

GENE EXPRESSION PROFILES ASSOCIATED WITH BEEF CATTLE RESISTANCE TO
RHIPICEPHALUS TICKS

By

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Declaration

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Abstract

Tick resistance is a complex trait influenced by numerous environmental, physiological and genetic factors. The length of the association between cattle breeds and tick species may play a vital role in the potency of the immune responses generated by the host post-infestation. The genetically determined components of host resistance, which may have evolved due to long periods of evolution of breeds in the presence of specific tick species, are regarded the most important factors of host resistance to ticks. The isolation and characterisation of genes associated with natural host resistance may provide a low-cost, environmentally sound and sustainable chemical-free alternative for tick control through gene introgression and improved accuracy of selection in breeding programs. This study examined the tick burdens and associated gene expression profiles in two ancient (Nguni – *R. decoloratus* and Brahman – *R. microplus*) and four modern (Nguni – *R. microplus*, Brahman – *R. decoloratus*, Angus – *R. decoloratus* and Angus – *R. microplus*) host-tick associations following artificial infestation. Approximately 100 unfed tick larvae of a single species were used to infest each animal, thereafter tick counts were enumerated 18-days post-infestation. Skin biopsies, from which RNA was extracted for use in the gene expression analyses, were collected pre-infestation from non-parasitized sites and 12-hours post-infestation at visible tick-bite sites. The panel of genes analysed comprised of cytokines (*TLR5*, *TLR7*, *TLR9*, *TRAF6*, *CD14*), chemokines and their receptors (*CCR1*, *CCL2*, *CCL6*), toll-like receptors (*IL-18*, *CXCL8*, *IL-10*, *TNF*) and other candidate genes (*BDA20*, *OGN*, *TBP*, *LUM*, *B2M*) whose expression was normalized against *RN18S1* (or β -actin-like). Custom 96-well RT² Profiler PCR arrays, fitted with primers designed and optimised by Qiagen, were used for real-time PCR analyses using RT² SYBR[®] Green dye and an ABI 7500 Standard real-time PCR cycler. The effects of breed, tick species and breed by tick species interaction on tick count were analysed using XLSTAT (2016) and SAS Enterprise Guide (2016). The fold regulation/change values were generated via the online RT² Profiler PCR Array Data Analysis Web-portal (SABioscience - Qiagen), using the $\Delta\Delta C_T$ method. The effects of breed, tick species and breed by tick species interaction on the differential gene expression of each gene were analysed using XLSTAT and SAS (2016). The expression levels of *LUM*, *B2M*, *TRAF6* and *TPB* showed significant breed variations. The Nguni and Angus differed for *TBP* and *TRAF6*, while the Brahman and Angus differed for *LUM* and *B2M*. *LUM* and *B2M* displayed significantly higher expression levels in the Brahman and Nguni cattle. Significant breed, tick species and breed by tick species interaction effects were detected from the tick count data, with the Brahman carrying less ticks than both the Angus and Nguni cattle, while the *R. microplus* resulted in heavier tick burdens than the *R. decoloratus* ticks. In both experiments, there was a lack of evidence of any breed by tick species interaction which would implicate the effect of length of association between breeds and tick species in the host response to tick challenge in respect with gene expression and tick burden.

Opsomming

Bosluis weerstand is 'n komplekse eienskap wat beïnvloed word deur verskeie omgewings-, fisiologiese- en genetiesefaktore. Die lengte van die assosiasie tussen rasse en bosluis spesies mag 'n essensiële rol speel in die sterkte van die immuun reaksie gegeneer deur die gasheer na besmetting. Die geneties bepaalde komponente van gasheer weerstand, wat kon ontwikkel het as gevolg van lang periodes van evolusie van rasse in die teenwoordigheid van spesifieke bosluis spesies, word beskou as die mees belangrikste faktore van gasheer weerstand tot bosluis. Die isolasie en karakterisering van gene geassosieer met natuurlike gasheer weerstand kan 'n lae koste, omgewingvriendelike en volhoubare chemiese-vrye alternatief lewer vir bosluis beheer deur geen introgressie en verbeterde akkuraatheid van seleksie in teelprogramme. Hierdie studie het die bosluis lading en geassosieerde geenuitdrukking profiele na kunsmatige besmetting ontleed in twee antieke (Nguni – *R. decoloratus* en Brahman – *R. microplus*) en vier moderne (Nguni – *R. microplus*, Brahman – *R. decoloratus*, Angus – *R. decoloratus* en Angus – *R. microplus*) gasheer-bosluis assosiasies. Ongeveer 100 ongevoerde bosluis larwe van 'n enkele spesie was gebruik om elke dier te besmet, waarna bosluis tellings 18 dae na besmetting geneem is. Vel biopsies, waaruit RNS geïsoleer is vir gebruik in die geenuitdrukking ontledings, was gekollekteer voor infestasië van af areas vry van parasiet besmetting en 12 ure na besmetting vanaf areas met sigbare bosluis bytplekke. Die paneel gene wat ontleed is het bestaan uit sitokiene (*TLR5*, *TLR7*, *TLR9*, *TRAF6*, *CD14*), chemokiene en hulle reseptore (*CCR1*, *CCL2*, *CCL6*), tolgagtige reseptore (*IL-1β*, *CXCL8*, *IL-10*, *TNF*) en ander kandidaat gene (*BDA20*, *OGN*, *TBP*, *LUM*, *B2M*) wat se uitdrukking genormaliseer was teen *RN18S1* (of β -aktien-agtige). Pasgemaakte 96-well RT² Profiler PKR arrays, toegerus met primers ontwerp en geoptimaliseer deur Qiagen, was gebruik vir ware tyd PKR ontledings met die gebruik van RT² SYBR[®] Groen kleurstof en 'n ABI 7500 Standaard ware-tyd PKR cyclus. Die effek van ras, bosluis spesie en ras by bosluis spesie interaksie op bosluis telling was ontleed deur gebruik te maak van XLSTAT (2016) en SAS Enterprise Guide (2016). Die vou regulasies/veranderingswaardes was gegeneer via die aanlyn RT² Profiler PCR Array Data Ontledings Webportaal (SABioscience - Qiagen), deur gebruik te maak van die $\Delta\Delta CT$ metode. Die effek van ras, bosluis spesie en ras by bosluis spesie interaksie op die differensiële geen uitdrukking van elke geen was geontleed deur gebruik te maak van XLSTAT and SAS Enterprise Guide (2016). Die uitdrukkingvlak van *LUM*, *B2M*, *TRAF6* en *TPB* het beduidende ras variasie getoon. Die Nguni en Angus het verskil vir *TBP* en *TRAF*, terwyl die Brahman en Angus verskil het vir *LUM* en *B2M*. *LUM* and *B2M* het beduidende hoër uitdrukkingvlakke in die Brahman en Nguni beeste getoon. Beduidende ras, bosluis spesie en ras by bosluis spesie interaksie effekte was waargeneem van die bosluis telling data, met die Brahman wat minder bosluis dra as beide die Angus and Nguni beeste, terwyl die *R. microplus* gelei het tot swaarder bosluis ladinge as die *R. decoloratus* bosluis. In beide eksperimente was daar geen bewys

van enige ras by bosluis spesie interaksie nie. Dit kan aandui dat die lengte van assosiasie tussen rasse en bosluis spesies geen effek op gasheer reaksie tot 'n bosluis uitdaging ten opsigte van geen uitdrukking en bosluis lading kan hê nie.

Dedication

To my father, a man who has worked tirelessly and without a single complain from the day I was born to support my dreams and aspirations when most discouraged him by saying that my dreams were out of reach. He retired from work only to return six years later to support my ambitions; for he felt that his contribution to my success had not yet be exhausted. To my late mother, may the peace of the Lord be with her resting soul: "Death ends a life not a relationship". I appreciate all the sacrifices she made when she was still with us, all of which paved the way to my becoming a first generation university student in my immediate family. In more ways than one the memory of my mother which lives within me has granted me the strength to strive for only the best even when I was at the lowest points of my life, feeling incapable of completing a Master's Degree. I owe every bit of my current success and all else from here on forth, to my wonderful family who did not allow poverty to drive them to discourage what seemed like a young underprivileged girl's overly ambitious goals. I would also like to dedicate this thesis to all my nieces and nephew. The cycle of poverty, "black-tax" and lack of further education beyond matric stops with my generation. I have now paved the way as the first generation university student in our family for you to see that is possible multiply whatever little you come from to prosper and built well founded legacies for your own children to come.

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Definitions of Key Terminology

Acaricide: A substance poisonous to mites or ticks

Agent: Thing that takes an active role or produces a specific effect

Antigen: A toxin or other foreign substance which induces an immune response in the body, especially the production of antibodies

Artificial: Made of produced by human beings rather than occurring naturally, often to simulating natural occurrences

Biopsy: An examination of tissue removed from a living body to discover the presence, cause, or extent of a disease

Biotype: A group of organisms having an identical genetic constitution

Bovine: An animal of the cattle group, which also includes buffaloes and bison

Ectoparasite: A parasite, such as a flea or a tick, which lives on the outside of its host.

Fold change: A measure describing how much a quantity of a specific gene changes going from an initial (pre-infestation state) to a final value (post infestation state)

Fold regulation: A measure describing how much a quantity of a specific gene changes going from an initial (pre-infestation state) to a final value (post infestation state) with negative value equivalent to $- (1/\text{fold change value})$

Host: An animal or plant on or in which a parasite or commensal organism lives

Infectious: Liable to be transmitted to organisms and capable of causing infection

Infestation: The act of inhabiting or overrunning in numbers or quantities large enough to be harmful, threatening, or obnoxious:

Ixodidae: The family of hard ticks, one of the two big families of ticks, consisting of over 700 species. They are known as 'hard ticks' because they have a scutum or hard shield, which the other big family of ticks, the soft ticks (Argasidae), lack

mRNA: The form of RNA in which genetic information transcribed from DNA as a sequence of bases is transferred to a ribosome

Pathogen: A bacterium, virus, or other microorganism that can cause disease

Parasite: An organism which lives in or on another organism (its host) and benefits by deriving nutrients at the other's expense

Primer-dimers: Potential by-products in PCR, consisting of primer molecules that have attached (hybridized) to each other because of strings of complementary bases in the primers

Real-time PCR or qPCR: A laboratory technique of molecular biology based on the polymerase chain reaction (PCR), which monitors the amplification of a targeted DNA or RNA molecule during the PCR, i.e. in real-time, and not at its end, as in conventional PCR

Resistance: The ability to not be affected by something, especially adversely, especially as a result of continued exposure or genetic change

Reverse transcription: The reverse of normal transcription, occurring in some RNA viruses, in which a sequence of nucleotides is copied from an RNA template during the synthesis of a molecule of DNA

Semi-arid: A climate or place that is partially arid (has little or no rain and too dry or barren to support vegetation), or semi-dry and has less than 20 inches of rain each year

Subtropical: Relating to the regions of the Earth bordering on the tropics, just north of the Tropic of Cancer or just south of the Tropic of Capricorn

Sustainable: Able to be maintained at a certain rate or level; conserving an ecological balance by avoiding depletion of natural resources

Tick: A parasitic arachnid which attaches itself to the skin of terrestrial vertebrates from which it sucks blood, leaving the host when sated, sometimes even transmitting disease causing pathogens to the host animal

Transcriptome: The sum total of all the messenger RNA molecules expressed from the genes of an organism

Tropical: Region of the Earth surrounding the equator that are delimited in latitude by the Tropic of Cancer in the Northern Hemisphere and the Tropic of Capricorn in the Southern Hemisphere, which are very hot and humid

Vaccine: An antigenic substance prepared from the causative agent of a disease or a synthetic substitute, used to provide immunity against one or several diseases

List of Abbreviations

<i>A. hebraem</i>	<i>Amblyomma hebraem</i>
ANOVA	Analysis of Variance
ARC-API	Agricultural Research Council-Animal Production Institute
<i>B. indicus</i>	<i>Bos indicus</i>
BoLA –DQ	Bovine Leukocyte Antigen DQ
<i>B. taurus</i>	<i>Bos taurus</i>
CNVs	Copy number variants
C_T value	Threshold cycle value
DAFF	Department of Agriculture, Forestry and Fisheries
FMD	Foot and mouth disease
gDNA	Genomic deoxyribonucleic acid
GLM	General Linear Model
H₀₍₁₎	Null hypothesis 1
H₀₍₂₎	Null hypothesis 2
H_{a(1)}	Alternative hypothesis 1
H_{a(2)}	Alternative hypothesis 2
Kg	Kilograms
L	Litres
LW	Live weight
MHC	Major Histocompatibility Complex
mRNA	Messenger RNA
qPCR	Quantitative polymerase chain reaction
QTLs	Quantitative trait loci
<i>R. appendiculatus</i>	<i>Rhipicephalus appendiculatus</i>
<i>R. decoloratus</i>	<i>Rhipicephalus decoloratus</i>
<i>R. microplus</i>	<i>Rhipicephalus microplus</i>
RNA	Ribonucleic acid
Rpm	Rotations per minute
RT² PCR	Reverse transcriptase real-time polymerase chain reaction
SNPs	Single nucleotide polymorphisms
TTBDs	Tick and tick-borne diseases
TBDs	Tick-borne diseases
UFL	Unfed larvae

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background

Beef cattle breeds, being mostly extensively managed and pasture-fed, are constantly challenged by external parasites. Roughly 70% of the beef production systems worldwide are located in areas recorded as hosting the highest population numbers of cattle ticks (Porto Neto *et al.*, 2011). Ticks pose the risk of inflicting deleterious effects on production traits by hindering the growth and weight gain, productivity, fertility as well as the meat quality of cattle (Untalan *et al.*, 2007; Marufu, Chimonyo, *et al.*, 2011). Subsequently the profitability of the beef cattle industry may be notably compromised due to the fact that numerous successful beef enterprises maximise their profit margins by concentrating more on fertility and a high weaning weight (Nel, 2015). Tick infestations produce losses commonly identified in beef enterprises as blood loss, tick worry, hide damage and toxin introduction into the herds (De Castro, 1997). Ticks together with their associated tick-borne diseases (TTBDs) are arguably the biggest impediment responsible for the elevated costs of production in the beef cattle production systems in semi-arid, tropical and subtropical areas worldwide (Gasbarre *et al.*, 2001; Rajput *et al.*, 2006; Morris, 2007; Kongsuwan *et al.*, 2010). The lagging expansion of beef cattle production behind other livestock production industries may also be ascribed to TTBDs manifestations (Mapholi *et al.*, 2014). This is exacerbated by the inability of the conventional tick control methods, which include the use of acaricides and vaccines, to successfully eradicate ticks, thus compromising overall cattle health (Wambura *et al.*, 1998; Gasbarre *et al.*, 2001; Marufu, Chimonyo, *et al.*, 2011). Alternative tick control measures that are sustainable and cost effective should, therefore, be developed and implemented.

Tick resistance among cattle breeds is variable, with the Nguni breed exhibiting a higher level of resistance to numerous tick species than the Bonsmara and Angus breeds (Jonsson, 2006; Muchenje *et al.*, 2008; Marufu *et al.*, 2011). Tick resistance in the Brahman cattle breed has been extensively studied in comparison to both the Nguni and the Angus breeds. Some studies have described the Brahman as possessing a superior degree of resistance to the *R. microplus* ticks species, while the Nguni and the Angus exhibited intermediate resistance and susceptibility, respectively (Porto Neto *et al.*, 2011; Manjunathachar *et al.*, 2014). Other studies have demonstrated an inverse resistance ranking order, with the Nguni displaying the highest level of resistance to various tick species (Rechav & Kostrzewski, 1991; Marufu, Qokweni, *et al.*, 2011). This presents an opportunity to exploit the host's resistance to ticks in developing more sustainable and efficient tick control programs. The number of ticks that an animal can carry is indicative of its level of tick resistance. This suggests that tick-resistant

animals will carry fewer ticks when compared to susceptible animals. Therefore, TTBDs may be controlled by rearing tick-resistant cattle breeds in tropical and subtropical regions (Marufu *et al.*, 2014).

Some discrepancies are apparent in literature, questioning the accuracy of using adult tick counts from live animals as a direct representation of the animal's true tick burden and tick resistance. Jonsson (2006) defined tick resistance as "the percentage of larval ticks which fail to survive to maturity following artificial infestation with a known quantity of larvae". According to Bonsma & Pretorius (1943), it was established that with successive infestation of the same host the level of tick resistance increases accordingly compared to the level at first resistance as a result of the animal's innate or acquired immunity. Conversely, Madder *et al.* (2011) reported that host resistance prolongs the female ticks' parasitic phase. Further contributing to these discrepancies are the outcomes of the research by Nyangiwe *et al.* (2013), which highlighted that it is virtually impossible to collect all adult ticks from the various attachment sites of the animal. However, this statement may be deemed valid only in the case of tick counts taken from animals which have experienced natural infestations, but not necessarily so in the case of studies which utilise the artificial infestation approach. This is because technique can be manipulated to allow for the controlled distribution of the tick larvae on the animal's body. These inconsistencies in literature validate the need for gene expression studies which work towards determining the gene expression profiles which constitute bovine tick resistance; a characteristic currently accepted to be represented phenotypically by the number of ticks successfully feeding on the animal under consideration.

Tick bites trigger immune regulatory and effector pathways in the host animal's body, which not only act by mediating the infiltration of the tick-bite site with innate immunity cells, but also by releasing specific proteins that fight infection at the site of infection (Wikel, 1996; Marufu *et al.*, 2014). These involve the activation of an array of biologically active molecules including cytokines, antibodies, B- and T-cells, and granulocytes among others (Wikel, 1999). The Bovine Leukocyte Antigen DQ (BoLA-DQ) lysozyme, cytokeratin or cytokines, interferon γ and tumour necrosis factor α have been identified as candidate systems and gene markers for tick resistance (Morris, 2007). The double amino acid residue motif marker (glutamic acid serine), located on the bovine major histocompatibility complex (MHC) of axon class II BoLA-DBR3 gene as well as on the PCR-RFLP alleles of BoLA-DBR3.2, DRB1 and DRBP1 (Martinez *et al.*, 2006; Mapholi *et al.*, 2014) has received increased attention over time. This suggests that responses to tick infestations may be under genetic control. By identifying the genes responsible for tick resistance, a better understanding of the variation that exists in tick resistance between and within cattle breeds may be generated.

Many of the recent studies which investigated the biological mechanisms of bovine tick resistance, as well as host-tick associations have been aided by the application of revolutionary molecular genetics technologies and bioinformatics (Porto Neto *et al.*, 2011). Extensive research has been centred on the biological information contained in the skin as the primary source required when reviewing the biological mechanisms, gene expression profiles and key pathways of host resistance in cattle (Porto Neto *et al.*, 2011). Single-nucleotide polymorphisms (SNPs) and copy number variations (CNVs) together with the release of the bovine genome sequencing project and the HapMap project, have paved the way for determining “natural” host resistance, leading to the preliminary isolation of genes and gene markers using ultra high density SNP chips ((Parizi *et al.*, 2009). These platforms led to gene expression studies that identified 18 and 48 genes, which had been expressed at higher levels in Hereford Shorthorn cattle formerly characterised as high-resistance and low-resistance, respectively (Wang *et al.*, 2007). Furthermore, a variety of differentially expressed candidate tick resistance genes, namely keratin-related genes, extracellular matrix genes and immunoglobulin- associated genes were identified (Wang *et al.*, 2007). Piper *et al.* (2008) observed significant differences in the genes associated with several toll-like receptors (TLR5, TLR7, TLR9), chemokines together with their receptors (CCR1, CC12, CCL26), as well as cytokines (IL-1 β , IL-2R α , IL-2, IL-10, TNF- α , Traf-6, NFkBp50), while Kongsuwan *et al.* (2008) identified a total of 138 differentially expressed genes and three fundamental pathways that were expressed in tick-resistant Brahman cattle. These genes were linked to pathways involved in cell-mediated immune responses, fluctuating intracellular Ca²⁺ levels and the structural integrity of the dermis. In addition, a number of host defence genes, acute phase protein components, transcription factors and lipid metabolism genes were identified (Kongsuwan *et al.*, 2008).

Given the variation that exists in tick species, tick resistance for a particular breed may be species-specific. This is because the variation is manifested in the characteristics on the tick species, which ranges from the mouthparts to the bioactive molecules in the saliva as well as other physiological properties (Marufu *et al.*, 2014). Consequently, the immune responses of a particular animal may vary depending on the biting tick species. For this reason, the severity of the effects of the tick-induced suppression of the animal’s immune system will depend on the degree to which its immune system has evolved in its ability to generate vigorous responses in defence against the biting tick species (Marufu *et al.*, 2014).

Breeds which may have experienced a long period of evolution in the presence of a particular tick species, and are resistant to that tick species, are suspected to have accumulated genes affecting resistance to that tick species (Frisch, 1999; Marufu *et al.*, 2014). Host-tick relationships can be classified into ancient and modern, which may significantly influence the rate at which the host

acquires resistance to a particular tick species. The ancient associations include the Brahman-*R. microplus* and Nguni-*R. decoloratus* associations, while the modern associations include the Angus-*R. microplus*, Angus-*R. decoloratus*, Brahman-*R. decoloratus* and Nguni-*R. microplus*. The South African indigenous Nguni cattle should thus be expected to be more resistant to the South African indigenous *Rhipicephalus decoloratus* (*R. decoloratus*) tick species than to *R. microplus*, the Asian counterpart. Similarly the Brahman is expected to exhibit superior resistance to the *R. microplus* than the *R. decoloratus* tick. The thought-provoking subject appears to be centred on the basis of understanding whether the superior tick resistance fashioned in Nguni and the *Bos indicus* cattle as a whole can be attributed to an uncharacterised unique genetic make-up or whether it is merely due to the long term association between the breed and the tick species over the years. Studying these host-tick associations with a high level of accuracy may aid in tick control programs.

1.2 Problem Statement

Tick infestations, together with the manifestation of associated tick-borne diseases, are arguably the biggest impediment responsible for the elevated costs of production in the beef cattle industries. Tick infestations are an even bigger problem in semi-arid, tropical and subtropical areas, such as South Africa (SA). Current control methods, which are mainly acaricides and vaccines, are ineffective in completely eradicating ticks. Furthermore, they have undesirable effects on both products produced by the animals and the environment where production takes place. Exploitation of the host's resistance is a possible alternative, where much of the available research has been focussed on the characterisation of the phenotypic aspects of tick resistance in cattle. Thus, improvement of the host's resistance to ticks is a cost effective and environmentally sound way to control ticks. The effectiveness of the improvement depends on the accuracy of identifying resistant animals; hence gene expression studies for tick resistance increases the accuracy of identifying animals with desirable genes underlying tick resistance.

1.3 Significance of the Research

Tick resistance gene expression studies have been conducted in earlier studies in beef cattle (Piper *et al.*, 2008; Brannan *et al.*, 2014). However, gene expression studies across different host-tick associations are lacking, at least in the tropical and subtropical regions. By studying the differential expression of a panel of candidate genes in different cattle breeds, a better understanding of the genes and pathways involved in tick resistance will be generated to explain the variance observed which cannot be ascribed to other factors. The mode of infestation used in the study was artificial infestation. The larval or free-living stage in the tick's life cycle is very vulnerable to fluctuations in environmental conditions, such as temperature, humidity and species of the grass (Kumar *et al.*, 2011).

As such, artificial infestations simulate field conditions while minimizing environmental effects and predation to ensure equal opportunity for each larval tick to attach. Therefore, the tick counts data as well as results obtained from the gene expression study is reliable as predominantly influenced by the genetic architecture of the animal. There is also limited understanding of the different host-tick associations, which can be elucidated by the gene expression studies for tick resistance. Understanding the host-tick associations aids in explaining the variation that exists in resistance to the different tick species. A better understanding of the variations in the different host-tick associations may, therefore, aid in developing genetically-based tick control measures. These may complement the use of acaricides and vaccines to facilitate the development of more sustainable, environmentally sound and targeted alternatives for tick control.

1.4 Hypotheses

The following null hypotheses were generated for the study:

- 1) $H_{0(1)}$: There is no significant difference in the gene expression profiles of the Nguni, Brahman and Angus cattle which have been infested with *R. microplus* and *R. decoloratus* tick species. Therefore, one breed does not exhibit superior resistance over the other breeds as a result of a unique genetic make-up.
- 2) $H_{0(2)}$: There is no difference in the level of resistance between the different ancient (Brahman-*R. microplus* and Nguni-*R. decoloratus*) and modern (Angus-*R. microplus*, Angus-*R. decoloratus*, Brahman-*R. decoloratus* and Nguni-*R. microplus*) host-tick associations. A co-evolutionary status between a specific breed and tick species does not render that breed more resistant to that particular tick species. Therefore, all three breeds exhibit similar tick burdens on day 18 post-infestation for both tick species.

The following alternative hypotheses were compiled for the study:

- 1) $H_{a(1)}$: The breeds possess significantly different gene expression profiles which render some breeds more resistant to *R. microplus* or *R. decoloratus* or both tick species as compared to the other more susceptible breeds.
- 2) $H_{a(2)}$: There is a difference in the level of tick resistance between the modern and ancient host-tick associations. Ancient host-tick associations result in low tick counts, while the modern host-tick associations produce significantly higher tick counts. Therefore, cattle breeds which may have experienced long periods of evolution in the presence of a particular tick species are more resistant to that tick species.

1.5 Objectives

The broad objective of the study was to collect tick count data as a measure of host tick resistance and susceptibility combined with gene expression data to describe gene expression profiles associated with the ancient (Brahman-*R. microplus* and Nguni-*R. decoloratus*) and modern (Angus-*R. microplus*, Angus-*R. decoloratus*, Brahman-*R. decoloratus* and Nguni-*R. microplus*) host-tick interaction. The specific objectives of the study were to use gene expression data together with tick count data to:

- 1) Compare tick counts as a measure of host tick resistance in the ancient (Brahman-*R. microplus* and Nguni-*R. decoloratus*) and modern (Angus-*R. microplus*, Angus-*R. decoloratus*, Brahman-*R. decoloratus* and Nguni-*R. microplus*) host-tick associations;
- 2) Conduct quantitative real-time PCR analyses which describe the different gene expression profiles underlying the various host-tick interactions, thereby enabling the characterisation of a panel of inflammation-related genes actively involved in triggering robust immune responses in naturally tick-resistant biotypes.

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

The gradually increasing global human population has been predicted to increase by 72% by the year 2050 (Hajdusek et al., 2016). While urbanisation increases, the demand for animal protein in the growing markets of Brazil, India, Russia, China and South Africa is equally escalating beyond the livestock producers' supply capacity (De Castro, 1997; Scholtz & Theunissen, 2010; Mapholi *et al.*, 2014). This is partly due to the deleterious effects of ticks and tick-borne diseases (TTBDs), which are socio-economic threats hampering livestock production on a global scale. Affecting an estimated 80% of the world's cattle population, TTBDs are considered one of the biggest threats facing beef production longevity (Rajput *et al.*, 2006; Marcelino *et al.*, 2012; Manjunathachar *et al.*, 2014). In South Africa, the *Rhipicephalus microplus* and *R. decoloratus* tick species have been ranked as the major health and production constraints in low-input farming systems that seldom have the economic means to finance chemical tick control strategies (Mbatia *et al.*, 2002; Mapiye *et al.*, 2009; Marufu *et al.*, 2014). Tick and tick-borne diseases cause economic losses in terms of increased livestock mortality rate, production losses i.e. damaged skin and hides, low milk yield, decreased dressing percentage and most importantly the cost of control methods.

The current tick control methods have not been successful in completely eradicating ticks from cattle herds. Moreover, the consumer demands for chemical-free reared animals and high quality animal products have intensified the global commitment towards the rational selection of parasite and disease resistant beef cattle breeds (Regitano *et al.*, 2008; Kongsuwan *et al.*, 2010). It has proven even to be more challenging to meet the increased demand and individual consumer preferences for beef without exhausting resources. Exploiting the host's resistance to ticks is one possibly cost-effective and sustainable approach for tick control that can be used to complement the existing control methods. The variation in tick resistance that exists between and within breeds makes the application of breeding practices to control ticks possible.

It is well documented that *B. indicus* cattle are more resistant to external parasites than *B. taurus* breeds and that resistance is likely to be improved through selection (Frisch, 1999; Machado *et al.*, 2010; Porto Neto *et al.*, 2011). The infiltration of superior genetics into the gene pool of the breeding stocks bears the potential to grant cross-protection against the economically important tick species co-infesting beef cattle (Machado *et al.*, 2010). However, the criteria for selection is yet to be

formulated in order to fully exploit the benefits of crossbreeding in controlling ticks, since the basis of resistance is still not known to be genetically based or a result of co-evolution. With the current major threat facing the beef industry being the manifestation of TTBDs and the lack of sustainable methods to control them, the studies of gene expression profiles associated with tick resistance are well justified.

An array of studies has been conducted on a wide selection of cattle breeds to examine the within- and between-breed variations in investigating tick resistance. Disappointingly, it is still not determined whether naturally tick-resistant breeds, such as the Nguni (*B. taurus africanus*) possess superior resistance to ticks as a result of long term association with the tick species or if it is due to a unique uncharacterised genetic makeup (Jonsson, 2006; Mapholi *et al.*, 2014). While numerous genes and pathways have been isolated and examined for their association with tick resistance or susceptibility in the bovine species, only a few have been identified as candidates for tick resistance (Morris, 2007). Numerous assumptions of host resistance have been generated based on the biological mechanisms in rodent model systems, however, there is still a lack of information which maps the association between candidate gene expression profiles and specific pathways to generate the observed characteristic of resistance to ticks in cattle (Gasbarre *et al.*, 2001). It is, therefore, imperative to characterise the fundamental transcriptomic components of the bovine genome, which are associated with resistance to the economically important *R. microplus* and *R. decoloratus* tick species.

2.2 Economic Implications of Tick Infestations

Throughout the decades, the reported economic implications connected to TTBDs have been alarming. However, it has been difficult to accurately estimate the magnitude of the trauma experienced economically resulting from TTBDs. Minjauw & Mcleod (2003) and Nyangiwe *et al.* (2013) mapped the importance, distribution, cattle populations affected and the costs associated with the economically important TTBDs in Southern Africa. However, reliable global data that accurately records the epidemiology of tick infestations and the costs involved in controlling them is still required (Jongejan & Uilengberg, 2004; Mapholi *et al.*, 2014). The majority of the quantifiable economic costs sustained are primarily estimated from the acaricide and vaccine treatments required to control tick burdens in the cattle herds. The early estimated costs were within the regions of US\$ 8.43 for plunge dipping, US\$ 13.62 for hand spraying and US\$ 21.09 for pour-on treatments per animal per year (De Castro, 1997; D'Haese *et al.*, 1999). In mitigating economic losses incurred by the beef production industry, it is equally important to acquire a comprehensive understanding of the factors influencing tick infestations and the spread of tick-borne diseases. A substantial amount of pressure has been placed on animal breeders and geneticists to identify specific alleles in the genotypes of beef cattle

breeds which constitute the functionality of innate immunity and biological mechanisms involved in tick resistance or susceptibility in beef cattle.

The estimated production losses associated with tick infestation are presented Table 2.1. Furthermore, Biswas (2003) cited by Ghosh *et al.* (2007) equally implicated damages produced by tick bite marks as the reason for the downgrading of skin and hides in the manufacturing of good quality leather. This diminishes the normal market value of livestock skin and hides by approximately 20 to 30%. Although *Rhipicephalus (Boophilus)* ticks have short mouthparts, the abundance of the ticks during infestations inflicts considerable damage to the skin and hides of animals since their preferred feeding sites are often on sections of the host's body with good leather potential (Jongejan & Uilenberg, 1994; Jongejan & Uilenberg, 2004). This is particularly problematic in Nguni cattle, where post-production value greatly relies on the quality of their hides.

Table 2.1: Annual production losses incurred as a results of ticks and tick-borne diseases (TTBDs)

Amount (US\$)	Reason	Region	Reference
13.9 – 18.7 billion	Total annual production losses	Global	De Castro (1997) and (Hajdusek <i>et al.</i> , 2016)
1.6 billion	1 million cattle fatalities	Africa	Olwoch <i>et al.</i> (2008) and Donovan (2015)
700 million	4 billion litres of milk lost	Brazil	Kristjanson <i>et al.</i> (1999) and Machado <i>et al.</i> , (2010);
600 million	390 million kg of meat lost	Brazil	Kristjanson <i>et al.</i> (1999) and Machado <i>et al.</i> , (2010);
498.7 million	Total production losses	India	Manjunathachar <i>et al.</i> , (2014) and Playford <i>et al.</i> (2005)
184 million	Total production losses	Australia	Minjauw & Mcleod (2003) and Playford <i>et al.</i> (2005)
1 billion (*92 million)	Annual loss due to trypanosomiasis	Africa (*SA's contribution)	Mapholi <i>et al.</i> , (2014)

Jonsson (2006) indicated a reduction of 8.6g in the weaning weight of the infested Nguni cattle, while the Bonsmara experienced a lower 8.0 reduction and the Hereford suffered an 8.9g loss as could be expected (Mapholi *et al.*, 2014). In the mixed bushveld farm in Mpumalanga Province of South Africa, the weaning weights of calves from dams predominantly infested with *R. decoloratus*, were reduced by an average of 8g per female tick engorged with approximately 1mm of blood on the cow (Madder *et al.*, 2014). In addition, mid-lactation Holstein-Friesian cows experienced a reduction in milk yield

and body weight gain and it could be estimated that each engorged female tick resulted in reductions of 8.9ml in milk yield and 1.0g reduction in body weight gain.

The impact of tick infestations and the diseases they transmit is more severe in developing countries, where resources are more limited than in developed countries (De Castro, 1997). One such place is the Eastern Cape province in South Africa, where tick infestations pose major challenges for small scale farmers (Masika *et al.*, 1997). Approximately 65% of the beef cattle produced in this province are reared under communal grazing systems. This amounts to an estimated national contribution to the beef industry of about 3.1 million cattle (Nyangiwe *et al.*, 2013). With practically all the economically important tick species known to infest cattle distributed in the communal grazing areas of this province, nearly a quarter of South Africa's beef cattle population is threatened (Nyangiwe *et al.*, 2013). If left uncontrolled, the TTBDs in this one province may ultimately result in an astounding national decline in beef production.

A notable amount of funds within the beef production industries is designated to TTBD control. However, it has been proposed that by finding the balance between maintaining animal health and administering emergency treatment, through continuous monitoring of the animals' health statuses, the sustainability of beef enterprises may be enriched (Odendaal, 2015). This methodology can be adapted from the intensive livestock production industries, such as the poultry industry, which uses blood tests to determine the levels of resistance to internal and external stressors (Odendaal, 2015). The necessary adaptation in the beef industry would require many more gene expression studies to be conducted, which explore the barely understood mechanisms of tick resistance in cattle, to develop techniques by which animals can be accurately screened for economically important characteristics such as tick resistance.

Although there is currently adequate scientific knowledge to support progressive tick control strategies, policy-makers have continued to fail to understand the national perceptions of the need for tick control (Pegram *et al.*, 1993; Jongejan & Uilengberg, 2004). This has resulted in failure to establish cost-benefit estimates of tick control measures in beef production enterprises (Pegram *et al.*, 1993). This was based on previous conventions that indigenous beef breeds required the same degree of applied control measures as the imported breeds. As a result, numerous populations of resistant cattle continue to undergo regular dipping routines because of the minority susceptible imported breeds leading to a continued increase in the costs of production with increasing TTBDs threats. Therefore, TTBDs remain arguably the biggest impediments gradually crippling the beef production industry. The production losses associated with ticks underline the need to develop a more comprehensive understanding of the mechanisms of tick resistance in beef cattle.

2.3 Conventional Tick Control Methods

The most commonly used tick control methods in cattle herds include vaccine and acaricide application, generally referred to as chemical treatments. The relative cost of control, which is a factor of frequency and duration of treatment, is dependent on the ecoclimatic conditions as well as the breed of the cattle and the tick species posing the challenge (Pegram *et al.*, 1993; Jongejan & Uilenberg, 2004). Additional differences in the type and cost of tick control method used exist in commercial farming systems as opposed to communal farms (Mekonnen *et al.*, 2002; Marufu *et al.*, 2011a). While commercial enterprises rely on acaricide usage, low-input production systems rely on the use of traditional medicines (Hesterberg *et al.*, 2007). Strategic pasture management, rotational grazing and pasture burning are alternative strategies often employed in low-input systems in most African countries and Australia, as they are thought to reduce larval tick abundance in the grass (Morris, 2007; Manjunathachar *et al.*, 2014). Chief among these has been the use of cross breeding systems to infiltrate breeding pools with superior tick resistance genes. Unfortunately, none of these methods have been developed to optimise breed-specific tick resistance or the animal's natural resilience to ectoparasite challenges. As a consequence of the continued administration of chemical treatments, previously tick-resistant cattle have gradually lost both the ability to resist ticks and their enzootic stability to tick-borne diseases (TBDs) (Pegram *et al.*, 1993).

2.3.1 Chemical acaricide approach

Chemical acaricide usage, including but not limited to regular dipping and spraying, has formed the backbone of tick control for many years in the beef production industry (Jongejan & Uilenberg, 1994). The most commonly used acaricides are composed of organophosphates, amidines, synthetic pyrethroids, avermectins, and flouzuron (Righi *et al.*, 2013). Amitraz, ivermectin and fipronil were later introduced to circumvent the reduced efficacy of the aforementioned active ingredients, however, reports have emerged highlighting resistance to these chemicals in certain tick species (Wyk & Baron, 2016). These have progressively replaced formulations largely made up of the more toxic chlorinated hydrocarbons (De Castro, 1997).

The prolonged and indiscriminate usage of acaricides, without rotation, has resulted in *Rhipicephalus* (*Boophilus*) tick species producing an endless array of strains which exhibit widespread multi-acaricide resistance (Li *et al.*, 2004; Morris, 2007; Reck *et al.*, 2014; Vudriko *et al.*, 2016). Consequently, the treatment of several economically important cattle tick species with acaricides has become ineffective. Rotational acaricide application techniques are recommended, where no single treatment is used for a prolonged period of time. The correct application of a formulation of flouzuron 2.5% and flumenthrin 1% twice monthly yielded a significant decrease in both *R. microplus* and *R. decoloratus*

tick loads; hence limiting the number of larvae roaming in pastures seeking hosts (Fourie *et al.*, 2013). Where ticks have already established resistance against the frequently used organophosphate and chlorinated hydrocarbon acaricides, the most effective rotation often includes the use of carbamate acaricides (Donovan, 2015b).

These chemical treatment methods of tick infestations have become increasingly expensive and less effective (Wambura *et al.*, 1998). Additionally, an amplified degree of anxiety has been generated among consumers with regards to the livestock industry claiming to maintain a so-called “chemical-free” production environment when using acaricides and other chemical treatments. The use of acaricides and other chemical treatments have been shown to have significant environmental implications (Gasbarre *et al.*, 2001; Morris, 2007). The basis of consumer concerns stem from the possible chemical contamination of meat, milk and all other animal products along with the contamination of the environment with chemical residues (Marufu *et al.*, 2011a; Regitano & Prayaga, 2011; Ibelli *et al.*, 2012). Therefore, widespread negative implications associated with acaricide usage have warranted the call for alternatives tick control measures that are not only cost-effective, but also environmentally-friendly.

2.3.2 Vaccination programmes

The skin is the first line of defence and according to Kongsuwan *et al.* (2010) it may possible to deal with external stressors in beef cattle by manufacturing anti-tick vaccines which strengthen the activity of the protective proteins in the skin barrier. Various attempts have been made to develop vaccines composed of recombinant tick antigens as a cost-effective and uncomplicated alternative tick control method in beef cattle (Wambura *et al.*, 1998; de la Fuente *et al.*, 2007; de la Fuente, 2012). Vaccine resistance by ticks is thought to evolve at a much slower pace than resistance against acaricides (Willadsen, 1997). Therefore, vaccine administration is the most recommended approach, especially in calves, where exposure to TTBDs has been insufficient to establish immune stability (Frisch, 1999). Nonetheless, it is also recommended that the use of vaccines be coupled with partial acaricide treatment as a means of short term tick control (Mapholi *et al.*, 2014). This is due to the slow-acting element of vaccines which generally means that they may take longer to set in and elicit their effects on the ticks.

Anti-tick vaccines derived from tick antigens have been extensively investigated. The *R. microplus* is a well-documented acaricide-resistant tick species for which a cost effective and environmentally sound vaccine was developed in Australia in the 1990s using the tick antigen Bm86 (De Castro, 1997; de la Fuente *et al.*, 2007; Donovan, 2015b). The Bm86 vaccine was prepared from internal tissues extracted from the midgut of the *R. microplus* ticks, which act to induce anti-tick immunity in the host cattle to

which the treatment was administered (Imamura *et al.*, 2005). Cattle which received the Bm86 tick antigen vaccine endured lesser tick burdens as a result of reduced larval infestations. This was predominantly due to the reduced number of engorged ticks and their post-bloodmeal weight as well as an estimated 90% reduction in the reproductive capacity of the feeding female ticks (Imamura *et al.*, 2005). However, the effects on the tick mortality and vector capacity were insignificant (Willadsen, 1997, 2006; de la Fuente *et al.*, 2007). It was postulated that consecutive treatments on the same animal would result in a reduction in the number of larvae on the animal over successive generations, ultimately disrupting the tick's breeding cycle (Donovan, 2015b). Challenging the optimism towards the use of Bm86 as a viable vaccine antigen is its variable efficacy on different *R. microplus* strains in different geographic locations as well as its inability to grant universal cross-species protection (Antunes *et al.*, 2014).

Designed specifically to overcome the drawbacks of Bm86 is a new class of vaccine targets called Ferritins, among which the intracellular iron-transporter ferritin 2 (FER2) is highlighted (Hajdusek *et al.*, 2010; Parizi, *et al.*, 2012a). Recombinant FER2 induces infertility and drastic reduction in tick feeding and post-bloodmeal tick weight in various tick species (Hajdusek *et al.*, 2016). A multi-antigen cocktail containing glutathione S-transferase from *Haemaphysalis longicornis* (GST-HI) and vitellin-degrading cysteine endopeptidase (VTDCE) and boophilus yolk pro-cathepsin (BYC) from *R. microplus* was recently verified to provide partial protection against the *R. microplus* species. This resulted in significantly higher body weight gains in vaccinated cattle (Parizi, *et al.*, 2012b).

Proteomic studies, using RNA interference functional analyses, have identified recombinant tick proteins Subolesin (SUB), SILK and TROSPA as good candidate vaccine antigens (Merino *et al.*, 2013; Antunes *et al.*, 2014). Cattle that received the SUB-MSPIa antigen containing vaccines showed significant reduction in tick burdens and tick-borne diseases (Merino *et al.*, 2013). In addition, an investigation of the potential anti-tick immunity induction properties of three cDNAs, encoding immunodominant 29 and 34kDa salivary gland-associated proteins and midgut-derived serine protease inhibitor 1 and 2, produced significant results (Imamura *et al.*, 2005).

The major limitation with currently available vaccines lies in the inability of one vaccine to protect against multiple tick species, thus lacking the capacity to serve as a stand-alone solution for tick control, particularly in extensive pastoral systems (Parizi *et al.*, 2012a; Parizi *et al.*, 2012b). This is a result of the differences that exist in tick physiological processes as well as variations among the host populations, breeds and nutritional status of the hosts (Parizi *et al.*, 2012a). This drawback together with a combination of numerous commercial and technical glitches - including the vaccines' efficacy, manufacture, application and stability - has led to the limited use of vaccines in beef cattle enterprises

(Willadsen, 2006; Machado *et al.*, 2010). Therefore, there is a need for tick control methods that will be able to protect cattle against multiple tick species.

There is currently fragmented knowledge regarding the various tick antigens which can be incorporated into new cattle tick vaccines. Furthermore, little is known about the tick and host's biochemistry to comprehend the mechanisms which make some tick species more susceptible to vaccination than others. However, the field of genomics continues to reveal resources from which new candidate antigens can be extracted in order to protect against tick infestations (Jongejan *et al.*, 2007).

Chemical acaricide and vaccine usage are currently considered the best weapon against tick infestations. However, the concept of natural host resistance as a chemical-free alternative for tick control is also being explored. Nonetheless, previously conducted studies have only scraped the surface of envisaged potential. This has driven the global resurgence of studies which examine the genetic constitution of tick resistance in tick resistant cattle in order to either formulate efficacious vaccines or develop tests which recognize host resistance before chemical treatments are administered.

2.3.3 Crossbreeding

The use of tick-resistant *B. indicus* cattle breeds has been extensively practised worldwide over the years as a low-cost and highly-effective alternative for tick control. The South African indigenous Nguni cattle breed is a tick-resistant breed of growing interest with regards to tick control. This breed has evolved and become well-adapted to withstand and prevail under harsh pedoclimatic and socio-economic conditions offered by communal farming systems, thus providing a cheap, effective and sustainable alternative tick control approach for beef production systems (Marufu *et al.*, 2011b). As a result, a significant proportion of South African farmers have shifted towards altering their herd compositions as an alternative approach for tick control. This alternative approach offers a solution for cattle herds to withstand heavy tick burdens in variable climatic conditions. This is achieved through the introduction of Nguni and Bonsmara cattle breeds and lowering the population numbers of the more susceptible breeds in the herd. The resilience and hardy nature of the Nguni cattle breed has already been studied in depth (Muchenje *et al.*, 2008; Marufu *et al.*, 2011a; Marufu *et al.*, 2011b). The Nguni cattle can be reared on natural pasture with the use of conventional parasite control measures and dietary supplementation (Muchenje *et al.*, 2008). The Bonsmara on the other hand, exhibits outstanding productivity in variable climate yet displays less resilience when challenged by tick-borne diseases, thus making it less suitable for rearing in tick infested semi-arid rangelands (Marufu *et al.*, 2011a).

An advance in the selection and breeding of tick-resistant beef herds emerged from the Belmont National Breeding Station (Frisch, 1999). A new *B. taurus* line was bred following the reciprocal crossing of the Hereford and the Shorthorn to give the remnant breed which was identified as the Belmont Adaptaur (De Castro, 1997; Frisch, 1999; Manjunathachar *et al.*, 2014). This crossbred line was described by De Castro (1997) as possessing, from early in life, an exceptional and absolutely stable degree of resistance to *R. microplus* ticks even in the presence of additional environmental stressors. A linear reduction rate in tick burdens of 7 ticks/year was achieved, ultimately resulting in a mean reduction of 275 ticks/animal/year in 1983 to 40 ticks/animal/year in 1998 (Regitano & Prayaga, 2011). The causative factor is thought to be the presence of the anti-tick gene, which lasts the lifetime of the animal and induces total or near-total resistance as a homozygous pair (Frisch, 1999). The gene has, however, not yet been properly characterised. This is different to the extreme hypersensitivity reaction which is often produced by European breeds to temporarily protect against perceived pathogenic tick antigens. Nonetheless, the Adaptaur has yet to develop a notable amount of polygenic resistance since the breed has only been exposed to *R. microplus* tick challenge (Frisch, 1999).

While crossbreeding with Zebu breeds (e.g. Brahman, Sahiwal, Nguni and Afrikaner cattle), that exhibit superior resistance to ticks, has revolutionized tick control against *R. decoloratus* and *R. microplus*, accurate methods to screen individual animals with the desired genotype are yet to be developed (Scholtz & Theunissen, 2010). Gene expression studies equipped with the potential to isolate candidate tick resistance genes may thus be an invaluable tool in compiling an accurate criterion for selection. Attention needs to be focussed on educating farmers and their advisors about the benefits of improved tick control as a factor of preserved enzootic stability, which is achieved through enhanced host resistance and immune responses. There is also a need to access and accept the true benefits of using different cattle breeds as this may institute alternative tick control regimes with sound economic thresholds.

Although the mechanisms of tick resistance are not yet well understood, it is accepted that uncontrolled infestations in optimal climatic conditions, where *R. microplus* and *R. decoloratus* are implicated are generally more detrimental for European breeds than Zebu cattle (Jongejan & Uilengberg, 2004). The indigenous cattle breeds which have had restricted association with a particular tick species tend to also regress towards susceptibility. It is thus uneconomical to keep European breeds as well as tick naïve indigenous breeds of cattle unless proper and adequate tick control infrastructures are implemented. Therefore, the choice lies between the continued administration of expensive and intensive tick control methods and forging novel crossbreeding programs that exploit the unique genetic makeup of tick resistant *B. indicus* cattle to improve tick susceptible European cattle populations.

2.4 Variations in Tick Resistance

The complexity of host resistance to ticks is influenced by numerous environmental factors together with physiological factors including climate change, sex, age and nutritional status of the animal (Ashton *et al.*, 1968). Despite this, the degree of resistance against different tick species varies between cattle breeds as well as among individuals of the same breeds.

2.4.1 Between- and within-breed variations

The Zebu or *B. indicus* breeds of cattle are the most adaptable and acquire tick resistance relatively faster and more effectively than the European or *B. taurus* and some African indigenous or Sanga cattle breeds (Porto Neto *et al.*, 2011). The *B. indicus* breeds of cattle were domesticated in harsh environmental conditions, which are thought to have dictated the progressive development of superior natural resistance against external stressors and parasites (Machado *et al.*, 2010). Therefore, the *B. indicus* breeds can be significantly distinguished from the *B. taurus* breeds of cattle for their ability to rapidly adapt to tick infestations and mount adequate immune responses (Morris, 2007). Jonsson (2006) studied the levels of tick resistance exhibited by the different breeds of cattle, which had been infested with *R. microplus* ticks. The purebred Brahman cattle (*B. indicus* breed) exhibited 99% resistance, while the 50% *B. taurus* × 50% *B. indicus* crossbred cattle and the *B. taurus* cattle showed 95-97% and 90% resistance, respectively.

Variations were also detected in the levels of tick resistance exhibited by the different cattle breeds when infested with different tick species. A study conducted using Brahman, Boran and Tuli cattle breeds showed a greater level of resistance in the Brahman and Boran breeds than the Tuli breed when infested with *R. microplus* ticks (Frisch & O'Neill, 1998). However, the Brahman's level of resistance against *R. decoloratus* ticks was significantly lower than that of the Nguni cattle (Rechav & Kostrzewski, 1991), as was its ability to resist *Amblyomma hebraem* (*A. hebraem*) ticks when compared to the Zimbabwean Sanga cattle breed (Norval *et al.*, 1996).

The recorded mean tick counts per breed in a study using *R. decoloratus* were 37.4, 24.1 and 5.3 for the Hereford, Bonsmara and Nguni breeds, respectively (Jonsson, 2006). As a result, the Nguni cattle breed was expected to have suffered less production losses than the other two breeds. A certain degree of inconsistency exists in the accuracy of using tick counts as the sole measure of bovine tick resistance. This is because the female ticks' parasitic phase is prolonged in resistant animals, which may result in resistant animals retaining proportionally more adult female ticks than would be found on susceptible animals (Tatchell, 1987). Nonetheless, an exponential relationship was described between tick counts under heavy natural infestations and the proportion of Holstein-Friesian in Holstein-Friesian × Guzerat crosses, resulting in 25% reduction in milk yield in untreated animals

(Lemos *et al.*, 1985). As the proportion of Zebu increased the number of engorged ticks decreased (Regitano & Prayaga, 2011). In a separate study in Australia, the validity of using tick counts as a measure of resistance was supported (Madder *et al.*, 2014).

Wang *et al.* (2007) documented the within-breed variation, where some individuals within the same breed group exhibited superior resistance, while others regressed towards susceptibility in response to the same tick species. The fact that individuals within the same breed group exhibit different levels of resistance suggests that a complex assembly of mechanisms and multiple genes are responsible for host resistance to tick challenge (Regitano & Prayaga, 2011).

Testosterone may also be of fundamental importance in the reduced tick resistance as increased incidents of tick-borne disease transmission are observed in bulls when compared to both cows and heifers (Hughes & Randolph, 2001). This was attributed to testosterone suppressing the expression of genes of both the innate and acquired inflammatory responses that are responsible for discouraging tick attachment and feeding. Moreover, the immunosuppressive properties of gestational hormones may be equally responsible for pregnant cows being more susceptible to tick infestations than non-pregnant cows (Mapholi *et al.*, 2014). More studies are required determine the validity of these conjectures and the contribution they make to within-breed variations.

Natural tick resistance is a trait highly influenced by the genetic makeup of the animals. Therefore, it is a trait under natural selection as a component of natural breed-specific fitness. This suggests that there is some genetic component which is linked to the expression of resistance in individual animals, thus possibly making it a heritable trait. Heritability estimates vary within breeds as well, ranging from low to high, as a result of both additive genetic variations for resistance and evaluation methods, whether artificially- or naturally-induced tick challenge (Regitano & Prayaga, 2011). Budeli *et al.* (2009) found heritability estimates ranging from 0.05 to 0.17 for tick count in South African Bonsmara cattle. Moderate heritability values for resistance, ranging from between 0.34 ± 0.06 and 0.41 ± 0.08 have been reported in other cattle breeds (Morris, 2007; Mackinnon *et al.*, 1991). This means that the resistance trait can undergo selection. However, the inability to accurately measure the trait continues to hinder breeding attempts for this trait (Mackinnon *et al.*, 1991; Morris, 2007; Regitano & Prayaga, 2011).

Since variations in tick resistance exist even under uniform management, this may suggest that tick resistance is under genetic control. Mapholi *et al.* (2014) and Regitano *et al.* (2008) highlighted the polygenic and multiloci nature of the tick resistance trait. Therefore, understanding the mechanisms of genetic control in interaction with the varying environmental conditions may help develop effective tick control programs.

2.5 Genetic Control of Tick Resistance

The fact that tick resistance is under genetic control suggests that it is possible to develop tick control strategies based on the genetic architecture of the animals (Regitano *et al.*, 2008). Attempts have been made to progressively alter the genetic composition of beef herds through rapid gene introduction into different regions (Morris, 2007). This technique encompasses either breed substitutions or more often crossbreeding and introgression with tick-resistant *B. indicus* breeds, or the least disruptive approach of within-breed genetic selection (Morris, 2007). Several approaches have been used to understand the mechanisms of genetic control of tick resistance.

2.5.1 Gene expression studies

Bovine tick resistance studies aimed at exploring the genetic constituents for within- and between-breed variations date back up to four decades (Porto Neto *et al.*, 2011). In the early 1980s, the research focus deviated from the genetic basis of tick resistance to the immunological aspects (Porto Neto *et al.*, 2011). This was driven by the urgency to accelerate the process towards developing not only a fast-acting, but also a long lasting chemical-free alternative for tick control. Many of the recent studies which investigated the biological mechanisms of bovine tick resistance, as well as host-tick associations have been aided by the application of revolutionary molecular genetics technologies and bioinformatics (Porto Neto *et al.*, 2011). However, there is still a limited amount of literature which documents the progress that has been made in understanding the DNA, RNA and cytokine expression profiles of tick resistance in the bovine species.

According to Porto Neto *et al.* (2011), extensive research has been centred on the biological information contained in the skin as the primary source required when reviewing the biological mechanisms, gene expression profiles and key pathways of host resistance in cattle. With time, genomic enhancements as well as the availability of the bovine genome sequence have resulted in a new spectrum of possibilities emerging to allow for more broad-based studies to be established. This has made it possible for researchers to isolate specific genes, which either work independently or in a collaborative manner to facilitate bovine tick resistance (Kongsuwan *et al.*, 2008). One such genetic enhancement is the mapping of the bovine genome through the incorporation of single-nucleotide polymorphisms (SNPs) and copy number variations (CNVs), which has paved the way for determining “natural” host resistance (Parizi *et al.*, 2009). The release of the bovine genome sequencing project, together with the HapMap project, have led to the preliminary isolation of genes and gene markers using ultra high density SNP chips (Machado *et al.*, 2010).

Studies have been conducted for well over 40 years to investigate the biological variants and serum-protein concentrations. Unfortunately, for most of those years it was thought that serum amylase C

was the only biological marker which could be significantly associated with tick resistant biotypes (Porto Neto *et al.*, 2011). Earlier, Ashton *et al.* (1968) observed a significant reduction in tick susceptibility in cattle which tested positive for serum amylase C. Conversely, recent studies in molecular genetics have focussed on the re-evaluation of acute phase proteins haptoglobin, serum amyloid A, alpha-1 acid glycoprotein and transferrin, for their potential contribution in eliciting tick resistance or susceptibility. Among these, haptoglobin and transferrin were identified as potential biomarkers, which could be incorporated in methods of monitoring tick infestations in cattle (Porto Neto *et al.*, 2011). Identification of specific genes responsible for tick resistance in beef cattle may aid in developing more targeted tick control strategies.

2.5.2 Characterised candidate gene for tick resistance

In a gene-expression study on cattle skin that had been infested with *R. microplus* larvae, Wang *et al.* (2007) found 18 and 48 genes, which had been expressed at higher levels in cattle formerly characterised as high-resistance and low-resistance, respectively. They further identified a variety of differentially expressed candidate tick resistance genes, namely keratin-related genes, extracellular matrix genes and immunoglobulin-associated genes. In addition, Piper *et al.* (2008) conducted a follow up study investigating the differential expression of 44 genes. Contrary to the observations of Wang *et al.* (2007), Piper *et al.* (2008) reported no significant difference in the expression of genes which were associated with either keratins or collagens in the tick-resistant Brahman and the susceptible Holstein cattle studied. Differences were observed in the genes associated with several toll-like receptors (TLR5, TLR7, TLR9), chemokines together with their receptors (CCR1, CC12, CCL26), as well as cytokines (IL-1 β , IL-2R α , IL-2, IL-10, TNF- α , Traf-6, NFkBp50) (Piper *et al.*, 2008, 2009). These genes displayed elevated expression in the parasitized sites of the susceptible cattle, whereas no variability was observed between the parasitized and non-parasitized sites of the Brahman (Porto Neto *et al.*, 2011). In addition, significant differential expression profiles were reported for genes IL-2 and IL8, which were downregulated and expression levels reduced, respectively, in Nelore calves following the first tick infestation with *Rhipicephalus sanguineus* ticks (Regitano *et al.*, 2008). On the contrary, IL-4 and IL-10 were upregulated in *Rhipicephalus sanguineus* mice (Ferreira & Silva, 1999). However, no significant differences were reported in the mature cattle suggesting that the role of the gene reported by Ferreira & Silva (1999) and Regitano *et al.* (2008) in tick resistance may not be significant.

Candidate systems and gene markers to date include Bovine Leukocyte Antigen DQ (BoLA-DQ) lysozyme, cytokeratin or cytokines, interferon γ and tumour necrosis factor α (Morris, 2007). Of greater significance is the double amino acid residue motif marker, commonly known as glutamic acid

serine. This marker is located on the bovine major histocompatibility complex (MHC) of class II BoLA-DBR3 gene as well as on the PCR-RFLP alleles of BoLA-DBR3.2, DRB1 and DRBP1 (Martinez *et al.*, 2006; Mapholi *et al.*, 2014). The BoLA gene marker was found on five different DBR3 alleles in 60% of the cattle, which were suspected to be susceptible and induced 96% susceptibility to tick challenge (Morris, 2007). Evidently, in a study conducted on dairy cattle in Brazil, the BoLA marker DBR alleles 3.2, 18, 20 and 27 were significantly associated with bovine resistance to *R. microplus* ticks (Martinez *et al.*, 2006). Furthermore, the BoLA class I alleles w6.1 and w7, located on BTA23q2.1, contributed to the generation of immune responses, which defend the host animal against tick fixation. There appears to be an inconsistency in the studies conducted to examine the association between BoLA alleles and increased tick resistance in cattle. A similar trend can be observed in the evidence which illustrates the role of any of the genes in the MHC in association with tick burdens (Porto Neto *et al.*, 2011). This further stresses that it is imperative to conduct further investigation to support the currently available data before proceeding to develop alternative methods of tick control in cattle.

In a study conducted by Kongsuwan *et al.* (2008), a total of 138 differentially expressed genes and three fundamental pathways were expressed in tick-resistant Brahman cattle. The pathways comprised of the development of cell-mediated immune responses, fluctuating intracellular Ca^{2+} levels and the structural integrity of the dermis. In addition, a number of host defence genes, acute phase protein components, transcription factors and lipid metabolism genes were identified (Kongsuwan *et al.*, 2008). Keratinocyte proliferation was found to be retarded by both enhanced calcium influx and increased transcriptional activation of the calcium signalling gene, while keratinocyte differentiation was intensified to facilitate activation of transglutaminase enzymes (Menon, 2002; Kongsuwan *et al.*, 2010). These enzymes work to irresistibly crosslink the keratin fibres and protein molecules, thus creating a robust and impenetrable sac around the skin lesion created by the feeding tick (Menon, 2002; Kongsuwan *et al.*, 2010).

There is sufficient evidence suggesting that tick burdens are dependent on the annual climate cycles (Willadsen, 2006). Tick infestations are deemed 56% worse in summer than in winter (Mbatia *et al.*, 2002). Thus, expression of the various genetic components was thought to be related to the seasonal variation in coat length and thickness, nutritional status, circadian rhythm or heat stress (Regitano *et al.*, 2008). For this reason, several genes and quantitative trait loci (QTLs) have been under investigation. It was established that the genes producing seasonal variation in tick resistance in cattle were located on the bovine chromosome 23 (BTA 23) (Mapholi *et al.*, 2014). The QTLs could be traced back to the genomic region that contained the BoLA genes, which were confirmed to play a crucial role in inducing tick resistance (Machado *et al.*, 2010; Mapholi *et al.*, 2014). The results of a chromosome-wide significance test, $P_g < 0.05$, indicated that BTA 23 was of greater importance as it

was the only chromosome that exhibited significant QTLs in both the rainy and dry season (Machado *et al.*, 2010). The QTLs on BTA 23 explained 5.9% of the phenotypic variation in the dry season and 5.7% of the variation in the wet season. Figure 2.1 shows the five additional BTAs, which were profiled by Machado *et al.* (2010). BTA 2 influenced variation in the dry season and BTA 10 explained 4% of the total phenotypic variation observed in the dry season. On the other hand, BTA 5, BTA 11 and BTA 27 accounted for 5.50%, 5.26% and 3.31% of the phenotypic variations in the rainy season, respectively. In addition, Porto Neto *et al.* (2010) identified a QTL positioned close to the ITGA11 gene on BTA10 affecting tick burdens. However, further studies are required to confirm the significance of the association. Genomic regions BTA 2, 13 and 19 contained 20 candidate genes, which required further investigation to determine their roles in influencing tick resistance (Porto Neto *et al.*, 2011).

Piper *et al.* (2009) found some suggestive results regarding the leukocyte-cytokine spectrum in tick-infested Brahman cattle. However, like many other studies, a great deal of research still needs to be done to validate the assumptions and theories. This creates a platform to identify markers and genes related to cytokine expression profiles at the tick bite sites in order to isolate genes that underlie tick resistance in cattle. This can be done efficiently through mRNA detection studies. The mRNA levels, however, do not necessarily reflect the abundance of the protein they encode and this could influence the outcomes of host-tick interactions (Jensen *et al.*, 2007).

Ongoing research is working towards establishing the characteristics of the biological components for the multigenic control system(s) of tick resistance or susceptibility in beef cattle. Such studies have yet to be conducted in South Africa, particularly to investigate the expression profiles produced by the different host-tick associations, which may be responsible for the differential expressions of various tick-resistance-related genes.

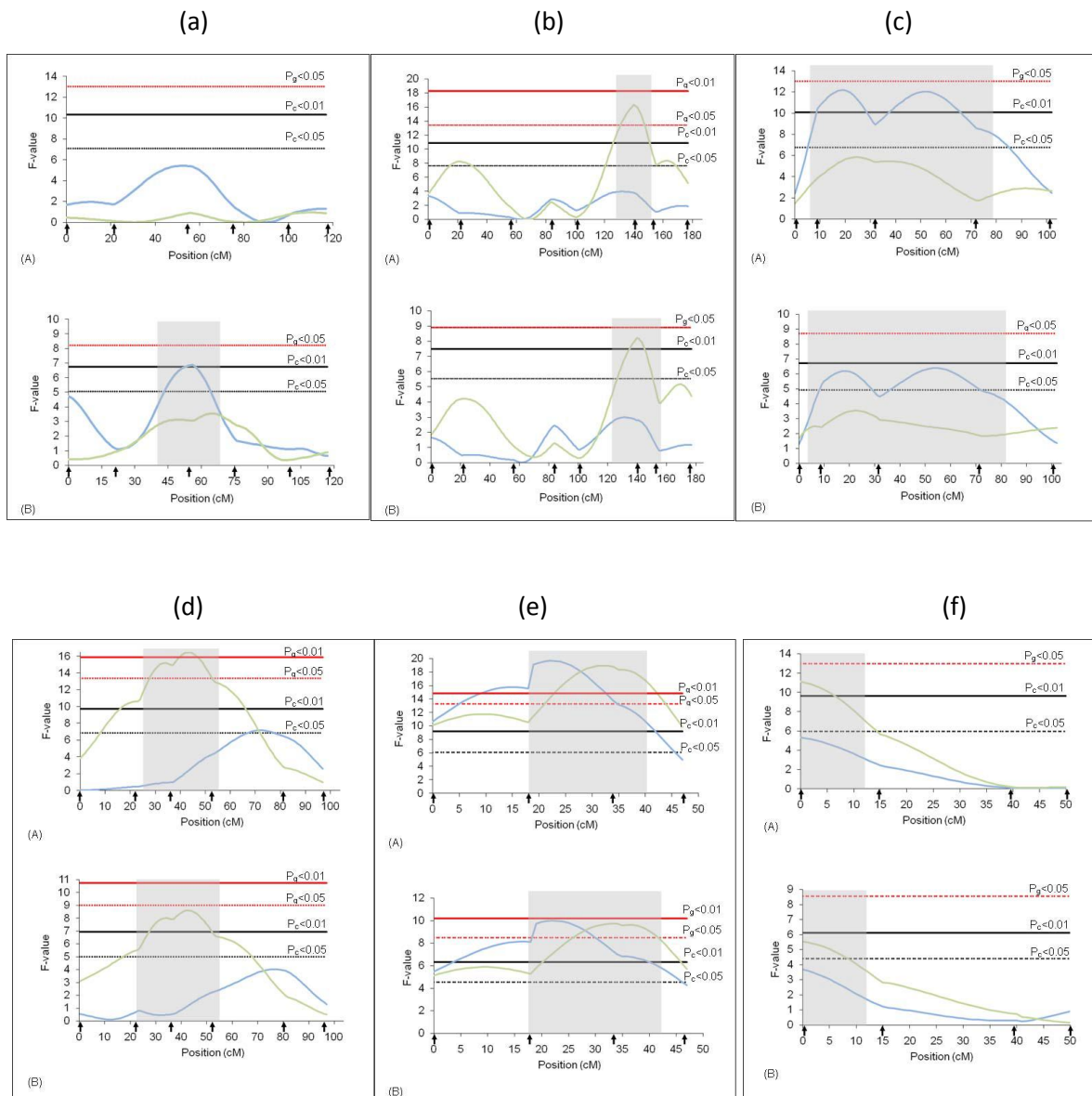


Figure 2.1: The F-statistic profiles for tick resistance generated from additive + dominant models. The x-axis indicates the relative position in the linkage map. Arrows indicate marker positions. Green line indicates rainy season and blue line indicates dry season. Grey bar indicates QTL confidence interval. P_g = genome wide significance threshold and P_c = chromosome wide significance threshold. (a) Analyses results of BTA 2, (b) analyses results of BTA 5, (c) analyses results of BTA 10, (d) analyses results of BTA 11, (e) analyses results of BTA 23 and (f) analyses results of BTA 27 (Machado et al., 2010)

2.6 Physiological Mechanisms of Tick Control

The primary site of immunological responses in the host body to tick bites is the skin. This reaction is mediated by polymorphonuclear cells, lymphocytes, mast cells, plasma cells and histamine secretion, which then induces hypersensitivity at the site of invasion, as well as in the arteriovenous anastomoses in the skin. This ultimately induces a grooming reflex by the host (Wambura *et al.*, 1998; Kongsuwan *et al.*, 2010). The rate at which the local skin generates an immune response to tick bites has been identified as a significant element for tick resistance, with a fast response time being linked to a higher degree of tick resistance (Mattioli *et al.*, 1993). The skin response is initiated by signalling molecules found in the subcutaneous layer of the skin and these include c-myc, Notch and CCAAT-enhancer binding protein (C-ERB) and p63, as represented in Figure 2.2 (Kongsuwan *et al.*, 2010).

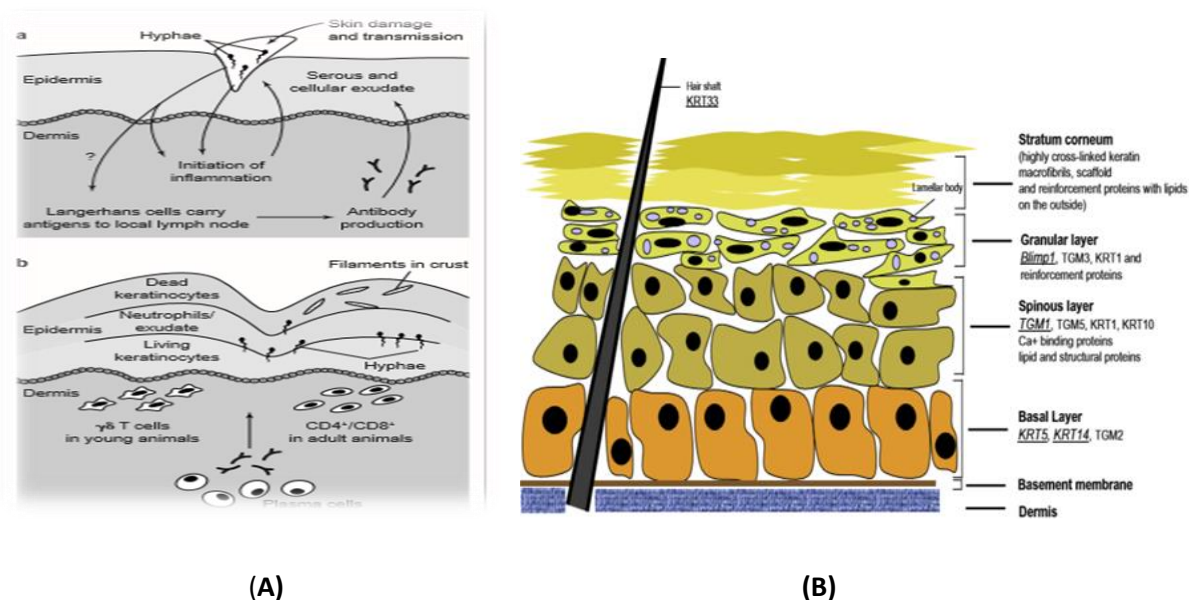


Figure 2.2. (A) The sequence of events following infection of a host animal with *Dermatophilus congolensis*. **(a)**. Hyphae grow from cocci, spreading into the epidermis and releasing antigens that might be acquired by Langerhans cells and presented to T cells in lymph nodes draining the infection site. Crusts are evident by Day 7 after infection **(b)**. *Dermatophilus congolensis* proliferates in the epidermis to produce filaments. By 14 days post-infection, T cells are present in the upper dermis and plasma cells in the sub-dermis. After a primary infection, lesion resolution commences around Day 14 and is completed by Day 28. In tick-infested animals, lesion resolution fails T cells and plasma cells accumulate in the dermis. (Ambrose *et al.*, 1999). **(B)** Structure of the skin epidermis showing the different layers and locations where keratinocytes differentiate and component proteins are synthesised as described by Candi *et al.* (2005) and Magnusdottir *et al.* (2007) (Kongsuwan *et al.*, 2010).

It is thought that the tick-resistant N'Dama cattle breed is able to reject a greater number of ticks as a result of their rapid dermal protective immunity response time (Mattioli *et al.*, 1993). Furthermore, due to the coevolution of the N'Dama breed and several cattle tick species, the prolonged contact between the two parties may have influenced the development of more effective tick resistance mechanisms in the N'Dama breed when compared to the Zebu and British breeds (Mattioli *et al.*, 1993). This is similar to the Brahman and *R. microplus* co-evolutionary status which is believed to be responsible for the superior resistance to *R. microplus* observed in the Brahman. It has also been proposed that the lower tick infestations experienced by Zebu and Sanga (*B. indicus*) breeds may be a result of their acquired tick avoidance behaviour, heightened skin sensitivity and increased grooming activity and, probably to a lesser extent, the breeds' body weights (Meltzer, 1996; Marufu *et al.*, 2011b). Smaller bodied animals experienced lighter tick burdens in comparison to heavier bodied animals and this was associated to the larger surface area allowing for increased tick attachment opportunities in the latter animal types (Mapholi *et al.*, 2014).

The variability in tick load and resistance thereof among different cattle breeds is partly attributable to the differences in several morphological traits, namely coat colour, hair type, thickness and length as well as skin thickness (Regitano & Prayaga, 2011). These include differences in hair length, skin thickness and coat characteristics. Consequently, shorter hair, smoother, lighter coloured coats and thinner skins have been associated with fewer tick burdens in comparison to longer hairs, thicker and darker coloured coats and thicker skin. Breeds with shorter hairs create an unfavourable microclimate for tick proliferation, made up of enhanced conduction and convection heat loss, and expose ticks to harmful natural elements and predation by birds, while also facilitating more effective self-grooming activities (Mattioli *et al.*, 1993; Machado *et al.*, 2010; Marufu *et al.*, 2011b; Ibelli *et al.*, 2012). The self-grooming is essentially an aftereffect of eosinophils acting on the mast cells to stimulate the release of the histamine they enclose inside their cytoplasmic granules and its translocation to the site of the tick bites (Veríssimo *et al.*, 2008).

In a microarray analysis by Piper *et al.* (2008), it was found that Brahman cattle, which naturally possess superior tick resistance, expressed increased amounts of C-ERB as compared to the lesser resistant Holstein-Friesian cattle (Kongsuwan *et al.*, 2010). Furthermore, the evidence presented by Kongsuwan *et al.* (2010) suggested that the abundant presence of the hair keratin KRT33B and structural differences in the sweat glands in highly resistant cattle breeds may be responsible for the relative humidity in these cattle, which hampers tick larvae survival. The Zebu cattle have thick moveable hides, covered with short straight and nonmodulated hair coupled with the well-developed panniculus muscles. These have an efficient erector pili muscle, sensitive pilomotor nervous system

and high density sweat glands that contribute to hair erection and the secretion of sebum in the hair to repel ticks (Kiss *et al.*, 2012).

The relationship between tick infestation and skin or coat characteristics has not yet been fully established. It is, however, understood that the defence barrier set in place by the skin's epithelium makes it difficult for ticks to establish stabilised feeding in highly resistant hosts subsequently failing to survive after a day of no feeding (Roberts, 1971; Kongsuwan *et al.* 2010). The Nguni breed was described to possess favourable coat characteristics, namely a smoother coat and shorter hairs, highly responsive skin immunity and possibly an abundance of superior tick resistance genes (Marufu *et al.*, 2011b). Similar coat characteristics which assist in discouraging tick attachment were observed in Tswana, Simmentaler, and Brahman cattle breeds (Marufu *et al.*, 2011b). However, the observation that Nguni cattle continued to display superior tick resistance whilst exhibiting unfavourable coat scores suggested that Nguni cattle had more mechanisms at work than just coat characteristics (Marufu *et al.*, 2011a). It is, therefore, essential to study the genetic architecture of the Nguni and Brahman cattle breeds in order to gain a comprehensive understanding of the tick resistance mechanism at work.

2.7 Host-tick Associations

As represented in Figures 2.3 and 2.4, *R. microplus* and *R. decoloratus* tick species are two of the more economically important tick species devastating the African beef production industries (Madder *et al.*, 2014). The *R. microplus* is originally from South East Asia but has dispersed to the greater parts of Australia, East and Southern Africa, as well as South and Central America (Jongejan & Uilengberg, 2004). The *R. microplus* has been mapped in coexistence with the *R. decoloratus* species along the eastern coastal belt of Africa and in the summer rainfall northern regions of South Africa (Mekonnen *et al.*, 2002; Madder *et al.*, 2014), to which the *R. decoloratus* has been confined (Jongejan & Uilengberg, 2004; Horak *et al.*, 2009). Although these species exhibit a preference of feeding on a wide variety of wild ruminants, their impact has been the greatest in their cattle feeding endeavours. In South Africa, both species populate scattered regions of the Eastern and Western Cape Provinces together with KwaZulu-Natal and in the interior areas of the Mpumalanga and Limpopo Provinces (Madder *et al.*, 2014). The *R. decoloratus* can also be found throughout the wetter areas of the Gauteng and North West Provinces as well as the eastern regions of the Free State (Madder *et al.*, 2014).



Figure 2.3: *R. microplus* distribution pattern in Africa



Figure 2.4: *R. decoloratus* distribution pattern in Africa

Zebu (e.g. Sahiwal) and Sanga (*B. taurus* × *Bos indicus*) cattle breeds, indigenous to India and Africa, respectively, are generally more resistant to Ixodid ticks following their initial exposure, while European breeds often remain susceptible even after consecutive exposure (Manjunathachar *et al.*, 2014). Factors that induce variations in tick distribution and tick burdens include season, geographic location, vegetation type, breed and age of the animal (Mtshali *et al.*, 2004). Therefore, the length of the association between a breed and tick species under the same conditions may influence the level of resistance displayed by the host (Frisch, 1999). This is dictated by the genetic drift during the establishment of the breed; a breed that is selected from one environment may not be able to exhibit a similar level of resistance in another environment. As such, host-tick associations can be classified as either ancient or modern.

The nature of ticks as obligate hematophagous parasites means that they need to establish and maintain intimate associations with their hosts for a protracted period of time in order to complete their life cycle (Jongejan *et al.*, 2007). The abundance of genomic information has facilitated studies aiming to decipher the barely understood complexity of host-tick interaction (Jongejan *et al.*, 2007). These include the use of host microarrays in order to understand the complexity of the interactions. However, the diversity of tick species, cattle breeds and the mechanisms that facilitate these interactions has resulted in highly complex biological systems to study.

Over the years ticks saliva has evolved to produce a cocktail of molecules with immunomodulatory functions as well as the ability to destroy host tissue integrity and block host haemostatic cascades (Regitano & Prayaga, 2011). Consequently, cattle breeds which simultaneously evolved in the presence of a particular species may have had to equally evolve mechanisms which generate vigorous immune responses against the tick challenge thus rendering them more resistant to that particular

tick species. Such ancient host-tick associations are observed between the Asian indigenous Brahman cattle and the *R. microplus* species as well as between the South African indigenous Nguni cattle and the *R. decoloratus* tick species.

Tick species distribution is immensely influenced by host distribution, changes in regional tick control measures, climatic change specifically changes in seasonal rainfall and the ability of the species to evolve to produce acaricide resistant strains (Tønnesen *et al.*, 2004). On the other hand, tick species introduced into areas where they spread rapidly, successfully compete with indigenous tick species to form modern host-tick associations with the indigenous cattle breeds of the region. Also, in an attempt to maximise indigenous livestock productivity and boost socio-economic development in large beef enterprises, exotic germplasm is often introduced into indigenous cattle populations (Manjunathachar *et al.*, 2014). These exotic breeds, however, lack the appropriate tick resistance genetic architecture that their indigenous counterparts possess, which may be as a result of long periods of evolution in the presence of a particular tick species. Commonly known to be widely distributed throughout temperate regions, the European (*B. taurus*) breeds introduced to South Africa establish modern host-tick association with the indigenous *R. decoloratus* ticks, which are predominantly widespread in the rangeland of the Eastern Cape and Limpopo Provinces (Rechav, 1982; Horak *et al.*, 2009; Nyangiwe *et al.*, 2011, 2013; Porto Neto *et al.*, 2011). The *R. microplus* and *R. decoloratus* tick species thrive in the warm and wet climates of the tropics and subtropics, while the in temperate climate provide tick-free conditions for production with European breeds. Thus, exotic breeds display a diminished level of resistance to these tick species. Similar modern host-tick associations may be established between the exotic breeds and the *R. microplus* tick species, which prevails in tropical and subtropical regions rather than the more temperate regions (De Castro, 1997).

European breeds may exhibit increased susceptibility to tick infestation; however, their performance in other production traits is often superior to that of Zebu and Sanga breeds. This entails that the efficiency and productivity of beef production enterprises might not be improved by selecting predominantly for the tick resistance trait. Therefore, it is imperative that host-tick associations, which may influence the level of tick resistance, be studied in depth to determine if it is responsible for the variation in tick resistance. Although several studies compared tick loads and performance in different host-tick associations (Norval *et al.*, 1996b; Muchenje *et al.*, 2008b), the trend in resistance levels in these host-tick associations still remains unexplained (Marufu *et al.*, 2011b). Therefore, by thoroughly studying these associations, customised and more focussed interventions for tick control can be developed and implemented.

2.8 Summary

TTBDs are currently the biggest cause of the elevated costs of production in the beef production industry, causing significant losses in milk yield, carcass composition, hide market price reduction and many more production losses. However, a substantial proportion of the financial implications are incurred through tick control measures. While chemical acaricides and vaccines have been used for tick control in the past, their inability to effectively protect cattle against multiple tick species and the gradual emergence of acaricide-resistant tick strains, has driven the global resurgence towards developing chemical-free alternative tick interventions capable of offering the cattle protection against multiple tick species. This is deemed possible by exploiting the host's natural resistance since tick resistance is under genetic control. Numerous studies have been conducted in an attempt to understand the genetic mechanisms, which confer superior resistance in the *B. indicus* cattle as opposed to their British counterparts. However, more studies are still required to uncover the relationship between tick resistance in South African beef cattle and the differential expression of tick-resistance-related genes, where the economically important *R. microplus* and *R. decoloratus* tick species are concerned. While crossbreeding programs have been developed to introduce superior tick resistance genes into the gene pool of the susceptible British breeds, the accuracy of selection is still far beneath where it should be to successfully produce tick-resistant crossbred cattle. A comprehensive understanding of tick resistance as a product of the different host-tick associations may help in explaining whether tick resistance is enhanced by the long term association between a particular tick species and a specific breed of cattle. Candidate genes and pathways associated with tick resistance have been identified, nonetheless more gene expression studies which would aid in developing a more comprehensive understanding of the mechanisms of tick resistance in beef cattle are required.

2.9 References

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CHAPTER 3

COMPARISON OF TICK COUNTS IN THE ANCIENT (BRAHMAN-*R. MICROPLUS* AND
NGUNI-*R. DECOLORATUS*) AND MODERN (ANGUS-*R. MICROPLUS*, ANGUS-*R.*
DECOLORATUS, BRAHMAN-*R. DECOLORATUS* AND NGUNI-*R. MICROPLUS*) HOST-TICK
ASSOCIATIONS**Abstract**

The possibility of minimizing the deleterious effects of tick infestations is presented through natural host resistance to ticks. The strength of natural host resistance, thought to be a factor of the length of the association between the breed and the biting tick, lies in the animal's ability to increase premature tick mortality in turn reducing the number of engorging adult females. British breeds, originating from tick-free regions, form modern host-tick associations with most tick species. Since there are currently no known genetic markers for tick resistance in cattle, breeding values for tick counts are used to determine the resistance or susceptibility of different breeds for use in selection programmes. In the current study, tick count data obtained 18-days post-artificial infestation with *R. microplus* and *R. decoloratus* unfed larvae, were used to compare the levels of resistance of the Angus, Brahman and Nguni cattle. Approximately 100 unfed tick larvae were placed on the shaved portion on the animal's back and enclosed within a calico back attached and sealed to the shaved area. The Brahman carried significantly fewer ticks than both Nguni and Angus cattle, while no differences in tick burden were detected between the Nguni and the Angus cattle. Significant differences were also observed between the two tick species, with the *R. decoloratus* displaying a reduced ability to successfully attach and feed till day-18 than the *R. microplus*. Significant breed by tick species interaction was also observed, however, none that would suggest the role a degree of co-evolution in the level of host resistance to ticks. Therefore, in areas where *R. microplus* and *R. decoloratus* are prevalent, tick control should be centred on the Brahman breed. The increased tick burdens in all three breeds following *R. microplus* infestations indicates that this tick species may be economically more important than the indigenous *R. decoloratus* species. Thus, more focus should be aimed at controlling this species to a greater extent.

Keywords

Resistance, artificial infestation, tick burden

3.1 Introduction

Rhipicephalus microplus and *R. decoloratus* are two of the most economically important tick species prevailing in South African beef cattle populations (Jongejan & Uilengberg, 2004; Canales *et al.*, 2008; Nyangiwe *et al.*, 2013). This is because they are responsible for multiple economic losses. The *R. decoloratus* is indigenous to South Africa and it was postulated that the *R. microplus* was introduced to the country in 1896 through imported cattle from Madagascar (Tønnesen *et al.*, 2004). The fact that the tropical and subtropical climatic conditions of South Africa are especially conducive for optimal tick proliferation, underlines the need for effective tick control measures. The use of acaricides and vaccines has not been successful in completely eradicating ticks (Willadsen, 1997). On the other hand, the host's resistance to ticks may minimize the deleterious effects of ticks. The power of natural host resistance lies in the animal's ability to increase premature tick mortality, thereby reducing the number of engorging adult females (Seifert, 1971; Jonsson, 2006). Thus, exploiting the host's resistance to ticks may be an alternative that may be used to complement existing tick control measures.

Resistance to these species is either acquired or innate, or even both. One of the approaches to exploiting host's resistance is by crossing European breeds of cattle (*Bos taurus*), such as, the Angus with Zebu or *B. indicus* breeds, particularly the Brahman and Nguni (Scholtz & Theunissen, 2010). This concept has been advocated for decades to improve tick resistance in beef breeds in tropical and subtropical regions. Measures to enhance tick resistance in the Angus breed extend far beyond selective breeding practices. Early attempts of introgression of tick resistance traits were crippled by a combination of depressed heritability of the resistance trait and unfavourable conditions counteracting natural host resistance (Seifert, 1971). More recent attempts to operate selection-based breeding systems, solely based on tick count data, have proved to be promising but the low correlation of tick counts with productive and reproductive traits poses an unsurpassable hurdle (Shyma *et al.*, 2013).

Resistance to *R. microplus* and *R. decoloratus* varies between breeds (Riek, 1962; Rechav & Kostrzewski, 1991; Mapholi, 2015). Although not economically feasible in extensive beef cattle enterprises, tick resistance is ranked in terms of tick counts (De Castro & Newson, 1993; Wambura *et al.*, 1998; Mattioli *et al.*, 2000; Marufu, *et al.*, 2011a). Resistant cattle carry significantly fewer tick loads than susceptible individuals. There are currently no available genetic markers for tick resistance therefore, breeding values for tick counts are used instead to determine genetic resistance or susceptible of the different breeds to conduct selection programmes (Biegelmeier *et al.*, 2015). In a study that was conducted at the University of Fort Hare comparing tick loads in Nguni, Bonsmara and

Angus cattle, the Nguni cattle carried the lowest tick burdens, thus exhibiting increased resistance, than both the Angus and Bonsmara cattle which had significantly higher tick burdens (Muchenje *et al.*, 2008b). The Brahman cattle have been shown to exhibit higher resistance to ticks of various species under South African field conditions, thus harbouring significantly lower adult tick loads, when compared to the Herford and Simmentaler cattle (Rechav, 1982; Rechav *et al.*, 1990).

The potency of natural host resistance is thought to be a factor of the length of association between the breed and the feeding tick (Rechav & Kostrzewski, 1991). Thus, resistant breeds might have experienced a long period of evolution in the presence of the tick species they are resistant to and developed resistance to the tick species (Frisch, 1999; Marufu *et al.*, 2011). Therefore, host-tick associations, which may be responsible for differences in tick resistance, may be classified as ancient or modern. The ancient host-tick associations comprise of Brahman-*R. microplus* and Nguni-*R. decoloratus* association. On the other hand, the Angus-*R. microplus*, Angus-*R. decoloratus*, Brahman-*R. decoloratus* and Nguni-*R. microplus* host-tick associations constitute the modern host-tick associations. To fully understand the basis of differences in tick resistance among breeds, tick counts need to be studied in the different host-tick associations.

The objective of the current study was, therefore, to rank tick resistance in terms of tick counts per animal in ancient and modern host-tick associations in animals that were artificially infested with *R. microplus* and *R. decoloratus* unfed larvae.

3.2 Materials and Methods

3.2.1 Study site

The trial was conducted at the Agricultural Research Council Animal Production Institute (ARC-API) in Irene, which is located 25° 53' 59.6" S 28° 12' 51.6" E. The area receives summer rainfall that amounts to approximately 573 mm annually, with highest rainfall (140mm) in January and the lowest (10mm) in May till August (Holiday Weather, 2016). The average midday maximum temperatures range from 20°C in June and 29°C in January (Online World Weather, 2012; Holiday Weather, 2016). Maximum night temperatures, on the other hand range between 5°C in July and 18°C in January (Online World Weather, 2012; Holiday Weather, 2016). A total of 36 large stock feeding pens were made available to house the experimental animals individually for the duration of the trial. The pens consisted of concrete floors which were cleaned daily. Each pen was equipped with an animal-operated tap which supplied fresh, clean water to each animal ad lib. The facility provided a crush pen that served as a constraint for the animals during the processes of calico bag attachment, repair and removal, artificial infestation, tick counting and dipping.

3.2.2 Tick species

Two tick species from the genus *Rhipicephalus*, subgenus *Boophilus*, were obtained from Clin Vet International laboratory for use in the study. These were the *Rhipicephalus microplus* (*R. microplus*) and the *Rhipicephalus decoloratus* (*R. decoloratus*) unfed larvae harvested from aseptic colonies. This ensured that no disease causing pathogens were introduced into the animals' systems during the course of the trial. The tick larvae were starved to ensure instant attachment and commencement of feeding upon infestation.

3.2.3 Experimental cattle

A total of 36 cattle were sourced from a selection of extensively managed farms. The Nguni and Brahman cattle came from Mpumalanga while Angus came from the Free State Province of South Africa, both of which are areas where the *R. microplus* and *R. decoloratus* tick species can be found. As a result, they would have been exposed to *R. microplus* and *R. decoloratus* tick challenge prior to the commencement of the study. Consequently, the animals were expected to have built a stable state of immunity towards these infectious agents. The animals consisted of 12 Nguni bulls, 12 Brahman bulls, as well as a mixture of six Angus heifers and six bulls, all aged between 12 and 15 months with similar body conditions and body weights. The cattle were all treated with amitraz upon arrival at the ARC feedlot. The cattle were housed in individual pens for the duration of the trial and allowed *ad libitum* access to a standard commercial feedlot diet, which was mixed on site as well as *ad libitum* supply of fresh, clean water. The cattle were all fitted with ear tags displaying their identification number to facilitate accurate tissue sample labelling.

All animals used in the study underwent procedures approved by the Stellenbosch University Research Ethics Committee: Animal Care and Use and were in compliance with internationally accepted standard for animal welfare and ethics (Austin *et al.*, 2005). Furthermore, the South African Department of Agriculture, Forestry and Fisheries (DAFF) granted Section 20 ethical approval for the use of *R. microplus* and *R. decoloratus* on the Nguni, Brahman and Angus cattle.

3.2.4 Artificial infestation

Artificial infestation was chosen as the mode of tick challenge because it simulates field conditions while minimizing factors, such as predation, temperature and humidity which significantly affect the survival rates of free-living ticks (Regitano & Prayaga, 2011). Therefore, all larval ticks were given an equal opportunity to attach and feed on the exposed skin on the animals' backs. The tick count data reported was thus a product of the animal's natural resistance.

The unfed tick larvae (UFL) were counted under a light microscope and organised into groups of approximately 100 before being placed into tick-safe vials. Each breed group of the experimental animals was split in half, with six animals per breed undergoing artificial infestation with the *R. microplus* species, while the remaining six were infested with the *R. decoloratus* species. Subsequently, the Angus groups were further divided in terms of sex, with 3 of each sex undergoing infestation with *R. microplus* and the other three being infested with *R. decoloratus* ticks.

The cattle were each restrained in the crush pen, where the animals' dorsal-medial area of the skin, onto which the calico bag would be attached and the unfed larvae inserted for feeding, was shaved using heavy duty electrical clippers (Legend[®], Lister, South Africa). The area was cleaned using a cloth soaked in lukewarm water to remove any excess dirt, wax or residues of the short acting acaricide. The calico bags were secured to the shaved areas on the animals' backs using Alcolin Contact Adhesive (Alcolin[®], South Africa) and allowed a few hours to dry before infestation. Once the bags were completely dry, one tick-containing vial was placed inside the bag of each animal, opened and left inside the bag to liberate the larvae and allow them to start feeding. The open sock end of the bag was then folded and twisted shut then secured with one rubber castration ring by means of a rubber ring applicator.

After 24 hours, the bags were opened to remove the vials and the tick bite sites were visually inspected. The bags were inspected twice daily, for the 18-day period required for tick maturation to adult size, to ensure proper calico bag attachment to the animals, thus ensuring that neither the ticks nor the tick bite sites were exposed to the external environment.

3.2.5 Tick counting

Tick count data were collected from all animals on day 18 of the trial before the short-acting water soluble acaricide (amitraz) was applied. The animals were restrained in a crush pen to conduct post-infestation tick counts. The rubber ring was removed from the calico bag to expose the area on the animal's back, where the artificial tick challenge had been initiated. Tick counting was carried out by two trained enumerators, by thorough examination of the body surface on the animal's back where the calico bag was attached. Only the live, feeding and visibly engorged adult ticks, which were still attached, were counted. Once the bags were removed and packed for incineration, the animals were then all treated with amitraz to prevent unwanted dispersal of the ticks throughout the feedlot.

3.2.6 Statistical analyses

The tick count data were analysed using SAS Enterprise Guide 7.1 (SAS, 2016). Summary statistics were done to generate quantile plots of the tick count data. A distribution analysis was conducted to test the assumption of normality using the Shapiro-Wilk. Subsequently, the assumption of

homoscedasticity was tested using the Levene's test for equal variances. The hypothesis of independence was not rejected and regarded as valid since the events of tick counting were conducted in such a way that no one tick counting event affected another and the artificial infestations were conducted following a completely randomised design. Given that all animals were equally artificially infested with approximately 100 ticks; the data were analysed using a General Linear Model through which the proportions of the tick burdens were calculated. These analyses made use of maximum likelihood instead of a normal F-test. An analysis of variance (ANOVA) using linear models in XLSTAT 2016 was also conducted to investigate the percentages representing the interaction between breed and tick species. The SAS output was then compared to the XLSTAT output to examine similarities in the results produced to determine which approach was most suitable to describe the data. Furthermore, a summary statistics output was generated containing the mean and standard error values for each of the main effects.

3.3 Results

The inclusion of the full tick count data set produced results with a severe outlier. This leverage point negatively influenced the distribution of the data. As a result, tick count data from animal NM4, with a value = 0, was omitted from all statistical analyses. One Brahman animal (ID: BM6) escaped from the experimental setting and had to be excluded from the trial. Therefore, a total of 34 observations were used in total; six Angus – *R. microplus*, six Angus – *R. decoloratus*, five Nguni – *R. microplus*, six Nguni – *R. decoloratus*, five Brahman – *R. microplus* and six Brahman – *R. decoloratus*. However, following the exclusion of this point, the normality assumption was still rejected ($P < 0.05$) due to the fact that the data produced contained binary variables with a binomial distribution.

Given that the results produced by both SAS and XLSTAT were the same, with the exception that the XLSTAT ANOVA indicated a significant interaction ($P < 0.001$) between breed and tick species, the statistical results presented for this study were from the XLSTAT ANOVA. No significant differences ($P > 0.05$) were detected among all three *R. decoloratus* treatment combination groups. On the contrary, Table 3.1 indicates that significant differences were apparent between the Angus – *R. microplus* and the Brahman – *R. microplus* groups ($P < 0.001$) as well as between the Nguni – *R. microplus* and Brahman – *R. microplus* groups ($P < 0.001$).

Both breed and tick species had significant effects ($P < 0.001$) on the tick counts. The Bonferroni pairwise comparisons shown in Table 3.1 indicated that Brahman animals carried significantly lower tick burdens ($P < 0.001$) than both the Nguni and the Angus animals. Table 3.2 shows that the Brahman had an average tick resistance success rate of 80.82 (± 12.66) percent, while the Nguni and Angus cattle exhibited average tick resistance success rates of 58.36 (± 7.03) and 51.25 (± 12.74) percent,

respectively. The Nguni and the Angus cattle did not differ significantly from each other ($P > 0.05$). The *R. microplus* species resulted in significantly higher tick burdens than the *R. decoloratus* ($P < 0.001$) with an average success rate in attachment of 72.69 (± 6.64) percent in comparison to the *R. decoloratus* which had an average attachment success rate was 5.06 (± 1.34) percent of the larvae applied.

Table 3.1: Bonferroni comparison of the tick burdens in the different breed and tick species interactions

Association	Category	LS means (\pm Standard error)	Lower bound (95%)	Upper bound (95%)	Groups
Angus * <i>R. microplus</i>	Modern	90,167 \pm 4,458	81,036	99,298	A
Nguni * <i>R. microplus</i>	Modern	84,000 \pm 4,883	73,998	94,002	A
Brahman * <i>R. microplus</i>	Ancient	40,400 \pm 4,883	30,398	50,402	B
Angus* <i>R. decoloratus</i>	Modern	7,333 \pm 4,458	-1,798	16,464	C
Nguni * <i>R. decoloratus</i>	Ancient	6,333 \pm 4,458	-2,798	15,464	C
Brahman * <i>R. decoloratus</i>	Modern	1,500 \pm 4.458	-7,631	10,631	C

Table 3.2: The mean tick count per breed and tick species (\pm standard error)

Main effect	Mean tick count \pm SE (%)	Mean number of ticks not attached \pm SE (%) (Average tick resistance)
Breed		
Angus	48.75 \pm 12.75	51.25 \pm 12.74
Nguni	41.64 \pm 12.66	58.36 \pm 7.03
Brahman	19.18 \pm 7.03	80.82 \pm 12.66
Tick species		
<i>R. microplus</i>	72.69 \pm 6.64	27.31 \pm 6.64
<i>R. decoloratus</i>	5.06 \pm 1.34	94.94 \pm 1.34

3.4 Discussion

The Nguni breed was included in the study due to its long-term association and co-evolution with the *R. decoloratus* species as the indigenous breed and tick species of South Africa, respectively. It was hypothesized that this ancient association would result in the development of a more superior degree

of resistance in this breed of cattle against the *R. decoloratus* ticks. The Brahman breed was chosen for its long-term association and co-evolutionary status with the *R. microplus* species, being both of Asian origin. This association was also expected to result in superior resistance in the Brahman cattle against the *R. microplus* ticks. The Angus breed was included in the study due to its lack of co-evolution or long term association with either of the tick species that were used. Moreover, this breed has been highlighted in literature as one of the most susceptible breeds to *Rhipicephalus* tick species among the three fore-mentioned breeds and this is thought to be the result of the modern host-tick associations. It was therefore hypothesised that the Angus would exhibit susceptibility to both tick species shown by significantly higher tick burdens.

3.4.1 Breed and tick species interaction

The interaction between breed and tick species was significant. However, no pattern could be derived which presented increased resistance as a factor of shared evolutionary conditions or long-term associations between the interacting host and tick species. The significant interaction resulted in a rank order that was the same for both tick species. In essence, all infestations with the *R. microplus* species resulted in higher tick counts in all the three breeds, while the *R. decoloratus* had the lowest tick counts in all the three breeds in the same order of breeds (Angus > Nguni > Brahman). Therefore, in this case the presence of an interaction between breed and tick species was attributable to the differences in tick species.

The Brahman cattle were initially thought to display the predicted increased resistance to *R. microplus* as a result of the ancient host-tick association between the breed and tick species in question. Piper *et al.* (2008) reported similar results, where the Brahman carried significantly fewer ticks when ranked against Holstein-Friesian (*B. taurus*) cattle following *R. microplus* tick challenge. On the contrary, the heightened, though not significant, resistance level found in the Brahman – *R. decoloratus* association suggested that the levels of resistance were not attributable to type of host-tick associations. Further validating this observation was the intermediate level of resistance to both tick species displayed by the Nguni. Rechav & Kostrzewski (1991) reported having had observed superior resistance in the Nguni cattle against the *R. decoloratus* tick species when compared to five other breeds in their study. Contrary to expectations, the results produced in this study from the Nguni interaction with the *R. decoloratus* tick species were divergent from the hypothesis that the Nguni would display increased resistance to the *R. decoloratus* due to the long term association between the breed and tick species.

The higher resistance to *R. decoloratus* by all the three breeds may have been due to the presence of *R. microplus*, which might have enhanced resistance levels to *R. decoloratus* (Tønnesen *et al.*, 2004). Although, the two tick species were not liberated for feeding simultaneously on the same animal, the

animals had exposure to both tick species prior to the study. While the two species may have co-existed in the field, they may have also co-fed on the animals in the natural habitat. Inherently, the animals would have acquired improved immune responses to the *R. decoloratus* as inflicted by the presence of the *R. microplus*.

3.4.2 Breed differences

Bos indicus cattle are, on average, more resistant to various tick species than both the African indigenous and European breeds (Frisch & O'Neill, 1998; Wambura *et al.*, 1998; Shyma *et al.*, 2013). However, reports of significant differences between the Brahman and the Nguni breeds in particular are lacking. The results of the present study exposed the presence of significant breed differences between the Nguni and Brahman cattle used, with the Nguni cattle carrying twice as many ticks as the Brahman. The Brahman demonstrated a superior level of resistance by having the lowest tick counts for both tick species. These results were consistent with previous studies in which the Brahman showed 99% superior resistance to *R. microplus* ticks, whereas the *B. taurus* European breeds lagged behind with 85% resistance following consecutive infestations (Wagland, 1975; Utech *et al.*, 1978; Jonsson, 2006). Bonsma & Pretorius (1943) reported an increase in the level of host resistance with successive infestations. Because the cattle used had prior exposure to both tick species, the deduction made by Bonsma & Pretorius (1943) explained the superior level of resistance observed in the Brahman cattle to *R. decoloratus*. Therefore, the hypothesis of enhanced acquired immunity as a result of long term association between the host and the infesting tick is once again not rejected in the Brahman breed.

There is a uniform understanding that large differences in tick resistance exist between the *B. indicus* and *B. taurus* breeds as well as between African indigenous and European breeds (Seifert, 1971). Therefore, the similarities between the Angus and Nguni cattle conflict with reports presented by other researchers who reported superior resistance in the African indigenous cattle in comparison to the European breeds (*B. taurus*) (Willadsen, 1980; Rechav & Kostrzewski, 1991).

The results are consistent with those from Kaiser *et al.* (1982) and Rechav *et al.* (1990), where breeds that exhibited superior resistance for one tick species were likely to express a similar level of resistance to other tick species. The situation is further exacerbated when comparing two closely related tick species, such as the *R. microplus* and the *R. decoloratus*, as was done in the current study. Therefore, it explains why the Brahman showed greater resistance to both tick species, when the Nguni was expected to experience the lowest tick burdens of *R. decoloratus*.

Observations made in Australian cattle populations demonstrated that animals which had not been dipped due to sickness or injury endured light tick burdens (Riek, 1962). A contradictory observation

was made in the Nguni cattle used in this study which suffered from acaricide poisoning prior to the start of the trial. Although intermediate to an extent, the tick burdens on the Nguni did not differ significantly from those of the susceptible Angus cattle. These results do not conform to previous studies of a similar nature. This was possibly indicative of a compromised immune system which weakened the natural host resistance.

3.4.3 Tick species differences

Certain tick species tend to co-exist and occasionally co-feed (Kopp *et al.*, 2009). However, this often results in the inevitable displacement of one species by another. Detailed studies have been conducted in South Africa mapping the adverse displacement of the indigenous *R. decoloratus* species by the Asian *R. microplus* species (Tønnesen *et al.*, 2004; Horak *et al.*, 2009; Nyangiwe *et al.*, 2011, 2013). While there is a vast number of publications, which describe the epidemiology of the *R. microplus* tick species on beef cattle, the number of reports which outline the density of *R. decoloratus* population on cattle of various breeds are still limited.

Distinct differences were observed between the infestation success rates of the two tick species. The *R. microplus* predominantly resulted in high tick burdens in all three breeds. On the contrary, *R. decoloratus* attachments were significantly resisted in all three breeds. This conforms to studies which classify *R. microplus* as a successful invasive species that achieves displacement of the indigenous *R. decoloratus* through increased success in feeding attempts simultaneously mating with *R. decoloratus* females to produce sterile hybrid eggs (Spickett & Malan, 1978; Madder *et al.*, 2011). Consequently, more female *R. microplus* than *R. decoloratus* ticks are able to complete their feeding. In so doing they are able to continue their development stages which results in laying fertile eggs (Norval & Short, 1984). This, however, contradicts the notion that the long term association between breed and tick species would result in improved resistance to the specific tick species. If the notion would have been applicable within the studied cattle population, then significantly lower tick counts would have been observed in all breeds following *R. microplus* challenge due to the long term association between all three breeds and the *R. microplus* species in South Africa.

Ticks have been demonstrated to display preferential attachment on the animal's body (Seifert, 1971; Mattioli *et al.*, 1993). This was observed in this study where the *R. microplus* ticks showed a clear pattern of preferential attachment. They were mostly found attached under the green ring of the calico bag, away from exposure to light. The *R. decoloratus* on the other hand were attached in plain sight. In a pasture setting this would then expose the *R. decoloratus* ticks to more predator attacks consequently reducing their engorgement success rates. Therefore, in a pasture setting it is probable that neither ancient nor modern host-tick associations are responsible for differences in tick counts

between these two tick species. This was consistent with the report by Short *et al.* (1989) where significantly more active movement of the *R. microplus* larvae than the *R. decoloratus* larvae in search of suitable environmental conditions that were likely to offer greater survival chances was observed; hence engorgement success.

3.5 Conclusions

The Brahman exhibited superior resistance and the Angus exhibited susceptibility, while the Nguni showed intermediate resistance to both the *R. microplus* and *R. decoloratus* artificially induced challenges. *R. microplus* and *R. decoloratus* are very closely related and this may have masked the detection of differences as a result of ancient and modern host-tick associations. Although both tick species were given an equal opportunity to successfully attach to the shaved area on the animals' backs, the tick burdens observed from *R. microplus* infestation were significantly higher than those of the *R. decoloratus* in all three breeds. This implies that the *R. microplus* had a greater ability to combat host resistance mechanisms than the *R. decoloratus* did. It is, however, clear that the groups of cattle used in this study was not an accurate representation of other Angus, Brahman and Nguni populations, which are exposed to field challenges whereby tick infestations manifest through preferential attachment and feeding. As a result, it was difficult to conclude with certainty that the differences observed in tick burdens were significantly influenced by the established ancient and modern host-tick associations.

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CHAPTER 4

DIFFERENTIAL EXPRESSION OF TICK RESISTANCE RELATED GENES FOLLOWING ARTIFICIAL INFESTATION WITH *R. MICROPLUS* AND *R. DECOLORATUS* TICKS**Abstract**

The differential expression of candidate genes is associated with subsequent suppression of cell-mediated inflammatory responses and the activation of antibody induction at the tick bite site. The objective of the current study was to conduct gene expression analyses using real-time PCR data of RNA extracted from skin biopsy samples collected 12-hours post-artificial-infestation with *R. microplus* and *R. decoloratus* ticks. The expression profiles of 17 previously identified inflammatory and immune function-related genes were studied in the different ancient (Brahman-*R. microplus* and Nguni-*R. decoloratus*) and modern (Angus-*R. microplus*, Angus-*R. decoloratus*, Brahman-*R. decoloratus* and Nguni-*R. microplus*) host-tick associations at the host-tick interface of the host skin. The data was normalised against one internal control (*RN18S1* commonly known as β -actin-like) and three quality controls. The panel of genes included cytokines (*TLR5*, *TLR7*, *TLR9*, *TRAF6*, *CD14*), chemokines and their receptor (*CCR1*, *CCL2*, *CCL6*), toll-like receptors (*IL-1 β* , *CXCL8*, *IL-10*, *TNF*) and other candidate genes (*BDA20*, *OGN*, *TBP*, *LUM*, *B2M*). There were differences among breeds in their genetic response to tick challenges, with the expression level displayed by the Brahman cattle differing significantly from those of the Angus cattle for genes *LUM*, *TBP*, *TRAF6* and *B2M*. Most of the differences were not of genes encoding products of the adaptive immune response but included genes of the extracellular matrix primarily involved in tissue repair. Important among which was *LUM*, and to a lesser extent *B2M*, which had expression levels significantly higher in the Brahman and Nguni cattle as opposed to the Angus cattle, thus presenting *LUM* as a potential biomarker for tick resistance. No tick-specific differences were detected. Furthermore, there was no evidence of breed by tick species interaction, which linked the gene expression profiles to the degree of co-evolution of the breeds and tick species. This implied that the effect host-tick association was not responsible for the gene expression post infestation.

Keywords

Gene expression, cytokines, chemokines, toll-like receptors, Brahman, Nguni, Angus.

4.1 Introduction

The devastating economic effects and environmental aftermaths resulting from infestations by the cattle ticks have been the driving force behind the global movement of the beef industry towards developing strategies with the potential to effectively combat tick burdens. While acaricides and vaccines have been the dominant tick control methods (Jongejan & Uilenberg, 1994), the host's resistance to ticks offers an opportunity that can be exploited to develop alternative tick control methods that can complement the existing methods. Resistance to tick challenge is significantly influenced by several other factors including annual climatic cycles, pasture management and the host's morphological coat characteristics (Willadsen, 2006; Machado *et al.*, 2010; Mapholi *et al.*, 2014). Understanding these factors may result in more sustainable and environmentally sound approaches for tick control (Kongsuwan *et al.*, 2008).

In previous studies, where tick counts were used, the *Bos indicus* exhibited a higher degree of resistance than the *Bos taurus*, while the *Bos taurus africanus* had intermediate resistance (Wilkinson, 1955; Frisch & O'Neill, 1998; Mapholi *et al.*, 2014). Also, the South African Bonsmara cattle were less resistant when compared to the Nguni cattle (Marufu, *et al.*, 2011b). Artificial infestation has been the method of choice in some of these studies. This is because artificially created host-tick associations yield more intense expression of acquired resistance than naturally occurring host-tick associations (Marufu *et al.*, 2014). Artificial infestations simulate field conditions, while minimizing factors such as predation, temperature, and humidity, which significantly affect the survival rates of free-living ticks under natural infestation conditions (Regitano & Prayaga, 2011). Artificial infestations grant an equal opportunity to all larval ticks to attach and feed on the animal. Given that tick resistance is under genetic control and is considered polygenic (Morris, 2007; Machado *et al.*, 2010; Mapholi *et al.*, 2014), artificial infestations are suitable for revealing the genetic variation for tick resistance that exists among breeds.

The genetic variation in tick resistance that exists among breeds provides an opportunity to improve tick resistance through genetic selection. To improve the accuracy of genetic selection, genomic technologies are available and have been extensively used in other industries, such as the poultry, swine and dairy. While these industries have effectively developed and deployed genomic strategies to optimise production efficiencies, the beef industry has failed to implement effective genomic tools to combat the unique challenges their production systems encounter daily (Rolf *et al.*, 2014). The complexity of the genetic components of tick resistance prevent host animals from exhibiting 100% resistance to all tick species (Morris, 2007). Genetic enhancements which describe the incorporation of single-nucleotide polymorphisms (SNPs) and copy number variants (CNVs) in the mapping of the

bovine genome could generate robust biological approaches for tick control using natural host resistance (Parizi *et al.*, 2009). A few studies have been conducted to characterise tick resistance in cattle, where some beef breeds were included. Wang *et al.* (2007) studied the low- and high-resistance Hereford Shorthorn cattle and reported significant within-breed variations in the expression of the extracellular matrix genes OBP, BDA20 and dendritic cell protein HFL-B5. Subsequently, Piper *et al.* (2008; 2009) conducted a follow-up gene expression study comparing the Brahman and Holstein-Friesian cattle and reported significant between-breed differences for toll-like receptors (TLR5, TLR7, TLR9, NFKBp50, MyD88, Traf-6, CD14 and IL-1b), chemokines and chemokine receptors (CCL2, CCL26, and CCR-1). These genes are responsible for tick antigens recognition and the activation and chemotaxis of inflammatory response cells to the site of inflammation. The majority of these genomic studies were primarily focused on the genes of the Major Histocompatibility Complex (MHC), rather than transcriptomic studies (Turner *et al.*, 2011). Transcriptome analyses provide a global picture of the cell function following infestation by profiling coding and non-coding transcriptional activity and gene expression following infestations.

Previous bovine gene expression studies have focused on the differential gene expression in different cattle breeds resulting from challenge with one tick species. This is, however, contrary to reality, where cattle are exposed to multiple tick species. Thus, the level of resistance of a particular breed may depend on the biting tick species. This may be attributed to the variation that also exists in tick characteristics, which include mouthparts, bioactive molecules of the saliva and other physiological characteristics (Marufu *et al.*, 2014). The length of the association of a given breed and a particular tick species may thus influence the level of resistance in the breed (Frisch, 1999). Therefore, breeds which may have experienced a long period of evolution in the presence of a particular tick species, and are resistant to that tick species, are suspected to have accumulated genes affecting resistance to that tick species (Frisch, 1999; Marufu *et al.*, 2014). Thus, host-tick associations can be categorised as ancient, where the breed and tick species co-evolved over a long time. Conversely, the host-tick association can be characterised as modern by co-evolution over a short time. There are currently very few studies which document the differential expression of the selected panel of genes in the different ancient and modern host-tick associations.

The Nguni breed was included in the current study due to its long term association and co-evolutionary status with the *R. decoloratus* species in South Africa. The Brahman breed, on the other hand, formed part of the study due its long-term association and co-evolutionary status with the *R. microplus* in Asia. Therefore, it was hypothesized that these ancient host–tick associations would result in the development of a more superior degree of resistance in these breeds against the particular tick species of common origin. This would be attributed to better-evolved genetic architectures and to a lesser

extent the improved acquired inflammatory responses. Lastly, the Angus breed was included in the study due to its lack of coevolution or long-term term association with either of the tick species that were used. Moreover, this breed was highlighted in literature as one of the most susceptible breeds to *Rhipicephalus* tick species among the three breeds to be included in the study. Therefore, the study investigated the gene expression profiles generated at the host-tick interfaces of two closely related tick species in association with three cattle breeds of various different origins.

4.2 Materials and Methods

The research site, experimental animals, and tick species were described in Sections 3.2.1 to 3.2.3.

4.2.1 Skin evaluation

The animals were restrained in the crush pens and examined for the presence of engorged ticks that may have attached prior to the commencement of the study and survived dipping. The animals were also examined for tick-related dermatitis and wounds, as well as other non-tick related wounds, which would require treatment.

4.2.2 Artificial infestation

The unfed tick larvae (UFL) were counted under a light microscope and organised into groups of approximately 100 before being placed into tick-safe vials. Each breed group of the experimental animals was split in half, with six animals per breed undergoing artificial infestation with the *R. microplus* species while the remaining six were infested with the *R. decoloratus* species. Subsequently, the Angus groups were further divided in terms of sex, with three of each sex undergoing infestation with *R. microplus* and the other three being infested with *R. decoloratus* ticks.

The animals were each restrained in the crush pen where the area on their backs, where the calico bag would be attached and the unfed larvae inserted for feeding, was shaved using heavy duty electrical clippers (Legend[®], Lister, South Africa). The area was cleaned using a cloth soaked in lukewarm water to remove any excess dirt, wax or residues of the short acting acaricide. The calico bags were secured to the shaved areas on the animals' backs using Alcolin Contact Adhesive (Alcolin[®], South Africa) and allowed a few hours to dry before infestation. Once the bags were completely dry, one tick-containing vial was placed inside the bag of each animal, opened and left there to liberate the larvae and allow them to start feeding. The open sock end of the bag was then folded and twisted shut then secured with one rubber castration ring by means of a rubber ring applicator.

After 12 hours, the bags were opened to remove the vials and the tick bite sites were visually inspected for any actively feeding ticks. Once the bags were removed and packed for incineration, the animals

were then all treated with a water-soluble short-acting acaricide (amitraz) to prevent unwanted dispersal of the ticks throughout the feedlot.

4.2.3 Skin biopsy collection

The animals were restrained in a crush and lightly sedated with 0.2 ml/50kg body weight xylazine (Rompun[®], Bayer, South Africa) administered intramuscularly in the rump. The dose was reduced to 0.1 ml/50kg in the Nguni because of the Nguni's below average body conditions. The Brahman breed has increased sensitivity to the sedative, thus these animals were closely monitored thereafter. A local anaesthetic injection, at a dose of 0.1 ml/site of 2% lignocaine hydrochloride injection (Lignocaine[®], Bayer, South Africa), was administered subcutaneously around the punch biopsy site to desensitise the area upon biopsy collection.

Using a disposable 5 mm biopsy punch, three skin biopsies were taken with a 5 mm diameter and a depth of 10mm. The three skin biopsies were, two from non-parasitized skin prior to infestation and one from parasitized skin from identifiable tick feeding sites 12 hours post-infestation. The biopsies were then directly placed into 15 ml tissue protect, RNase- and DNase-free tubes (CELLSTAR[®] tubes, Greiner Bio-One) and completely immersed in 5 ml RNeasy[®] RNA stabilization Reagent (Qiagen). Then, the tubes were incubated overnight in the reagent at 2-8°C, then transferred to -80°C for archival storage in the reagent. Once the biopsies were collected, the biopsy site was treated with chlorfenvinphos 0.48% (Supona Aerosol Spray[®], Zoetis, South Africa) and oxytetracycline (Terramycin Wound Powder[®], Fivet, South Africa) to prevent bacterial infection and wound myiasis.

4.2.4 RNA extraction

Great precision, speed and accuracy were employed in handling the RNA samples because the RNA was between transitions making it extremely volatile and challenging to preserve in terms of quality and integrity. All the equipment and machines used during the isolation process were sterilized in 70% ethanol and sprayed with RNase-away spray to prevent any RNase digestion of the samples. The forceps used were soaked in 70% ethanol between sample removals from the tubes and a new blade was used for cutting each sample to prevent cross contamination of one sample by another.

4.2.4.1 Sample preparation

Both the RNeasy[®] stabilized and liquid nitrogen snap-frozen biopsy samples were removed from the tissue protect tubes using sterilized forceps. Roughly 50-100 mg of each tissue sample was weighed off for RNA isolation. RNA isolation was conducted following the TRIzol[®] Reagent protocol (Ambion, Life technologies[™]). Each biopsy was placed in a specialized 2ml screw cap tube containing 2 ceramic beads and 500 µl of TRIzol reagents. The tissue samples were macerated, disrupted and homogenized using the Geno/Grinder 2010[®] (SPEX[®] SamplePrep, Vacutec) machine set to run for 15 minutes at a

speed of 1750 rpm. An additional 500 μ l of TRIzol reagents was added to the lysate, then briefly vortexed and incubated at room temperature for 10 minutes.

4.2.4.2 RNA precipitation

The lysate was transferred to 2ml Eppendorf tubes using 19G syringes to prevent any debris from being transferred to the new tubes. A total volume of 200 μ l of chloroform was added to the filtered lysate and vortexed for 15 seconds and then incubated at room temperature for 15 minutes. The resulting mixture was centrifuged at 12 000 \times g for 15 minutes at 4°C. Centrifugation resulted in the separation of the mixture into three distinct phases: the first phase at the bottom of the tube consisted of a red organic phase concentrated with protein, the second or middle phase consisted of an interphase of DNA and the third and uppermost colourless aqueous phase that was primarily made up of RNA. The aqueous phase was transferred to a fresh 2ml Eppendorf tube containing 200 μ l of isopropanol and vortexed for 15 seconds. This was followed by a 10-minute incubation phase at room temperature and centrifugation of the mixture at 12 000 \times g for 10 minutes at 4°C to allow the RNA precipitate to form a pellet on the side and bottom of the tube.

4.2.4.3 RNA wash

The supernatant was carefully removed from the tubes leaving behind only the pellet. The pellet was washed by adding 1000 μ l of 75% ethanol to the pellet and vortexing the samples briefly, then centrifuging the tube at 12 000 \times g for 5 minutes at 4°C. The RNA pellet was air-dried for 10 minutes, then resuspended in 50 μ l of RNase-free water and incubated on a heating block for 15 minutes at between 55 and 60°C, tapping the tube every three minutes to facilitate complete dissociation.

4.2.4.4 RNA clean-up and gDNA contamination removal

The Qiagen RNeasy® Mini Kit Quick-Start Protocol was followed to conduct total RNA clean-up and removal of genomic DNA (gDNA) contamination. One volume of 70% ethanol was added to the lysate and mixed well by pipetting, but not centrifuged. A total of 700 μ l of the sample was transferred to an RNeasy spin column placed in a 2 ml collection tube and centrifuged for 15s at 10 000 rpm. The flow-through was discarded and then the On-column DNase digestion protocol was followed for further RNA clean-up. This involved adding 350 μ l Buffer RW1 to the RNeasy column and centrifuging for 15s at 10 000 rpm, after which the flow-through was discarded. Subsequently, 80 μ l of DNase incubation mix was added directly to the RNeasy column membrane and allowed a 15 min resting period at 20-30°C. The incubation mix was prepared using 10 μ l DNase stock solution added to 70 μ l Buffer RDD (Qiagen). An additional 350 μ l Buffer RW1 was added to the RNeasy column and once again centrifuged for 15s at 10 000 rpm. Two cycles of 500 μ l Buffer RPE were added to the RNeasy spin column and centrifuged at 10 000 rpm for 15s for the first cycle and for 3 min for the second cycle, in order to dry

the membrane. Subsequent to each cycle, the flow-through was discarded. The RNeasy spin column was placed in a new 1.5 collection tube. Then 30µl RNase-free water was added directly to the spin column membrane and left to rest for 5 min. Lastly the tubes were centrifuged for 1 min at 10 000rpm to elute the total RNA. The tubes were stored at -20°C to prevent RNA degradation.

4.2.5 RNA quality and concentration check

The RNA concentrations were quantified using the Qubit® 2.0 Fluorometer together with the Qubit® RNA BR assay kits. The NanoDrop spectrophotometer (NanoDrop® DN-100, Thermo Fisher Scientific) was used to quantify concentrations higher than 600ng/µl for which no exact values could be generated using the Qubit fluorometer.

The NanoDrop spectrophotometer was used to check the purity of the RNA by generating the 260/280 values (Bustin *et al.*, 2009). All samples that produced 260/280 values ≥ 1.70 were considered suitable for real-time PCR analyses. The quality or integrity of the RNA was further verified using agarose gel electrophoresis. A 1% agarose gel was prepared using 1g of agarose powder in 100 ml 1× TBE buffer mixed with 3 µl ethidium bromide to make the bands visible in the gel. A mixture of 1 µl of 10X green loading buffer and 4 µl of RNA was loaded into each well of the gel and run against 5 µl green DNA ladder (positive control) in an RNase-free tank containing 1X TBE buffer. The machine was set to run for 45 minutes at 90V to facilitate adequate 28S and 18S band separation.

4.2.6 cDNA synthesis

Prior to the real-time PCR analyses, cDNA was synthesised using equal amounts of total RNA (Huggett *et al.*, 2005). The RT² First Strand Kit supplied by Qiagen was used according to manufacturer's protocol. To obtain optimal results, 400ng of total RNA per sample was used. In preparing the genomic DNA mix, 400ng of total RNA together with 2 µl of buffer GE and variable amounts of high-quality nuclease-free water (depending on the volume of RNA added) were mixed together in an Eppendorf tube to form a total volume of 10µl. The mixture was incubated at 42°C for 5 minutes then immediately placed on ice for at least 1 minute while the reverse-transcription mix was prepared. The reverse-transcription mix for 1 reaction made use of a master mix total volume of 10 µl which consisted of 4 µl 5X Buffer BC3, 1 µL Control P2, 2 µl RE3 Reverse Transcription mix and 3 µl RNase-free water. The 10 µl reverse transcription master mix was added to a tube containing the 10 µl genomic DNA elimination mix and gently mixed by pipetting up and down. The final mixture was incubated at 42°C for exactly 15 min then immediately incubated at 95°C for 5 minute to stop the reaction. The final reaction was stored in a -20°C freezer while awaiting real-time PCR analyses.

4.2.7 *Primer design and optimization*

The primers for each of the genes of interest were custom designed by Qiagen (WhiteHead Scientific, South Africa) using forward and reverse primer sequences associated with the GenBank and UniGene reference sequence numbers listed in Table 4.1.

4.2.8 *Quantitative real-time Polymerase chain reaction (qPCR) analysis*

Using real-time PCR analyses, the gene expression profiles of the panel of genes listed in Table 4.1 were examined. A PCR components mix was prepared in a 5ml tube for each sample. The mix comprised of 12.5µl 2x RT² SYBR[®] Green Mastermix, 11.5µl HIGH-QUALITY RNase-free water and 1µl cDNA synthesis reaction to make the required total volume of 25µl per well. In preparing the master mix for 24 wells 330µl 2x RT² SYBR[®] Green Mastermix, 304µl HIGH-QUALITY RNase-free water and 26µl cDNA synthesis reaction were prepared to give a total volume of 660µl, thus providing an access volume of 10% to facilitate pipetting errors. Each well of the Custom 96-well RT² Profiler PCR arrays (Qiagen, WhiteHead Scientific, South Africa) received 25µl of the components mix and was then tightly sealed with an Optical Thin-Walled 8-Cap Strips.

Custom 96-well RT² Profiler PCR arrays, which were used for the real-time PCR analyses, facilitated high-throughput focused expression analysis on the genes of interest. The arrays came equipped with the primers already placed in each well for each of the genes of interest. Each plate enabled the analyses of four samples at a time to generate amplification data for 17 genes of interest and four reference genes per sample.

The arrays were also fitted with primers designed to amplify three Qiagen recommended quality control parameters, namely Bovine Genomic DNA Control (BGDC), Reverse Transcription Control (RTC) and Positive PCR Control (PPC). The BGDC is a very sensitive assay that detects the unique non-coding region that is far removed from any transcriptional start site within the bovine genome. Any sample that produced C_T values below 35 was analysed carefully gene for gene, while those with C_T values lower than 30 had their RNA re-purified with genomic DNA removal, preferably using DNase I and a spin column. BGDC values <30 indicated that genomic DNA was likely to have been contributing signal to most if not all the genes of interest for that specific sample (Qiagen, 2015).

The PPC is a matrix that measured the PCR array reproducibility by measuring the technical variability of the PPC wells across all samples. The recommended difference between any two samples was not to be more than two from one another for suitable data to be produced for further data analysis (Qiagen, 2015).

Table 4.1: Description of the 17 genes of interest and their gene product functions

Gene Symbol	Gene name	RefSeq Number		Function of gene product
		UniGene	GenBank	
IL-1 β	Interleukin 1, beta	Bt. 4856	NM_174093	Pleiotropic; pro-inflammatory
CXCL8	Interleukin 8	Bt.49470	NM_173925	Chemo-attractant for effector blood cells
IL10	Interleukin 10	Bt.4723	NM_174088	Anti-inflammatory
CCL2	Chemokine (C-C motif) ligand 2	Bt.2408	NM_147006	Recruitment and activation of immune effector cells; inflammatory response
CCL26	Chemokine (C-C motif) ligand 26	Bt.23451	NM_001205635	Recruitment and activation of immune effector cells; inflammatory response
CCR1	Chemokine (C-C motif) receptor 1	Bt.62596	NM_00107739	Recruitment of immune effector cells to site of inflammation
TLR5	Toll-like receptor 5	Bt.66307	NM_001040501	Pathogen recognition and activation of innate immunity
TLR7	Toll-like receptor 7	Bt.111931	NM_001033761	Pathogen recognition and activation of innate immunity
TLR9	Toll-like receptor 9	Bt.12810	NM_183081	Pathogen recognition and activation of innate immunity
CD14	Cluster of differentiation 14	Bt.4285	NM_174008	Confers lipopolysaccharide sensitivity to neutrophils, monocyte & macrophages
TRAF6	TNF receptor-associated factor 6	Bt.9201	NM_001034661	Mediates signal transduction from the TNF receptor family
TNF- α	Tumor necrosis factor – alpha	Bt.12756	NM_173966	Cell signalling protein (cytokine) involved in systemic inflammation
OGN	Osteoglycin	Bt.5341	NM_173946	Corneal keratan sulfate proteoglycan; regulates collagen fibrillogenesis in skin
TBP	TATA box binding protein	Bt.22662	NM_001075742	General transcription factor
LUM	Lumican	Bt.2452	NM_173934	Collagen fibril organization; epithelial cell migration; tissue repair
B2M	Beta-2-microglobulin	Bt.64557	NM_173893	Formation of amyloid fibrils in some pathological conditions; presentation of peptide antigens to the immune system
BDA20	Bovine dander allergen 20	Bt.550	NM_174761	Weak inducer of both humoral and cellular responses

The RTC measured the efficiency of the reverse transcription across samples by detecting the artificial mRNA with a poly-A tail not homologous to any mammalian or bacterial sequence that is preloaded into the primer buffer of the RT² First Strand cDNA synthesis kit (Qiagen, 2015). The artificial mRNA is reverse transcribed with the messages in the samples and upon detection of this sequence; it was possible to determine whether the data from all the samples could be used for comparison. The RT efficiency was determined by calculating the ΔC_T (RTC-PPC) for each sample. The preferred difference between the C_T values was ≤ 5 above which the RNA was to be re-purified.

The threshold was set to one for all the arrays. This point lies slightly above the middle of the geometric phase of the amplification curve, where all the curves were straight and parallel to each other (Wong & Medrano, 2005). The baseline was set to range from 2-15 cycles since the earliest amplification was visible between cycle 12 and 18. This greatly improved the quality of the data by ensuring that a sufficient amount of the noise was subtracted so that it had no significant effect on the output of the curves.

4.2.9 Statistical analysis

The threshold cycle (C_T) values generated by the ABI real-time cycler were used to calculate the expression level of each gene using the RT² Profiler PCR Array Data Analysis Webportal (SABioscience - Qiagen). The fold change value of each gene was calculated using the $\Delta\Delta C_T$ method explained below (Livak & Schmittgen, 2001; Wong & Medrano, 2005):

$$\Delta C_T = C_{T(\text{Gene of interest})} - C_{T(\text{Reference gene})}$$

$$\Delta\Delta C_T = \Delta C_T(\text{Test group n}) - \Delta C_T(\text{Control group n})$$

$$\text{Fold change} = 2^{(-\Delta\Delta C_T)}$$

$$\text{Fold regulation} = \text{Fold change for values} \geq 1, \text{ and } \text{Fold regulation} = \left(-\frac{1}{\text{fold change}} \right) \text{ for values} < 1$$

In essence fold change is the ratio of the relative gene expression between the control sample and the test sample where ratios >1 indicate upregulation or increased gene expression and ratios between 0 and 1 indicate downregulation or decreased gene expression and ratios = 1 indicate no change in gene expression (Qiagen, 2015).

To facilitate ease of interpretation of the data, fold regulation values for each gene were used for all relative quantitation statistical analyses which measured variabilities in gene expression normalised

against the reference gene RN18S1 (Wong & Medrano, 2005). Fold regulation values >1 indicated upregulation while fold regulation values < 1 indicated downregulation of the gene of interest.

A distribution analysis of the data was conducted in SAS[®] Enterprise Guide 9.4 (SAS, 2016) to test the data for normality and homoscedasticity. Normality was tested using the Shapiro-Wilk test and P-P distribution plots per gene, while the Levene's test for homogeneity was used to test for homoscedasticity.

Employing XLSTAT 2016, an analysis of variance for two-way factorial designs was used to generate the P value for the interaction between the main effects, breed and tick species, for each of the genes. All genes which produced non-significant interaction values ($P > 0.05$) were tested for the significance of each of the main effects. Genes that exhibited significant values ($P < 0.05$) for either one of the main effects were further analysed using the Bonferroni pairwise test for Least Square (LS) means to determine which treatments differed from each other. A coefficient of determination (R^2) was generated for each gene to provide an indication of the amount of variation that was explained by the formulated model, as well as the contribution of the interaction and each of the main effects to the observed variation.

The mean and standard errors for each gene were generated in every treatment combination group, namely Angus-*R. microplus*, Angus-*R. decoloratus*, Brahman-*R. microplus*, Brahman-*R. decoloratus*, Nguni-*R. microplus*, and Nguni-*R. decoloratus*. From this output, it was then determined whether each of the genes were up- or down-regulated, with mean fold regulation > 1 indicating upregulation and mean fold regulation values < 1 indicating down-regulation of the specific gene (Qiagen, 2015). All mean fold regulation ≥ 2 were also classified as over-expressed, indicating that the gene's expression was relatively high in both the test and the control samples. While those that were < 0.5 were deemed under-expressed indicating that the gene's relative expression level was low in both the control and the test samples.

4.3 Results

4.3.1 RNA quality and concentration validation

Although all but one sample yielded high concentrations of mRNA (see Figure 4.1-C), in most of the samples the 18s and 28s bands were not clearly visible in the gel, but quite a bit of smearing could be seen. This was indicative of the presence of partially degraded mRNA within the samples. However, the samples were considered good enough quality for use in RT-qPCR analyses.

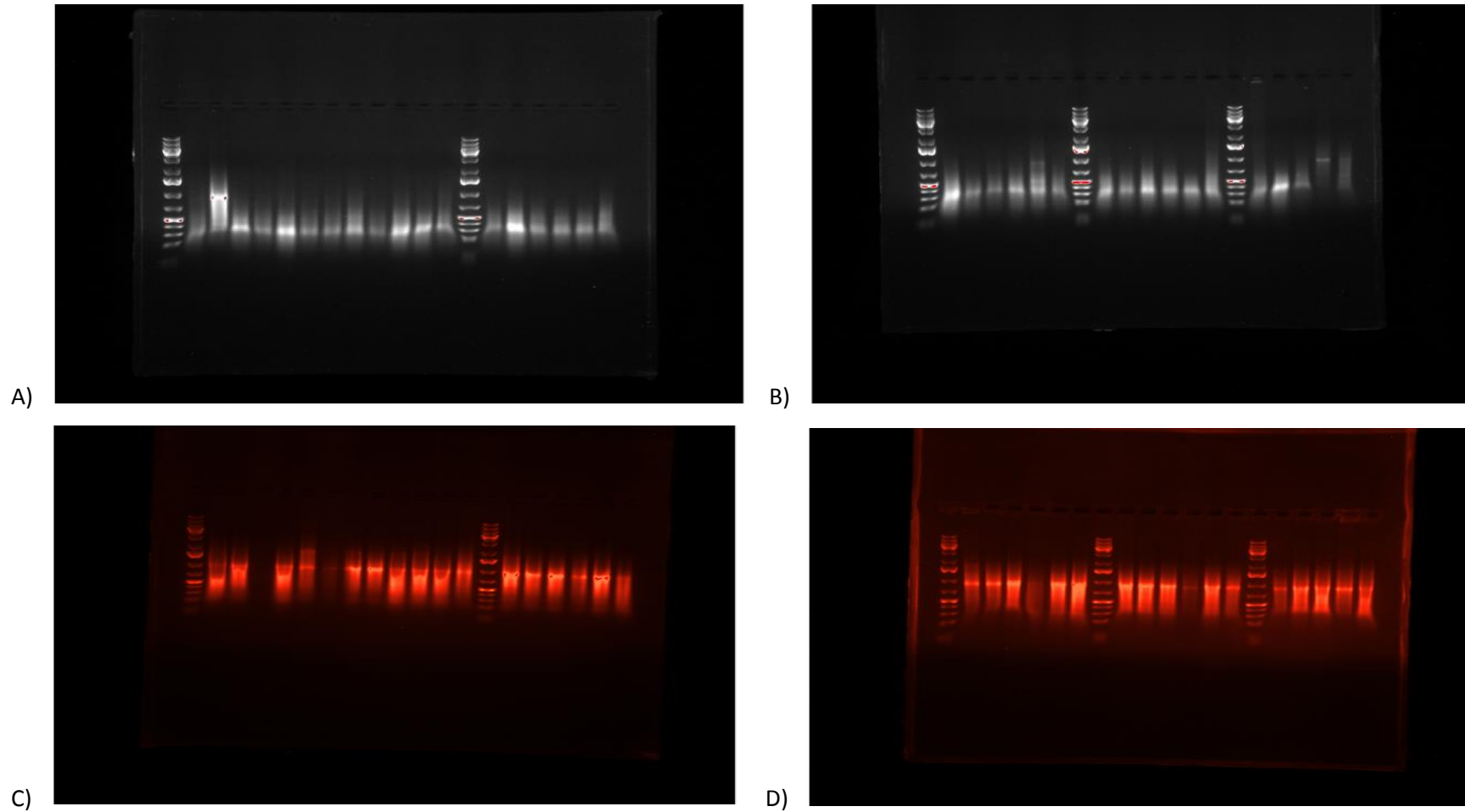


Figure 4.1: 1% agarose gel images. Reading lanes from left to right; A) Pre-infestation samples AD1-AD6 and AM1-AM6 and ND1-ND6; B) Pre-infestation samples NM1-NM6, BD1-BD6 and BM1-BM5; C) Post-infestation samples AD1-AD6 and AM1-AM6 and ND1-ND6; B) Post-infestation samples NM1-NM6, BD1-BD6 and BM1-BM5 (A, B and N are breeds Angus, Brahman and Nguni, respectively, and D and M represent *R. microplus* and *R. decoloratus*, respectively)

4.3.2 Statistical analyses using data filtered for outliers

Data from samples AD3, BD6, BM5, BM6 and ND4 were removed from the data set to avoid misrepresenting the data. The C_T values produced by sample BM5 could not be normalized using the selected reference gene (RN18S). Combined with the fact that the biopsy sample for this animal was not taken from a visible tick bite site, the presence of the oily substance on its skin which was thought to be remnants of the short-acting acaricide used, may have been the factor which distorted the data produced. Data from samples BD6 and ND4 were also omitted from further analyses due to the production of low and extremely high fold regulation values, respectively, which were outliers that significantly decreased the R^2 -value. Sample AD3 was also omitted due to yielding a very low mRNA concentration, which could not be salvaged for RT-qPCR analysis. No data could be retrieved for sample BM6 because the animal fled from the experimental setting and thus had to be excluded from the trial. The omission of the abovementioned data points significantly improved the goodness of fit of the model by increasing the coefficient of determination (R^2 -values) and reducing the Akaike Information Criterion (AIC). Therefore, statistical results for this study were based on data from the following sample groups: five Angus-*R. decoloratus*, six Angus-*R. microplus*, five Brahman-*R. decoloratus*, four Brahman-*R. microplus*, five Nguni-*R. decoloratus* and six Nguni-*R. microplus*.

4.3.3 Data normalisation through selection of suitable reference genes

The accurate averaging of the reference gene using either arithmetic or geometric means could not be conducted as the data did not contain replicates required for this approach (Vandesompele *et al.*, in press). Of the four candidate reference genes, which were investigated for variable expression in the different treatment groups, namely Ribosomal protein, large, P0 (*RPLP0*), 18S ribosomal RNA (*RN18S1*), Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and Beta-actin-like (*LOC616410*), only *RN18S1* could be used to normalise the data. The average C_T values for the reference genes were 24.153, 15.717 and 25.399 for *RPLP0*, *RN18S1* and *GAPDH*, respectively, while *LOC616410* yielded C_T values beyond the set RT-qPCR cut-off point of 40 cycles, and thus had to be omitted from consideration (Heid *et al.*, 1996). 18S ribosomal RNA was the least variably expressed with a C_T value range of 7.75 and an average of 1.575 in the difference between pre- and post-infestation C_T values. Ribosomal protein, large, P0 was the most variably expressed with a C_T value range of 10.089 and an average of 4.821 in the difference between pre- and post-infestation C_T values. As a result of the other reference genes yielding values significantly above the RT-qPCR assay manufacturer's recommendation of 1.5 in the average difference of the control and test C_T values, *RN18S1* was selected as the most suitable reference gene for data normalisation.

4.3.4 Normality and Homoscedasticity test

As represented in Figure 4.2, the hypothesis for normal distribution was rejected for the fold regulation data for all the genes except two, *TRAF6* and *TBP*, which had W-values of 0.983 ($P = 0.881$) and 0.881 ($P = 0.229$), respectively. The Levene's test for homogeneity indicated that the fold regulation values for all the genes of interest were homoscedastic ($P \geq 0.05$). The null hypothesis for equal variances was rejected for genes *TLR7*, *TLR9* and *LUM* due to their P-values being equal to 0.0497, 0.007 and 0.012, respectively. Following the comparison of the P-values obtained from the One-Way ANOVA and those from the Welch's ANOVA for genes *TLR7*, *TLR9* and *LUM* it could then be concluded that heteroscedasticity of the data for these genes would not affect the results due to the p-values from both tests being similarly insignificant as is shown in Table 4.2.

Table 4.2: Tests for homogeneity per gene of interest

Gene Symbol	Levene's Test for Homogeneity P-values	One-way ANOVA P-value	Welch's ANOVA P-value
IL1B	0.2681	0.162	0.1855
CXCL8	0.2227	0.4977	0.4054
IL10	0.2509	0.229	0.1204
CCL2	0.5502	0.4018	0.2966
CCL26	0.2007	0.1639	0.1228
CCR1	0.2825	0.4188	0.0311
TLR5	0.1512	0.7596	0.7781
TLR7	0.0497	0.172	0.0709
TLR9	0.007	0.1352	0.0561
CD14	0.4376	0.315	0.0802
TRAF6	0.3362	0.0395	0.0457
TNF	0.1658	0.5211	0.1979
OGN	0.1661	0.5277	0.6436
TBP	0.1965	0.0265	0.0258
LUM	0.012	0.006	0.0138
B2M	0.1647	0.229	0.0298
BDA20	0.211	0.1521	0.0342

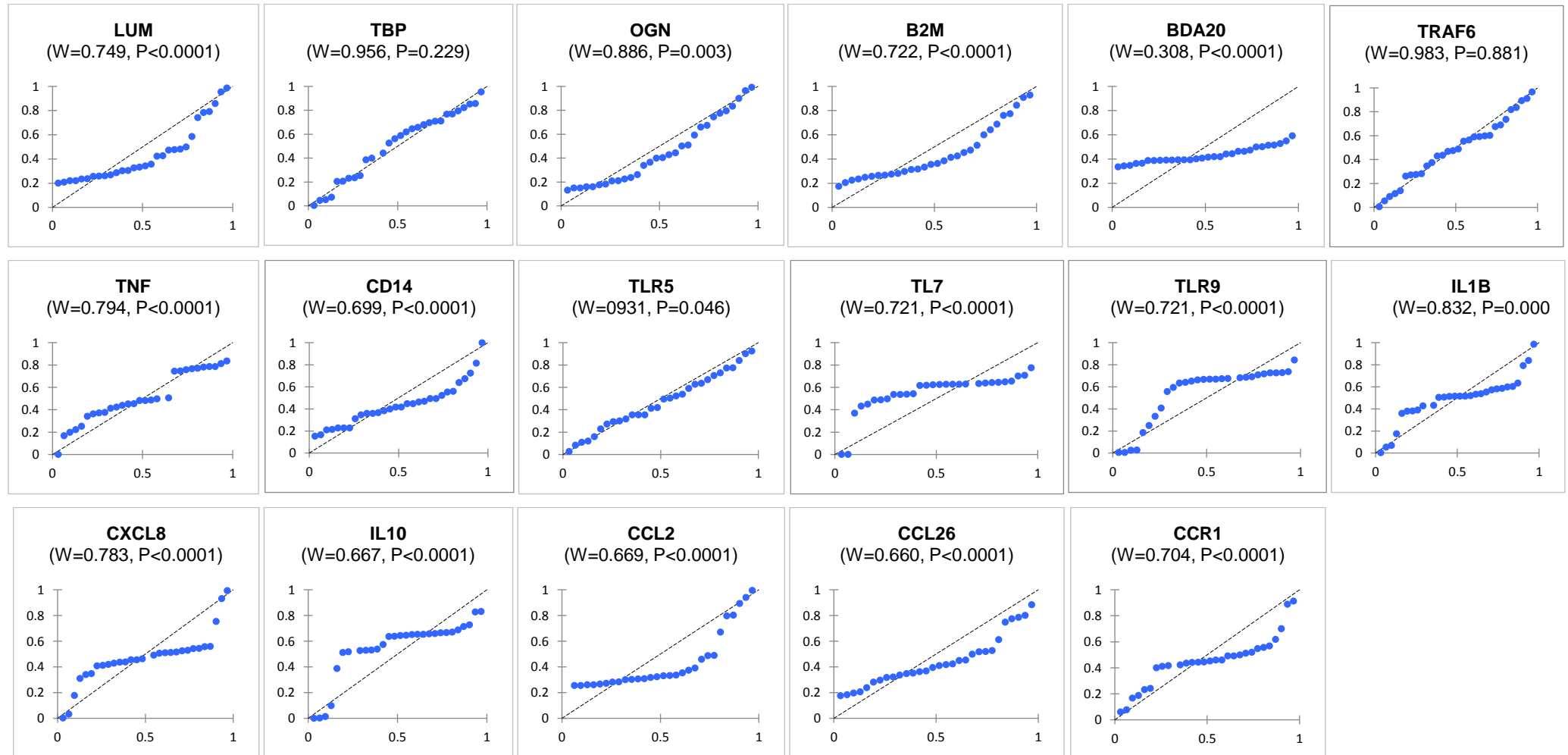


Figure 4.2: Fold regulation normal P-P distribution plots per gene of interest including Shapiro-Wilk (W) test and two-tailed p-values. Y-axis = Theoretical cumulative distribution and X-axis = Empirical cumulative distribution.

4.3.5 Expression levels

None of the genes expressed in the animal exhibited significant interaction between the main effects, breed and tick species. However, a Bonferroni pairwise comparison for the interaction between breed and tick species revealed a significant difference in the Least Square (LS) means of treatment Nguni-*R. decoloratus* (LS = 9.372) and Angus-*R. microplus* (LS = -0.405).

While the expression of the majority of the genes did not differ significantly according to breed, the expression levels of genes *TRAF6*, *TBP*, *LUM* and *B2M* were significantly different according to breed with P-values 0.039, 0.026, 0.012 and 0.023, respectively (Table 4.3). A Bonferroni pairwise comparison combined with a One-Way ANOVA of the breed types (Figure 4.3) revealed significant differences between the Nguni and Angus for *TBP* (P = 0.008) and *TRAF6* (P = 0.016), as well as between the Brahman and Angus for *LUM* (P = 0.003) and *B2M* (P = 0.007). None of the genes produced significant P-values (P > 0.05) for the main effect tick species as indicated in the pairwise test in Figure 4.4. The gene *TBP* on the other hand, had a P-value which was significantly lower than the rest of the genes and closely bordering significance and non-significance with P = 0.053.

Table 4.3: P-values and R² values produced by the general linear model for the gene of interest when investigated for the main effects breed and tick species.

Gene Symbol	P-values		R ² -value
	Breed	Tick species	
IL1B	0.162	0.244	0.122
CXCL8	0.498	0.841	0.049
IL10	0.229	0.113	0.100
CCL2	0.402	0.164	0.063
CCL26	0.164	0.190	0.121
CCR1	0.419	0.809	0.060
TLR5	0.760	0.182	0.019
TLR7	0.172	0.349	0.118
TLR9	0.135	0.487	0.133
CD14	0.315	0.626	0.079
TRAF6	0.039	0.909	0.206
TNF	0.521	0.263	0.045
OGN	0.528	0.659	0.045
TBP	0.026	0.053	0.229
LUM	0.012	0.472	0.280
B2M	0.023	0.351	0.236
BDA20	0.115	0.244	0.126

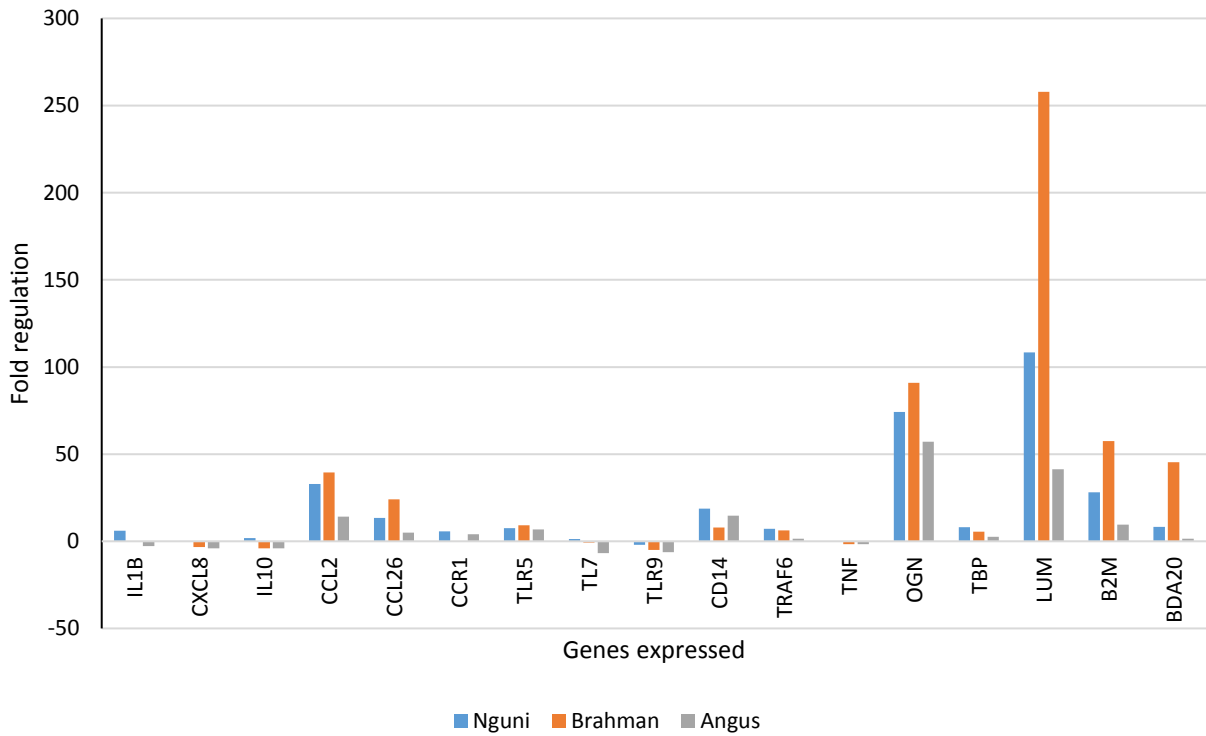


Figure 4.3: LS means, using fold regulation as a measure of the expression levels of 17 genes of interest in the Angus, Brahman and Nguni following tick infestations.

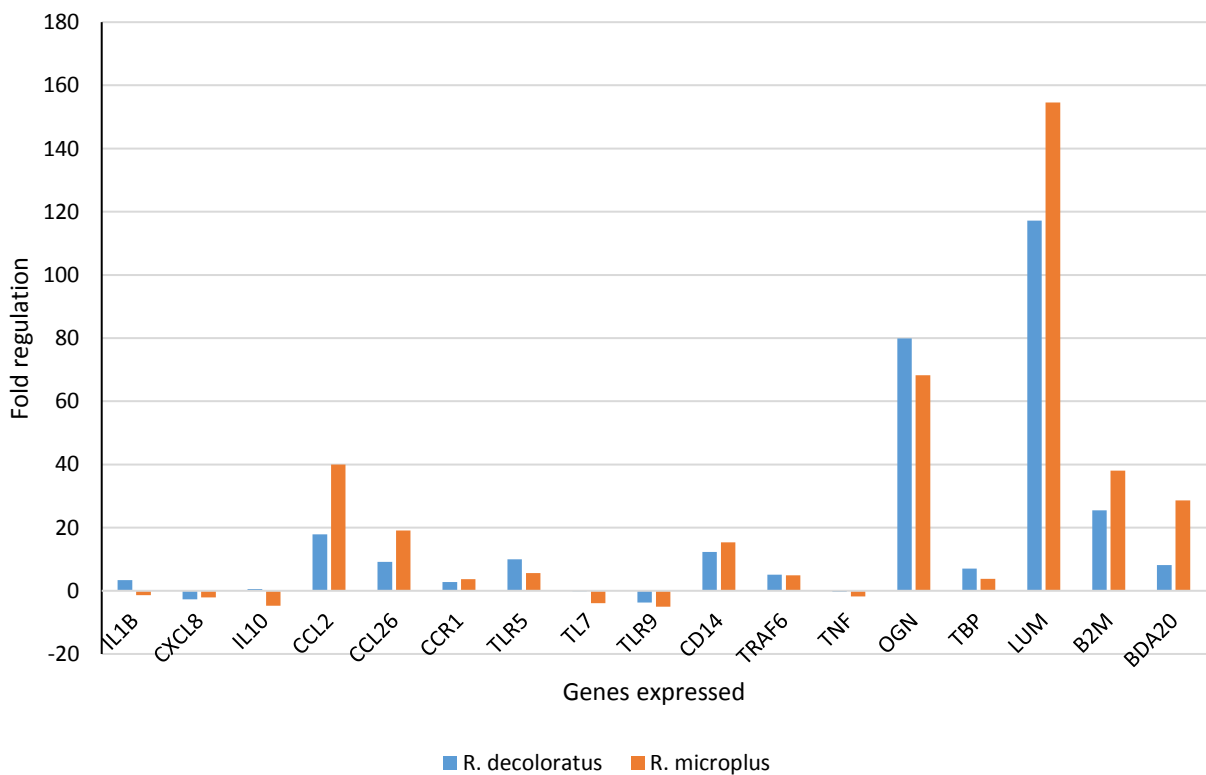


Figure 4.4: LS means of genes expressed in animals infested with *R. microplus* and *R. decoloratus*.

Table 4.4 lists the mean fold regulation values for each gene in the respective treatment groups. Table 4.5 shows a summary of the data presented in Table 4.4 to facilitate the identification of patterns of gene expression following challenge with ticks. There were increases in the expression levels of six genes (*CCL2*, *CCL26*, *CD14*, *OGN*, *LUM*, *B2M*) following challenge with ticks for all breed × tick species groups. Five genes (*CCR1*, *TLR5*, *TRAF6*, *TBP*, *BDA20*) increased expression or remained approximately equal after infestation with ticks for all groups. Conversely, completely mixed results were obtained in the breed × tick species groups for expression levels for the genes *IL1-β*, *TLR7*, and *TLR9*, while the expression levels of three genes (*CXCL8*, *IL10*, *TNF-α*) decreased or remained the same after tick challenge in all breed × tick species groups.

In the treatment group Angus-*R. decoloratus*, genes *IL1B*, *IL10*, *CCL2*, *CCL26*, *CCR1*, *TLR5*, *TLR7*, *CD14*, *TRAF6*, *OGN*, *TBP*, *LUM* and *B2M* were upregulated following infestation, with genes *CCL2*, *CCL26*, *CCR1*, *TLR5*, *CD14*, *TRAF6*, *OGN*, *TBP* and *B2M* showing over-expression. However, genes *CXCL8*, *TLR9*, *TNF-α* and *BDA20* were downregulated with *CXCL8* and *TLR9* showing under-expression. Treatment groups Brahman-*R. decoloratus*, Nguni-*R. decoloratus* and Nguni-*R. microplus* exhibited similar expression patterns to the Angus-*R. decoloratus* group. The exception was that genes *IL10* and *TLR7* were downregulated in the Brahman-*R. decoloratus* group, while *B2M* was downregulated in the Nguni-*R. decoloratus* and Nguni-*R. microplus* groups. Treatment group Angus-*R. microplus* had genes *CCL2*, *CCL26*, *CCR1*, *TLR5*, *CD14*, *OGN*, *LUM*, *B2M* and *BDA20* upregulated, among which *CCL2*, *CCL26*, *CCR1*, *CD14*, *OGN*, *LUM*, *B2M* and *BDA20* were over-expressed. The genes *IL1B*, *CXCL8*, *IL10*, *TLR7*, *TLR9*, *TRAF6*, *TNF* and *TBP* were downregulated with *IL1B*, *TLR7*, *TLR9* and *TNF-α* showing under-expression. An expression pattern similar to that of group Angus-*R. microplus* was observed in the group Brahman-*R. microplus* with the only difference being that gene *CCR1* was downregulated while *TRAF6* and *TBP* were upregulated in the latter group. Also presented in Table 4.4 is the trend that the Angus treatment groups, more specifically the Angus-*R. microplus* group produced the minimum expression values for all, but two (*CCR1* and *CD14*) of the genes of interest, while the Nguni groups produced maximum values for most of the genes with no specification of the tick species.

Table 4.4: Mean normalised fold regulation values for 17 genes of interest in each of the six treatment groups

	Angus-R. <i>decoloratus</i>	Angus-R. <i>microplus</i>	Brahman-R. <i>decoloratus</i>	Brahman-R. <i>microplus</i>	Nguni-R. <i>decoloratus</i>	Nguni-R. <i>microplus</i>
Host-tick associations	Modern	Modern	Modern	Ancient	Ancient	Modern
IL1B	0.578 ± 1.017	-6.075 ± 8.051	1.088 ± 1.215	-1.855 ± 2.799	8.600 ± 6.513 ^b	3.700 ± 2.434 ^b
CXCL8	-6.144 ± 2.724 ^a	-1.810 ± 5.707	-3.852 ± 1.447 ^a	-2.728 ± 0.384 ^a	1.960 ± 4.885	-1.682 ± 1.190
IL10	1.440 ± 1.565	-9.590 ± 5.718 ^a	-0.872 ± 1.159	-7.240 ± 8.456 ^a	1.042 ± 0.622	2.593 ± 1.140 ^b
CCL2	8.458 ± 1.137 ^b	20.008 ± 14.723 ^b	28.872 ± 9.652 ^b	50.195 ± 31.677 ^b	16.154 ± 7.806 ^b	49.570 ± 27.943 ^b
CCL26	5.622 ± 1.651 ^b	4.365 ± 6.819 ^b	13.610 ± 4.042 ^b	34.388 ± 25.918 ^b	8.236 ± 1.631 ^b	18.442 ± 3.575 ^b
CCR1	3.970 ± 1.309 ^b	4.038 ± 8.707 ^b	0.798 ± 1.487	-0.853 ± 2.191	3.540 ± 0.821 ^b	7.850 ± 2.579 ^b
TLR5	12.500 ± 6.521 ^b	1.123 ± 4.208	11.128 ± 2.790 ^b	7.095 ± 4.789 ^b	6.338 ± 1.309 ^b	8.713 ± 1.385 ^b
TLR7	0.516 ± 1.127	-13.965 ± 9.647 ^a	-0.844 ± 1.022	-0.518 ± 1.144	-0.112 ± 1.031	2.873 ± 0.767 ^b
TLR9	-2.544 ± 0.867 ^a	-10.005 ± 3.982 ^a	-6.338 ± 2.704 ^a	-3.585 ± 1.141 ^a	-2.320 ± 0.378 ^a	-1.547 ± 0.170
CD14	13.710 ± 3.127 ^b	15.575 ± 12.499 ^b	8.602 ± 1.865 ^b	7.240 ± 3.032 ^b	14.568 ± 1.864 ^b	23.092 ± 8.015 ^b
TRAF6	3.718 ± 1.896 ^b	-0.702 ± 2.893	5.274 ± 1.168 ^b	7.283 ± 5.018 ^b	6.378 ± 0.577 ^b	8.090 ± 1.993 ^b
TNF	-0.308 ± 0.910	-2.895 ± 2.791 ^a	-1.042 ± 1.053	-2.148 ± 0.468 ^a	0.122 ± 0.677	-0.448 ± 0.548
OGN	94.832 ± 49.478 ^b	19.308 ± 16.549 ^b	86.660 ± 33.819 ^b	95.198 ± 59.177 ^b	58.086 ± 4.828 ^b	90.150 ± 15.200 ^b
TBP	5.426 ± 1.216 ^b	-0.405 ± 2.497	6.416 ± 1.767 ^b	4.743 ± 3.702 ^b	9.372 ± 1.408 ^b	6.898 ± 0.952 ^b
LUM	49.326 ± 21.347 ^b	33.507 ± 15.499 ^b	218.752 ± 52.470 ^b	297.033 ± 172.467 ^b	83.512 ± 16.906 ^b	133.278 ± 46.208 ^b
B2M	7.108 ± 3.057 ^b	12.157 ± 6.034 ^b	45.954 ± 10.639 ^b	69.000 ± 43.197 ^b	23.478 ± 6.944 ^b	32.900 ± 14.198 ^b
BDA20	-1.156 ± 1.462	4.227 ± 1.706 ^b	17.444 ± 2.780 ^b	73.210 ± 67.850 ^b	8.176 ± 2.190 ^b	8.428 ± 3.377 ^b

*a: values below the cut-off threshold of -2 are categorised as under-expressed genes

*b: values above the cut-off threshold of +2 are categorised as over-expressed genes

Table 4.5: Relative change in expression for 17 genes of interest in each of the six treatment groups. Data are presented as arrows according to the magnitude of the normalised fold regulation values as follows: |fold regulation| <2 = ↔; fold regulation ≥ 2 = ↑; fold regulation ≥ 10 = ↑↑; fold regulation ≥ 100 = ↑↑↑; fold regulation ≤ -2 = ↓; fold regulation ≤ -10 = ↓↓; fold regulation ≤ -100 = ↓↓↓. Rows are coloured according to whether all groups showed an increase or equivalence of expression (light green) or a decrease or equivalence of expression (red/yellow), or were inconsistent (grey).

	<i>Angus-R. decoloratus</i>	<i>Angus-R. microplus</i>	<i>Brahman-R. decoloratus</i>	<i>Brahman-R. microplus</i>	<i>Nguni-R. decoloratus</i>	<i>Nguni-R. microplus</i>	
IL1B	↔	↓	↔	↔	↑	↑	
CXCL8	↓	↔	↓	↓	↔	↔	
IL10	↔	↓	↔	↓	↔	↑	
CCL2	↑	↑↑	↑↑	↑↑	↑↑	↑↑	
CCL26	↑	↑	↑↑	↑↑	↑	↑↑	
CCR1	↑	↑	↔	↔	↑	↑	
TLR5	↑↑	↔	↑↑	↑	↑	↑	
TLR7	↔	↓↓	↔	↔	↔	↑	
TLR9	↓	↓↓	↓	↓	↓	↔	
CD14	↑↑	↑↑	↑	↑	↑↑	↑↑	
TRAF6	↑	↔	↑	↑	↑	↑	
TNF	↔	↓	↔	↓	↔	↔	
OGN	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	
TBP	↑	↔	↑	↑	↑	↑	
LUM	↑↑	↑↑	↑↑↑	↑↑↑	↑↑	↑↑↑	
B2M	↑	↑↑	↑↑	↑↑	↑↑	↑↑	
BDA20	↔	↑	↑↑	↑↑	↑	↑	
Key	All increased	All decreased	All increased or no change	All decreased or no change	Mixed Inconsistent	Increased relative to other breeds	Decreased relative to other breeds

4.4 Discussion

Host-tick interactions in cattle, following *Rhipicephalus (Boophilus)* tick challenge, resulted in an array of complex differential gene expression profiles. Gene expression studies, using qPCR, have been considered to be one of the most effective approaches to advancing the comprehension of the genetic basis of tick resistance in beef cattle. Despite no significant interaction between breed and tick species, Rodriguez-Valle *et al.* (2013) reported significant differences in the expression of transcripts between *R. microplus* ticks that had fed on the tick-resistant Brahman, as opposed to those that fed on tick susceptible Friesian-Holstein cattle. This suggests that host-tick associations may have conferred the differences in the expression profiles of the genes of interest, although the variations were insignificant. In this study, the expression of 11 of 17 genes showed an increasing or stable level of expression in skin following challenge with ticks; three genes showed inconsistent patterns and three genes showed patterns characterised by decreasing or constant levels of gene expression after tick attachment. These results are broadly consistent with previous work (Piper *et al.*, 2008; Piper, 2010).

4.4.1 Tick species

No significant differences ($P > 0.05$) were observed in the expression patterns of all the genes of interest in cattle infested with either the *R. microplus* or the *R. decoloratus*. These two tick species are both from the subgenus *Boophilus* (The Center for Food Security and Public Health, 2007) and have been shown to share numerous morphological characteristics (Jongejan & Uilengberg, 2004). These might explain the lack of difference in their feeding signature which subsequently result in host gene expression profiles that are indistinguishable from each other between these two species.

Although, there is a lack of literature on the comparative genetics of the two species, a lot of work has been undertaken towards the sequencing of the genomes of the *Rhipicephalus* ticks (Willadsen, 2006). Studies have indicated that the wide variety of bioactive molecules contained in the tick's saliva may contain partially characterised immune-active proteins and lipids to induce vasodilatory, antihemostatic and immunomodulatory activities, to facilitate successful feeding (Wikel, 1996, 1999; Francischetti *et al.*, 2010; Oliveira *et al.*, 2010). Consequently, numerous candidate genes are differentially expressed and pathways are activated in the host animal as an attempt to re-establish homeostasis. Therefore, the feeding signatures of different tick species, as characterised by the differential host gene expression profiles, would be expected to differ among species shared morphological characteristics.

4.4.2 Breed differences

Piper *et al.* (2008) and Wang *et al.* (2007) support the data observed in the current study which shows significant differences in the expression profiles among breeds. Significant differences were observed

between the Angus and Brahman breeds for genes *LUM* ($P = 0.003$) and *B2M* ($P = 0.007$). In addition, significantly different expression levels were detected between the Nguni and Angus for genes *TBP* ($P = 0.008$) and *TRAF6* ($P = 0.016$). While this is in contrast with what other researchers have reported, Piper *et al.*, (2008) also reported to have observed breed-associated differential expression of gene *TRAF6*.

Noteworthy is the fact that all incidences of significantly higher fold regulation values were observed within the high and medium resistance breed groups, the Nguni and Brahman, respectively. With the exception of genes *TLR7*, *CXCL8* and *TNF*, none of the genes of interest were downregulated within the Nguni treatment combination groups. However, with the *Bos indicus* breeds, particularly the Brahman, often being classified as high resistance, it is rational to classify both the abovementioned breeds as high resistance (Jonsson, 2006; Wang, *et al.*, 2007; Machado *et al.*, 2010). Conversely, all but two (*CCR1* and *CD14*) of the genes of interest produced their lowest expression values and were often downregulated within the Angus-associated treatment groups, predominantly the Angus – *R. microplus* group. This directly contradicts the results of Piper *et al.* (2008) in which the low resistance animals had indications of high levels of inflammation. It was apparent in the current study that lower resistance cattle breeds had reduced inflammatory responses. This contradicts the perception that the increased level of tick resistance observed in tick-resistant animals is characterised by unique gene expression profiles rather than inflammatory responses.

4.4.3 Expression levels

The genes encoding the extracellular matrix constituents, most importantly *LUM* and *B2M*, were upregulated at much higher levels in the high (Brahman) and intermediate (Nguni) resistance breeds than the genes involved in immune system regulation and inflammatory responses. This was consistent the results by Piper *et al.* (2010), where a microarray study showed upregulation of genes encoding constituents of the extracellular matrix in the tick resistant Brahman cattle in comparison to the susceptible Holstein-Friesian cattle. Furthermore, Kongsuwan *et al.* (2010) highlighted the importance of the epidermal permeability barrier of the skin as an important component of resistance in cattle against ticks, which explains the heightened expression of these genes in the tick-resistant Brahman cattle. Although upregulated in the tick-susceptible Angus cattle, the effect of the inflammatory response genes in conferring tick resistance was minimal, however, this contradicted the study by Piper *et al.* (2008).

4.4.3.1 Other candidate genes

The genes within this class participated in tick resistance not by initiating host immune responses, but rather by promoting continued cellular regeneration, tissue repair and detoxification of the tick bite site. This activated the mechanism required to discourage long term supply of blood meal to the tick.

All the genes within this category (*BDA20*, *OGN*, *TBP*, *LUM*, *B2M*) were upregulated within all treatment combination groups, with three of them presenting significant between-breed differences, namely *TBP*, *LUM* and *B2M*. However, *TBP* and *BDA20* were downregulated in groups Angus – *R. microplus* and Angus- *R. decoloratus*, respectively. High values were detected for *LUM* within treatment groups Brahman – *R. microplus*, Brahman – *R. decoloratus* and Nguni – *R. microplus*. Furthermore, a high expression value was produced by *BDA20* in treatment group Brahman – *R. microplus*. The significance of the values produced by treatment group Brahman – *R. microplus* may be overrated resulting from the smaller number of samples retrieved for this group relative to the other groups. However, to validate the results detected for *BDA20*, Piper *et al.*, (2008) found differential expression for *BDA20*, while Wang *et al.* (2007) observed upregulation of this gene at much higher levels than the other genes of interest.

Of the four genes which were significantly differentially expressed between breeds, *LUM* was upregulated at a much higher level than all the upregulated genes. As a gene that encodes a member of the small leucine-rich proteoglycan (Weizmann Institute of Science, 2016a), *LUM* serves in conjunction with *OGN* to induce immune responses. Gene *OGN* similarly presented higher upregulation values than the rest of the genes of interest. Both *LUM* and *OGN* are capable of regulating fibril organisation and circumferential growth as well as epithelial cell migration in the process of tissue repair at the tick bite site (Weizmann Institute of Science, 2016a). The significantly high expression level of *LUM* in the Brahman animals more than the Angus suggested that the Brahman had a stronger capacity to prevent tick feeding through continuous tissue repair than Angus animals did. This was true for both tick species. Although *OGN* similarly produced high expression values, the between-breed differences were inconsequential. The results in this study were consistent with other studies by Piper *et al.* (2008, 2009) and (Kongsuwan *et al.*, 2008), therefore *LUM* shows potential as a biomarker for high host resistance to both *R. microplus* and *R. decoloratus* tick species.

Gene *B2M* has often been identified within the panel of housekeeping genes that exhibit mRNA expression stability in all cell types in a variety of tissues and under any environmental conditions. Conflicting results were obtained in this study in that *B2M* was differentially expressed at levels which were significantly different between the Brahman and Angus treatment groups. The gene *B2M* was upregulated in all the treatment groups, with much higher expression levels observed from both the

Brahman treatment groups. The *B2M* gene is a component of the MHC class I that is responsible for presenting peptide antigens (including tick antigens) to the immune system while simultaneously forming amyloid fibrils in pathological challenges (Weizmann Institute of Science, 2016b). Therefore, the significantly low *B2M* expression levels produced by the Angus animals imply that this breed's nucleated cells had a poor capacity to detect the tick antigens in order to prompt host immune responses.

The gene *BDA20* was upregulated in all treatment groups, displaying significantly higher expression levels in the group Brahman – *R. microplus* while a stable downregulation was observed in group Angus – *R. decoloratus*. *BDA20* has been thoroughly studied and characterized at sequence level (Prah et al., 1982; Rautianinen et al., 1997). However, there are limited records documenting the immunological functions of this gene, which warrants further studies of its role in tick resistance. *BDA20* was reported to share a wide range of immunological properties as *Bos d 2*, which is another lipocalin allergen with an affinity to bind IgE antibodies (Kinnunen, 2007). Confirmed to be the most predominant allergen in bovine dander belonging to the family of lipocalins (Mantyarvi et al., 1996), *BDA20* is presumed to participate in initiating first line inflammatory responses and detoxification of skin-related pathogenic challenges. It produces an allergic reaction to the tick antigen, consequently prompting the mobilisation of antibodies to the tick bite site. It was worth noting that *BDA20* was highly expressed in the Brahman animals while the Angus, which had large quantities of shedding dander-containing stratum corneum, produced low expression values and even downregulation of this gene. Furthermore, it was indicated that feeding ticks secrete lipocalins in their saliva to overwrite host immune responses (Wang et al., 2007). Interesting as it might have been to investigate the origin of the expressed *BDA20*, from the host or the tick saliva, such studies were beyond the scope of this study. However, *BDA20* also show potential as a biomarker for tick resistance in cattle.

Unlike *LUM*, the significant differences in the expression levels of *TBP* between the Nguni and Angus treatment group were unexpected. *TBP* is a component of the RNA polymerase III, as such it was expected to behave like a housekeeping gene that had expression levels that remained constant in all treatment combinations to facilitate continued cell growth; hence tissue repair regardless of the biological or environmental conditions (Vannini & Cramer, 2012). While *TBP* was upregulated in all treatment groups, the gene displayed a downregulated but stable expression level in group Angus - *R. microplus*. It is, however, evident that under stressful conditions, such as those inflicted by tick infestations, the regulatory protein *Maf1* may repress RNA polymerase III activity (Vannini et al., 2010). This would explain the downregulation of *TBP* in treatment group Angus – *R. microplus*, which in turn resulted in significant differences in expression levels between the Angus and Nguni groups, specifically between the Nguni – *R. decoloratus* and Angus – *R. microplus* treatment combinations.

4.4.3.2 Toll-like receptors

Toll-like receptors have been implicated as key role-players in a myriad of immune functions correlated to their ability to differentially express and initiate appropriate immune responses to various pathogenic invasions at the earliest stage of immune development (Kopp & Medzhitov, 1999; Menzies & Ingham, 2006). It is widely understood that toll-like receptors vary in abundance in response to the host's altered immune responsiveness upon detection of what is described by Menzies & Ingham (2006) as pathogen-associated molecular patterns (PAMP). This property was observed in this study supported by the significant differential expression of *TRAF6* between Nguni- and Angus-containing treatment groups. *TRAF6* is the only one within the *TRAF* family of proteins known to participate in signalling via *Toll/IL-1* receptors. *TRAF6* is activated by *IL-1 β* mediated stimuli (Kopp & Medzhitov, 1999), which explains the recognisable shared expression patterns observed between the two genes of interest. *TRAF6* produced a significantly lower fold regulation value in group Angus – *R. microplus* and was then classified as significantly under-expressed.

Gene *CD14* participates in host innate immunity following pathogenic challenge by conferring lipopolysaccharide sensitivity to neutrophils, monocytes and macrophages (Ibeagha-Awemu *et al.*, 2008). This gene encodes surface antigens, which recognise and bind monomeric lipopolysaccharide that are delivered to the *TLR4* complex to mediate cytokine secretion and inflammatory responses via *TRAF6* (Haziot *et al.*, 1996). *CD14* functions as a co-receptor to *TLR4* to confer lipopolysaccharide sensitivity to neutrophils, monocytes and macrophages (Ibeagha-Awemu *et al.*, 2008). This would explain the similar pattern of expression seen in the *CD14* gene with that of the toll-like receptors. Previous studies have reported a lack of evidence implicating circulating *CD14* levels in disease susceptibility (Haziot *et al.*, 1996). However, the expression of *CD14* on the surface of monocytes and neutrophils varied among Holstein cattle with different *CD14* genotypes (Ibeagha-Awemu *et al.*, 2008). The same findings were observed in this study in that *CD14* was expressed at different levels in the treatment groups with no significant effects in expression profiles attributable to tick species or breed.

Contrary to what Piper *et al.*, (2008) described, the panel of toll-like receptor associated genes analysed in this study exhibited expression patterns which were clearly different from each other. While genes *TLR5*, *TRAF6* and *CD14* were upregulated for all treatment combination groups, with the exception of stable but partial downregulation of *TRAF6* in group Angus – *R. microplus*, gene *TLR9* was downregulated in all treatment combinations and stable in group Nguni – *R. microplus*.

Judging from the combination of up- and downregulation levels, it was apparent that the expression profile of *TLR7* presented no affiliation for any breed or tick species. *TLR7* is one of the most abundant

TLR transcripts found in the skin of the bovine species (Menzies & Ingham, 2006). In this study, the only distinguishable trend in the expression profiles of *TLR7* was that the Brahman breed exhibited a slightly compromised ability to generate immune response to PAMPs via *TLR*-related mediums.

All the treatments exhibited heightened sensitivity to recognise and counteract target PAMPs through the activation of *TLR5*, *TRAF6* and *CD14*-associated responses. On the contrary, none of the treatment groups seemed to deploy *TLR9*-related responses. This may be the result of the gene's limited abundance among the 10 well-described *TLR* detected in bovine skin (Menzies & Ingham, 2006).

4.4.3.3 Cytokines

The role and genetic diversity of cytokines as signalling molecules in numerous physiological and immunological processes has been studied (Turner *et al.*, 2011). Initially, cytokines were described as orchestrators of the complex aspects of immunoregulation in murine species, however, with the co-evolution of tick and host species, ticks have developed strategies to modulate cytokine activity (Wikel, 1996).

The gene *TNF- α* was downregulated in all treatment combination groups, excluding the Nguni – *R. decoloratus* group in which it was upregulated. A stable pattern of expression was observed for *TNF- α* in all *R. decoloratus*-containing treatment groups. These results contradict data presented by Piper *et al.*, (2008, 2009) where overexpression of the gene *TNF- α* in both *Bos taurus* and *Bos indicus* breeds of cattle was observed. However, this could be attributed to the differences in the stages of inflammatory response between 12-hours post infestation (in the current study) and the 24-hours post infestation (Piper *et al.* (2008, 2009) study). *TNF- α* works in collaboration with *IL-1 β* to induce fever; thereby stimulating immune cell proliferation and differentiation following the parasitic invasion (Vilaek & Lee, 1991). It was interesting to note that in this study for most treatment groups the upregulation of *IL-1 β* was met by a downregulation of *TNF- α* . Similar to the finding of Wikel (1996), where a significant reduction in the release of *TNF- α* following the application of salivary-gland extracts from 0 - 9-day old engorging female ticks was observed, this study showed the downregulation of *TNF- α* in all treatment groups 12 hours-post infestation.

IL-1 β and *IL10* produced similar expression profiles to one another, except that in treatment group Brahman-*R. decoloratus* *IL-1 β* was upregulated whereas *IL10* was downregulated. Similar to *TNF- α* , gene *IL10* showed stable expression in all *R. decoloratus*-containing treatment groups. Oliveira *et al.*(2010) studied the effect of tick saliva on dendritic cells and similarly observed an increased production of the *IL10* and reduced synthesis of *TNF* following exposure to tick saliva. There was, however, no discernible affiliation for a specific breed or tick species in the present study. The multifunctional *IL10* is an immunoregulatory cytokine which, depending on the timing and site of

tissue collection during infection, may either aggravate or alleviate immunopathology through inhibition of Th1 cell, NK cell and monocyte-macrophage activity (Moore *et al.*, 2001; Couper *et al.*, 2008). Therefore, the upregulation of this gene is not always beneficial. Although no significant differences were observed in the expression profiles of *IL10* among the six treatment groups, a distinct difference was noted between the expression profiles of the Nguni and Brahman treatment groups. The Nguni cattle upregulated the gene following challenge with both tick species, while the Brahman downregulated the gene. *IL10* has been reported to limit the production and proliferation of proinflammatory responses by suppressing *IL-1 β* , *CXCL8* and *TNF* cytokine activity (Couper *et al.*, 2008). However, the expression trend described in this study does not comply with these reports. This could be attributed to the fact that the expression levels of neither cytokine was within a significantly high or low range. It is postulated that this may have been because the skin biopsy samples were collected 12 hours post infestation, potentially placing the inflammatory responses within the low to moderate range. Accordingly, the *IL10* activity in that regard would have resulted in moderate pathology prevention, ultimately allowing the tick antigen to temporarily escape immune control and facilitate continued feeding.

CXCL8 was also downregulated in all treatment groups. The host immune responses are generally downregulated by tick salivary gland extracts during feeding activities, although this occasionally benefits the host such as the inhibition of *CXCL-8* (Regitano & Prayaga, 2011). Contrary to a study by Regitano & Prayaga (2011), where the downregulation of the *CXCL8* gene was only observed in animals with a 50% Angus genotype, the gene was downregulated in all the breeds included in the current study. However, the results of the current study were in agreement with those conveyed by Hajnická *et al.* (2001) indicating the inhibition of *CXCL8* from binding to its receptors in humans, thus reducing the level of detection of the gene following exposure to salivary gland extracts from several ixodid tick species. These also included extracts from ticks belonging to the *Rhipicephalus* genus. Regitano *et al.* (2008) similarly implicated the presence of *CXCL8* binding proteins in the salivary gland extracts of some tick species as the culprit for the observed breed-specific pattern of *CXCL8* levels reduction following artificial infestation with *R. microplus*. The proinflammatory cytokine *CXCL8* mediates the activation of neutrophils and other effector blood cells and their chemotaxis and influx to the tick bite site (Cacalano *et al.*, 1994; Regitano *et al.*, 2008; Brossard & Wikel, 2016). Blocking this chemokine from binding to its receptors confers successful feeding by the tick (Brossard & Wikel, 2016). Therefore, none of the breeds exhibited tick resistance through the activation of the first line of defence of the innate immune response, which is mediated by the mobilisation of neutrophils when *CXCL8* binds to its receptors in response to the tick antigen at the tick bite site.

4.4.3.4 Chemokines together with their receptors

Inflammatory chemokines and their receptors are trophic molecules that play a crucial role in immune responses by inducing directed chemotaxis of localised effector immune cells to the tick bite site to re-establish homeostasis (Sarau *et al.*, 1997; Navratilova, 2006; Widdison & Coffey, 2011). Although the chemokines and chemokine receptors expression profiles are not yet been fully characterised in cattle, the receptors share numerous similar features with those of humans, albeit several species-specific immunome differences may exist (Widdison *et al.*, 2010; NCBI, 2016). The inherent robustness of the chemokine and chemokine receptor systems is thought to be on based the polyploidy and redundancy of the systems (Widdison *et al.*, 2010).

Numerous studies describe the up- and down-regulation of chemokines and their receptors in inflammatory and autoimmune diseases (Navratilova, 2006). The expression profiles observed for the two chemokine ligands (*CCL2*, *CCL26*) and the one receptor (*CCLR1*) forming the panel of genes were consistent with those reported by Piper *et al.* (2008, 2009). All the three genes were upregulated in all six treatment combination groups, with the exception of *CCR1*, which was downregulated but stable in the Brahman – *R. microplus* group and stable in group Brahman – *R. decoloratus*. Both chemokine ligands produced significantly higher fold regulation values in the Brahman – *R. microplus* group. Furthermore, significantly higher expression levels were detected for *CCL2* in the group Nguni – *R. microplus*. In contrast, Wang *et al.*, (2007) detected downregulation for *CCR1* and *CCL2*.

4.5 Conclusions

There were more similarities than differences in the gene expression profiles of the different breeds and tick species studied. Furthermore, no important tick species × breed interactions that would suggest differences according to the co-evolutionary history of tick species and cattle breeds were observed. The differential gene expression profiles of the genes of interest (*IL-1β*, *CXCL8*, *IL10*, *CCL2*, *CCL26*, *CCR1*, *TLR5*, *TLR7*, *TLR9*, *CD14*, *TRAF6*, *TNF-α*, *OGN*, *TBP*, *LUM*, *B2M* and *BDA20*) were not products of either ancient or modern host-tick associations. Therefore, the significant differences highlighted between some of the treatment combination groups were likely due to the breed effect. This view was supported by the observed significant differences between the Brahman and Angus, as well as the Nguni and Angus breed groups for genes *TRAF6*, *TBP*, *LUM* and *B2M*. As hypothesised, the Angus, being the low resistance breed, fell victim to the significantly low expression levels for the differentially expressed genes. It varied significantly from the Brahman and Nguni, demonstrating poor tick resistance as a result of compromised immune responses, while no significant differences were detected between the two high resistance breeds. It is likely that no significant differences in gene expression were found between the *R. microplus* and *R. decoloratus* treatment groups for any

of the genes of interest due to the high genetic and morphological similarities shared by these two tick species. Nonetheless, breed variations only accounted for approximately 30 percent of the observed variation in gene expression in all treatment groups. This suggests that the majority of the differential gene expression profiles produced in cattle post infestation with *Rhipicephalus (Boophilus)* ticks was likely due to a complex array of other factors in addition to variations in breed and tick species.

4.6 References

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CHAPTER 5

GENERAL DISCUSSIONS AND CONCLUSIONS

5.1 Summary of Findings and General Discussions

A number of differentially expressed candidate tick resistance genes have been identified as being associated with the tick resistance trait. These include the 18 genes in high-resistance cattle and 48 genes in low resistance Hereford Shorthorn cattle, associated with keratin, the extracellular matrix and immunoglobulin, that were identified by Wang *et al.* (2007) post-infestation with *R. microplus* ticks. In addition, Piper *et al.* (2008, 2009) identified several toll-like receptors (TLR5, TLR7, TLR9), chemokines together with their receptors (CCR1, CC12, CCL26), as well as cytokines (IL-1 β , IL-2R α , IL-2, IL-10, TNF- α , Traf-6, NFKBp50). Furthermore, the Bovine Leukocyte Antigen DQ (BoLA-DQ) lysozyme, cytokeratin or cytokines, interferon γ , tumour necrosis factor α and double amino acid residue motif marker (i.e. glutamic acid serine) were implicated in tick resistance (Morris, 2007). This was followed by identification of 138 differentially expressed genes and three fundamental pathways in a study by Kongsuwan *et al.* (2008).

In the current study, there were significant breed specific gene expression variations observed between the high-resistance Brahman and the low-resistance Angus cattle as well as between the Brahman and the intermediate-resistance Nguni cattle. While most of the 17 genes of interest, previously identified to influence tick resistance in the abovementioned studies, did not differ significantly according to breed, most of the breed-specific differences were not the core elements of the adaptive immune response, but included identified characteristics of the extracellular matrix, such as was described by Wang *et al.* (2007). These genes included *LUM*, *TBP*, *B2M* and *TRAF6*, which showed significant differences in their expression profiles in different breeds. These four genes have consistently appeared in literature as influencing tick resistance. These are mostly collagen-related genes, primarily involved in tissue repair at the tick bite site. Important among which was the *LUM* gene which had expression profiles that displayed a much higher degree of upregulation in the Brahman (*B. indicus*) than the Angus (*B. taurus*) cattle. *LUM* encodes products responsible for regulating collagen fibril organisation and circumferential growth, epithelial cell migration and cell repair at the tick bite site. It is likely that high-resistance cattle such as the Brahman have a stronger capacity to prevent continued tick feeding through continuous tissue repair unlike their low-resistance (Angus cattle) counterparts, in which the *LUM* showed diminished expression levels. Therefore, *LUM* shows potential as a biomarker for tick resistance in cattle. In addition, although insignificant differences were detected for BDA20 in the different host-tick associations, the higher expression

levels of this gene in the Brahman-*R. microplus* group in comparison to the Angus-*R. decoloratus* group make it a potential biomarker for tick resistance in cattle. The *TBP* gene may induce increased tick resistance in the Brahman cattle by collaborating with *LUM* to facilitate continued tissue repair despite biological or environmental conditions which may vary (Vannini & Cramer, 2012). On the contrary, the increased expression levels detected in the Brahman cattle for *B2M* suggested that the Brahman's ability to resistant ticks may be a factor of the increased capacity of nucleated cells to detect tick antigens so as to prompt host immune responses; a characteristic which might have been suppressed in the susceptible Angus cattle (Weizmann Institute of Science, 2016b).

There were differences among breeds in their ability to reject tick attachment and feeding till maturity. The Brahman cattle exhibited an increased ability to resist tick attachment following challenge with the *R. microplus* and *R. decoloratus* ticks in comparison to the Nguni and Angus cattle. No differences were detected in the tick burdens of the Nguni and the Angus, despite the Nguni carrying slightly less ticks than the Angus cattle 18-days post-infestation. The Nguni and Brahman breeds are known to offer an unmatched degree of resistance which is very quick to kick in when protection against ticks is needed. However, the tick count data and gene expression profiles obtained for the Nguni diverged from expectations.

None of the genes were significantly different with respect to type of tick species. Conversely, significant differences were detected between tick species in the host response with regards to the number of engorged ticks carried by the animal 18-days post-infestation. The *R. decoloratus* ticks had on average significantly lower attachment success rates than the *R. microplus* ticks in all three breeds.

There was no evidence of breed by tick species interaction that implicated a high degree of co-evolution in the gene expression profiles at the host-tick interface on the host's skin. Furthermore, although the breed by tick species interaction was significant with regards to tick counts, no pattern could be derived from the six treatment groups which would implicate the long-term co-evolutionary status of the breeds and tick species in the tick counts measured post-artificial infestation. This is likely due to the fact that the *R. microplus* and *R. decoloratus* tick species share numerous features which are likely to deem breeds that are resistant to one tick species equally resistant to the other tick species.

5.2 Recommendations for Future Studies

The diversity of tick species, cattle breeds and host-tick interactions continue to increase. Therefore, it is recommended that new genomic resources and new insight, in respect to the differential gene expression patterns conferred by the different tick species in the infested animals be acquired in

developing new chemical- free tick-infestation intervention strategies. The lack of information about the tick-specific effects on the gene expression profiles in the different host-tick associations warrants the need for in-depth tick count and gene expression comparative studies using more genetically and morphologically diverse tick species. Gene expression studies produce snapshots of the actively expressed genes following infestations. Therefore, transcriptomic studies which not only focus on a panel of previously identified genes of interest, but involve next-generation sequencing technology to profile thousands of expressed genes simultaneously, are required. This will provide a global picture of the cell activity at the host-tick interface in the different modern and ancient host-tick associations in an attempt to fully characterise the genetic components of tick resistance in cattle. RNA-sequencing studies, capable of producing sensitive and accurate transcriptome-wide coverage of gene expression changes pre- and post-tick-infestation, are recommended. This may aid in developing robust tick control approaches capable of purging barriers of tick control for purposeful execution against TTBDs in the beef production industry. Although gene expression studies are not likely to substitute the established approaches for studying tick resistance, they will certainly complement them in developing high-throughput modes of tick control.

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