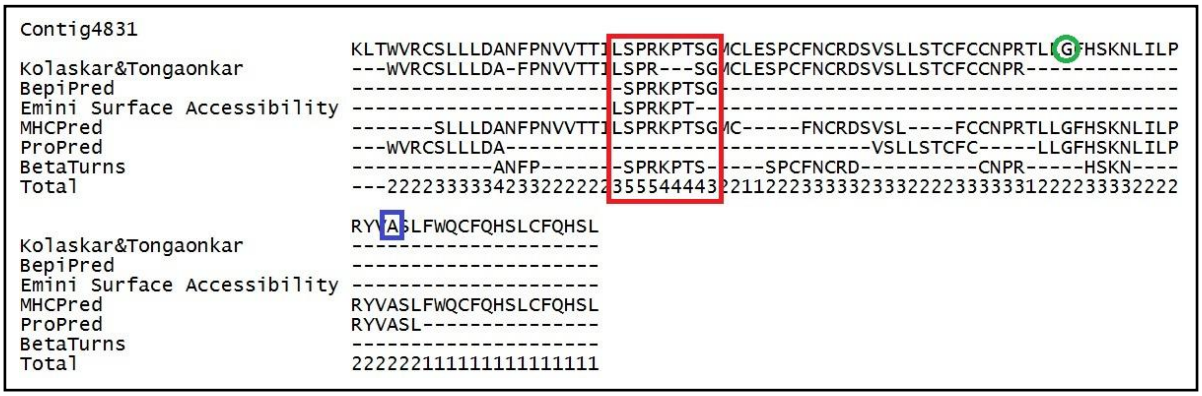

Figure 3.6. Predicted immunogenic peptides for Contig4831

Red box indicates the chosen peptide. Predicted GPI-anchoring sites are indicated with a green circle (Pierleoni et al., 2008) and blue box (Eisenhaber et al., 1999). Numbers indicate the combined score of the six programs for each amino acid.

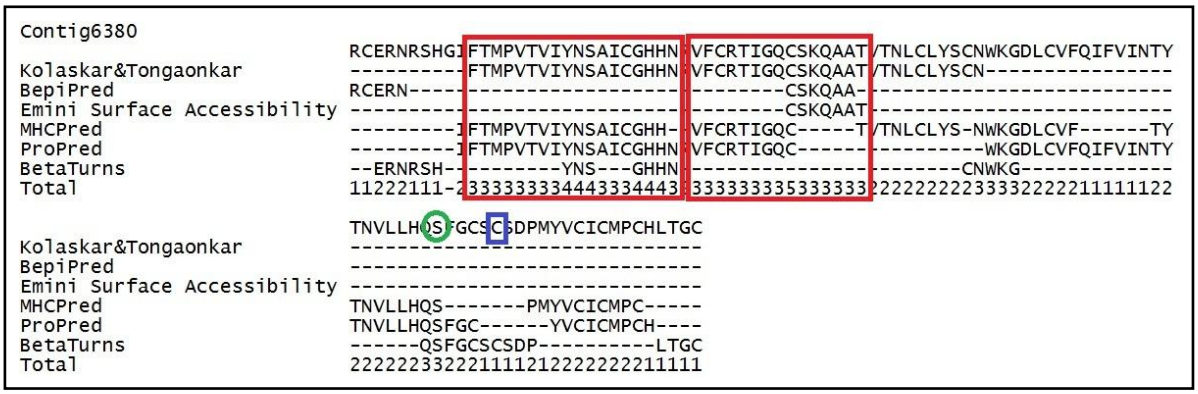

Figure 3.7. Predicted immunogenic peptides for Contig6380

Red boxes indicate the chosen peptides. Predicted GPI-anchoring sites are indicated with a green circle (Pierleoni et al., 2008) and blue box (Eisenhaber et al., 1999). Numbers indicate the combined score of the six programs for each amino acid.

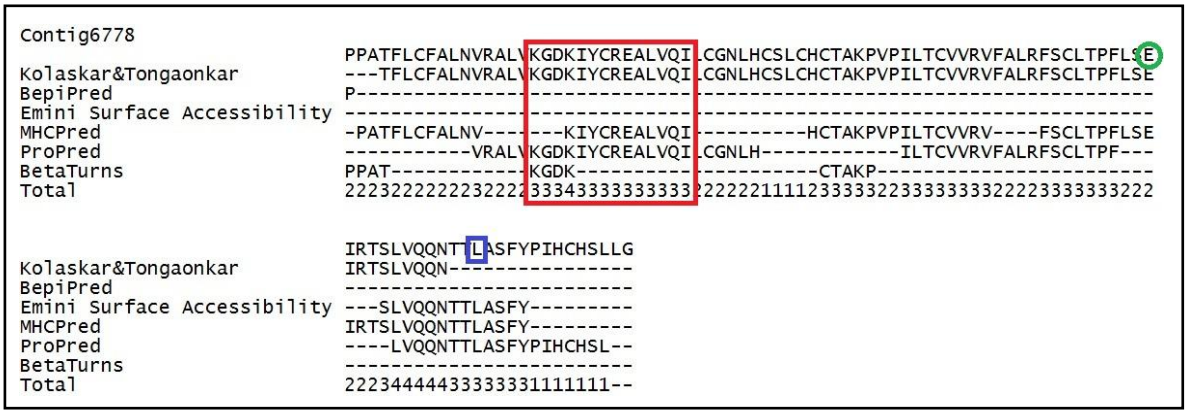

Figure 3.8. Predicted immunogenic peptides for Contig6778

Red box indicates the chosen peptide. Predicted GPI-anchoring sites are indicated with a green circle (Pierleoni et al., 2008) and blue box (Eisenhaber et al., 1999). Numbers indicate the combined score of the six programs for each amino acid.

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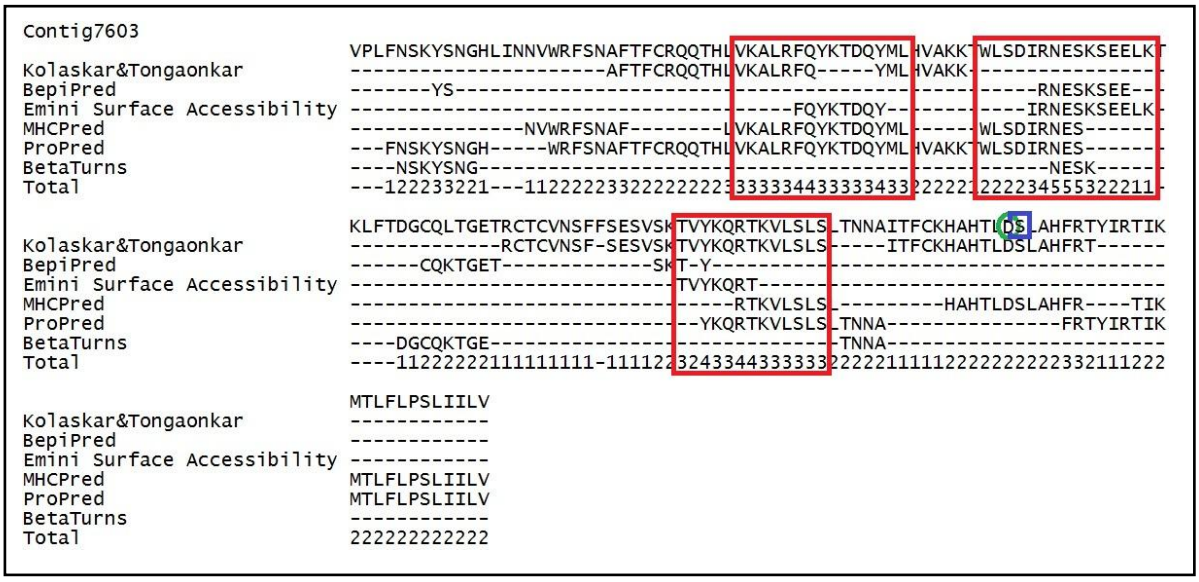

Figure 3.9. Predicted immunogenic peptides for Contig7603

Red boxes indicate the chosen peptides. Predicted GPI-anchoring sites are indicated with a green circle (Pierleoni et al., 2008) and blue box (Eisenhaber et al., 1999). Numbers indicate the combined score of the six programs for each amino acid.

Some chemical properties of each peptide are shown in Table 3.4.

Table 3.4. Chemical properties of predicted immunogenic epitopes

Predicted antigen	Peptide number	Sequence	Length	Nett charge at pH 7	Amino acid composition (%)		
					Hydrophilic	Hydrophobic	Neutral
Contig8501 (Bm86)	1	SSVCSDFGNEFCRNAGGGC	19	-1	16	16	68
	2	CDCGEWGAMNKTRRGGGC	18	0	22	11	67
	3	LSKHVLRKLQACEHGGGC	18	4	33	22	44
Contig4831	1	LSPRKPTSGGGC	12	2	17	8	75
Contig6380	1	FTMPVTVIYNSAICGHHNGGGC	22	2	9	32	59
	2	VFCRTIGQCSKQAATGGGC	19	2	11	16	74
Contig6778	1	KGDKIYCREALVQIGGGC	18	1	28	28	44
Contig7603	1	LVKALRFQYKTDQYMLGGGC	20	2	20	40	40
	2	WLSDIRNESKSEELKGGGC	19	-1	37	21	42
	3	TVYKQRTKVLSLSGGGC	17	3	18	29	53

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3.6.6 Membrane protein isolation and ELISA

Membrane proteins from *R. microplus* were isolated and analysed using SDS-PAGE. Apart from the apparent smear of proteins, clearly distinguishable bands were visible on the gel (Figure 3.10A).

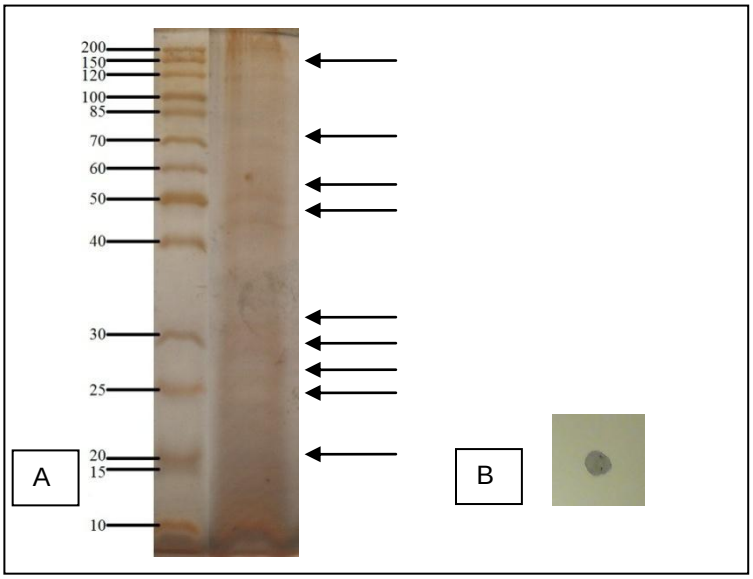

Figure 3.10. Isolation of membrane proteins from the midgut of *R. microplus* females

Isolated membrane proteins are shown in (*A*) and dot blot to indicate the presence of intact Bm86 is shown in (*B*). Arrows indicate high abundance proteins.

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The results for the ELISA using the pooled antiserum from three mice immunised with a crude extract containing membrane proteins from the midgut of *R. microplus* are shown in Figure 3.11. Peptides where the absorbance differed significantly with that of the highest-scoring Bm86 peptide (number 2) are indicated with asterisks. Peptides 1 from Contig6380, Contig6778 and Contig7603 differed highly significantly (*p*-value <0.001) with peptide 2 from Bm86 (Contig8501).

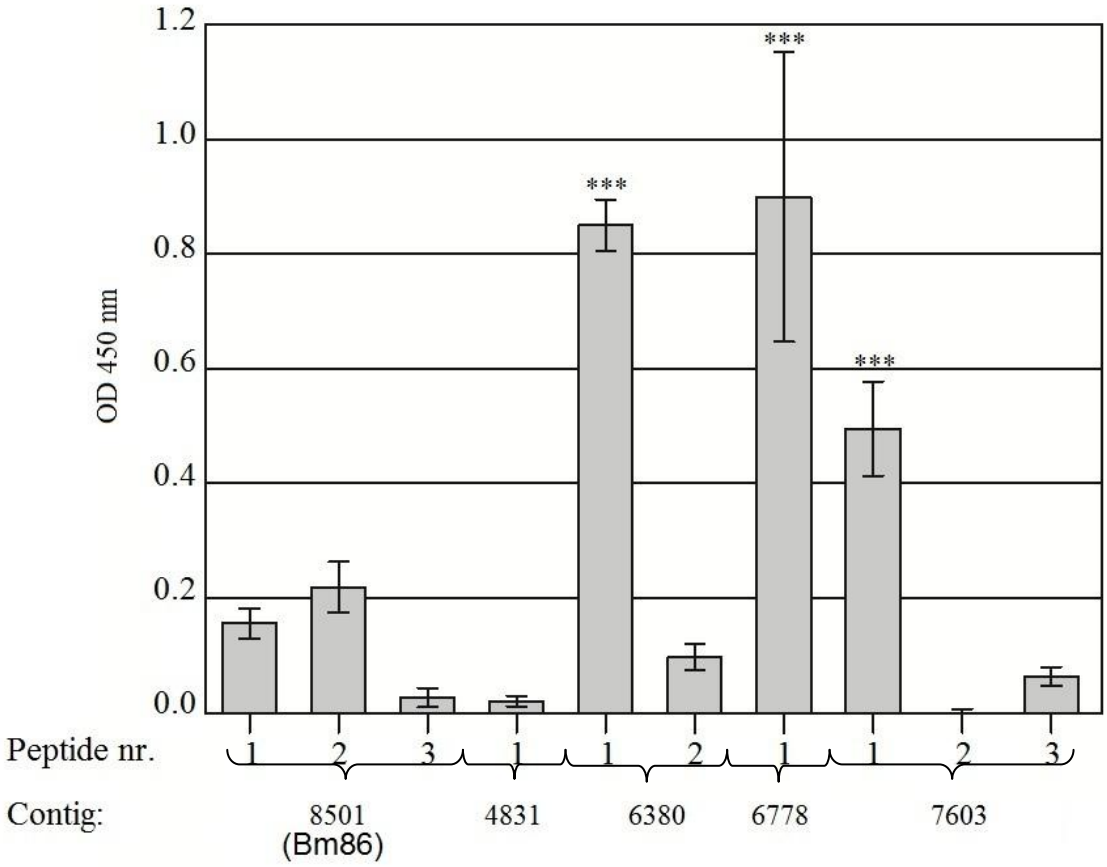

Figure 3.11. Mouse antiserum reactivity against the predicted epitopes

Immunodominant epitopes were predicted and used for ELISA. Antiserum was from mice immunised with membrane proteins from the midgut of *R. microplus*. Graph shows the average OD_{450nm} of four technical repeats performed using pooled antiserum. Error bars indicate ±1 standard deviation. Refer to Table 3.4 for peptide sequences.

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3.7 Conclusion

There is no shortage of scientific publications describing the lack of vaccine targets against *R. microplus*. This is not surprising, as the genome of *R. microplus* has not been fully sequenced and assembled. Fortunately, results from the *R. microplus* EST sequencing project have enabled the design of custom oligonucleotide microarrays. This has paved the way for researchers to study various processes in *R. microplus*, such as genes involved in acaricide resistance (Saldivar et al., 2008) and host-sensing (Rodriguez-Valle et al., 2010).

Advances in molecular biology and the advent of reverse vaccinology (Rappuoli, 2000) have changed the way vaccines against pathogens, or parasites in this case, can be developed. Reverse vaccinology has also been shown to be a viable alternative to vaccine discovery, especially in cases where traditional approaches have failed (Pizza et al., 2000).

Since the genome of *R. microplus*, and likely its proteome, is very complex, the large number of genes to be evaluated using a reverse vaccinology approach had to be reduced somehow. Based on the properties of Bm86, the only commercially available anti-tick vaccine, a set of criteria was developed.

Various computer programs were used in this study to identify immunodominant epitopes in proteins that are expressed throughout the life cycle of *R. microplus*. Their validity was ultimately demonstrated as antiserum from mice that had been immunised with membrane proteins from the midgut of *R. microplus* was able to recognise peptides that correspond to predicted immunodominant epitopes. The results also show that it is possible that, had only one peptide been tested per antigen, immunodominant epitopes would not have been identified. Peptides for both Contig6380 and Contig7603 were tested that showed very high and very low reactivity with the antiserum pool.

Peptides from three novel antigens were recognised to a greater extent than peptides from Bm86, possibly indicating that novel vaccine candidates have been discovered using this combined functional genomics and immunoinformatics approach. It is possible, however, that the peptides representing Bm86 were not the most immunogenic ones available, as they were identified using only one algorithm (Rodríguez et al., 1994). As can be seen in Figure 3.5, there are areas that had higher scores than the peptides selected by abovementioned researchers.

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Perhaps, before one could make claims as to the immunogenicity of the novel antigens that have been identified in this study, the immunodominant epitopes for Bm86 should be tested using the same antiserum. As it is also known that an antibody-mediated immune response cannot be directly correlated with protection after immunisation (Hope et al., 2010), cattle trials will need to be performed in order to validate these novel antigens as anti-tick vaccine candidates. The results do show, however, that immunisation using a crude membrane protein extract resulted in B cell activation and production of IgG against these proteins.

Reverse vaccinology appears to be an attractive solution for the identification of anti-tick vaccines but it does have its shortcomings, as there is the possibility of non-protein protective antigens, which are not evaluated with this approach. The application of a reverse vaccinology-based approach has, however, revealed a large number of potential vaccine candidates against *R. microplus*.

The best predicted peptide for Contig4831 did not yield good results after evaluation with ELISA (Figure 3.11). This might be due to various factors. For example, it is possible that an incorrect epitope was predicted, or that expression of this protein is very low in the midgut of feeding *R. microplus* females. For this reason, Contig4831 can by no means be excluded from future analyses because its immunogenicity has not been evaluated using recombinant protein. Therefore, depending on financial constraints, peptides could be designed for the rest of the proteins in Table 3.3, using the same methodology described in this chapter. Preference could then be given to those proteins whose peptides performed the best in the ELISA. Subsequent experiments would then involve their recombinant expression and evaluation for antigenicity using laboratory animals, followed by vaccine trials using cattle.
A fully sequenced and assembled genome, coupled with the development of genomic tools that focus on *R. microplus* (Bellgard et al., 2012) will aid the identification of full-length transcripts. Promising techniques such as RNA-seq will be of invaluable use in this regard.

Another form of identifying membrane proteins as potential targets for vaccine targets is by so-called protein shaving, where enzymes specific for glycosylphosphatidylinositol are used to isolate GPI-anchored membrane proteins (Rappuoli and Bagnoli, 2011). A comprehensive project analysing membrane proteins from the midgut of *R. microplus* is currently under way. Results from this study will provide invaluable insight into the mialome of *R. microplus.* In addition, results from this study could be used to compare expression levels, as measured using DNA microarray, with proteomic data.

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In this project, seven GPI-anchored potential vaccine candidates against *R. microplus* have been identified. They are expressed in the larvae, nymphs and midgut of feeding adult female ticks and this was determined using DNA microarrays, of which the results were confirmed using real-time PCR. Their true status as GPI-anchored proteins has not been confirmed but this could be performed as explained above. Using various computer programs, they were predicted to be immunogenic and this was evaluated by using antiserum from mice that had been immunised with membrane proteins from the midgut of *R. microplus* adult female ticks. The possibility of contaminating cytosolic proteins in this extract was not investigated further and the availability of antiserum raised against *R. microplus* subolesin, a cytosolic protein, would have enabled such a study. An alignment-free approach using VaxiJen was followed, in order to predict protective antigens. The true accuracy of VaxiJen could be evaluated by testing the protective capability of antigens predicted not to be protective antigens, but vaccine trials using antigens expected to fail would not be financially feasible. It is argued, however, that the identification of any true positives would be of great value regardless of the identification of false negative protective antigens. The proteins identified in this project also do not share sequence similarity with any mammalian sequences, suggesting that host proteins would most likely not become the target of an immune response raised against these novel proteins, but this would need to be confirmed experimentally.

Compared to the first example of reverse vaccinology, where hundreds of potential open reading frames were identified and evaluated as vaccine candidates, only seven proteins were identified in this study. This might be explained by the various criteria imposed on the thousands of genes that were evaluated. Compared to cytosolic and membrane-bound proteins, GPI-anchored proteins are in the minority (Table 3.2). This, coupled with the requirement that all proteins had to have a VaxiJen score of higher than 0.7698, despite the default VaxiJen requirement for a protective antigen to have a score of just 0.5, can explain why so few proteins were identified using this reverse vaccinology approach. Perhaps, the 570 predicted *N. meningitidis* open reading frames from the first reverse vaccinology article (Pizza et al., 2000) should be subjected to analysis using VaxiJen. It would be interesting to see whether the antigens that ultimately were evaluated in phase III clinical trials are predicted by VaxiJen to be protective antigens or not.

Future aims

1. The way forward for this research is therefore to recombinantly express the novel proteins identified in this study, preferably in *P. pastoris.* These proteins can then be

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used to immunise small experimental animals, such as mice, to test their immunogenicity.

2. Protein expression of numerous membrane and GPI-anchored *R. microplus* proteins is currently under way and other members of the research group will subsequently evaluate their ability to confer anti-tick protection to mixed breed (*B. taurus* x *B. indicus*) cattle. These trials are planned for commencement in the second quarter of 2013.

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Chapter 4

Concluding discussion

4.1 Rationale of the study

R. microplus is one of the most economically important ticks that parasitize the global cattle population. It affects cattle indirectly by transmitting *B. bovis*, *B. bigemina* and *A. marginale*, causing babesiosis and anaplasmosis, resulting in losses of milk and beef production (de la Fuente and Kocan, 2006; Wang et al., 2007). In South Africa, co-infestation of the some 14.1 million heads of cattle with *R. decoloratus* and *R. microplus* is widespread (DAFF, 2011; Terkawi et al., 2011). This presents a significant burden on the bioeconomy.

Acaricides remain the preferred method of tick control but suffer from numerous drawbacks. Immunological control of ticks, in the form of a recombinantly expressed vaccine, offers an attractive alternative approach. However, the only anti-tick vaccine, Bm86, has failed to solve the global problem with *R. microplus* and novel vaccine candidates need to be identified (Guerrero et al., 2012). At inception of this project, EST sequencing projects focusing on *R. microplus* had been established (Wang et al., 2007). This was a remarkable milestone for tick research but no information was available regarding the expression profile of the various identified putative genes.

Based on similarities between both their phylogeny and geographic distribution in South Africa, *R. microplus* and *R. decoloratus* ticks were studied using a custom oligonucleotide microarray. The tissue of choice was the midgut, which is the first site of encounter with the host's immune factors.

4.2 Findings of this study

With vaccine design in mind, considering genes that are expressed in the midgut of both tick species, 6730 genes were identified (Figure 3.4). It is hypothesised that membrane proteins are ideal vaccine candidates, as they come into direct contact with the host's immune factors

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(Rappuoli and Bagnoli, 2011). From the 6730 genes that are actively expressed in the midgut of both *R. microplus* and *R. decoloratus* females, 1224 are predicted to contain membrane-spanning helices. This is a significant improvement on the problem of identifying suitable anti-tick vaccine candidates (Nuttall et al., 2006).

Furthermore, global transcriptomic analyses of the fold change in expression between the two ticks revealed that 2476 genes were expressed at similar levels among the two tick species, whereas 1084 were found to be more highly expressed in the midgut of *R. microplus* females, compared to 136 genes that were more highly expressed in the midgut of *R. decoloratus* females. Using KOG annotation, it was noted that the three largest clusters of biological processes among the genes more highly expressed in both *R. microplus* and *R. decoloratus* were signal transduction mechanisms, lipid transport and metabolism and posttranslational modification, protein turnover and chaperones. Chi-square analysis indicated a significant increase in genes involved in lipid transport and metabolism (Table 2.7). This is a curious observation, because it has been reported previously that intracellular absorption of nutrients from the blood meal is primarily directed at haemoglobin (Sonenshine, 1991), which is a protein. One would therefore expect an upregulation in genes involved in the breakdown of protein instead. As mentioned earlier, however, one of the largest clusters of biological processes included protein turnover, which might be responsible for the breakdown of haemoglobin. Apart from the absorption of haemoglobin, lipid inclusions of varying size are found in digestive cells during the slow feeding phase (Sonenshine, 1991). Considering that the haemoglobin content per red blood cell (by weight) is approximately 100 times higher than that of either lipids or cholesterol (Weed et al., 1963), the primary source of energy from the blood meal might in fact be haemoglobin.

With vaccine design being one of the goals of molecular parasitology, a systematic pipeline was devised to identify a manageable number of proteins to be used as potential vaccine candidates. This pipeline consisted of functional genomics experiments to identify genes that are expressed in the midgut of both *R. microplus* and *R. decoloratus* females (Figure 3.4), as well as in larvae and nymphs of *R. microplus.* This was followed by using computational tools to predict the most likely reading frame of each gene, as well as tools to predict if each protein is membrane-bound or GPI-anchored. The most likely candidates as anti-tick vaccines were determined using an alignment-free approach for the prediction of protective

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antigens and ultimately, seven proteins were identified that were predicted to be both GPI-anchored and more likely than Bm86 to be protective antigens (Table 3.3). Finally, epitopes were predicted and corresponding synthetic peptides were evaluated using ELISA. This led to the identification of three epitopes that are recognized to a greater extent than previously published Bm86 epitopes, when using murine serum raised against membrane proteins from the midgut of *R. microplus*. These results are significant because they show that proteins in the membrane protein extract led to the activation of B cells, resulting in the production of IgG antibodies that recognize these epitopes.

This study was therefore relevant because numerous potential anti-tick vaccine candidates that are expressed in the midgut of both *R. microplus* and *R. decoloratus* were identified. Moreover, results indicate that genes involved in lipid transport and metabolism are upregulated in the midgut of feeding females. In addition, using a largely computational approach, epitopes from novel proteins that are recognized by murine serum raised against membrane proteins from the midgut of *R. microplus* were identified.

4.3 Limitations of this study

Based on similarities between the mitochondrial 12S and cytochrome c oxidase I sequences of *R. microplus* and *R. decoloratus* (Murrell et al., 2001), it was hypothesised that oligonucleotide microarrays based on *R. microplus* sequences could be used to study expression levels of genes in *R. decoloratus*. The ideal alternative would be to have oligonucleotide microarrays that comprise sequences from *R. decoloratus*. Unfortunately, however, very little sequence information is available for *R. decoloratus,* rendering this option unfeasible. Results from RNA-seq or EST sequencing experiments would have been necessary in order to design a microarray based on *R. decoloratus* sequences. Moreover, only four microarrays were available, limiting the number of possible experiments. Had more microarrays been available, comparisons could have been done between other tissues and life stages from *R. microplus* and *R. decoloratus*. It is believed, however, that the current methodology may have identified genes that are highly conserved between *R. microplus* and *R. decoloratus*, which would make ideal cross-protective vaccine candidates. Finally, previous research groups had used microarrays specific to *R. microplus* (Rodriguez-Valle et al., 2010; Saldivar et al., 2008) but these were not publicly available when this project started.

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As a result, comparing results from abovementioned studies with those from this study is far more time-consuming.

Due to their lack of similarity with sequences from other organisms, tick genes remain largely unannotated. This is a major problem for downstream analyses that rely on the predictive power of computational tools. Moreover, the lack of GO annotation tools for non-model organisms limits the analysis of data from functional genomics experiments.

Finally, the prediction of B and T cell epitopes needs to be improved for tick antigens that would get recognized by a bovine host. Fortunately, this is a subject of ongoing research (Nene et al., 2012), as insight into bovine MHC alleles remains limited.

4.4 Future prospects

The lack of sequence data for *R. decoloratus* could be addressed by performing RNA-Seq (Nagalakshmi et al., 2010), which would be advantageous by providing sequence data as well as information regarding expression levels. If deemed necessary, the sequence data could subsequently be used to design an oligonucleotide microarray specific to *R. decoloratus* for future experiments.

Proteomics experiments studying *R. microplus* proteins are limited but they offer the potential advantage of confirming the reading frame of putative genes (Kongsuwan-2010). Proteomic analyses of *R. microplus* by other members of the research group are under way, which would offer exciting answers that could be compared with results from this study to elucidate the relationship between expression level (expressed as intensity on a microarray) and protein level.

Results from Chapter 2 also indicate the importance of genes that are involved in lipid transport and metabolism. Gene knockdown using RNAi might reveal whether they are in fact essential for a tick's survival, after which their three-dimensional structures could be determined and used for the design of novel acaricides that impair lipid metabolism in ticks.

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Results from Chapter 3 indicate the validity of the novel methodology for the identification of anti-tick vaccine candidates. Their recombinant expression and evaluation in vaccine trials are currently under way. In collaboration with the developers of the alignment-free approach for the identification of protective antigens, results from these vaccine trials could also be used to improve the software.

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