The application of genomics and transcriptomics for the characterization of the genetic diversity of tick-resistance in Angus, Brahman, Nguni, and Santa Gertrudis cattle artificially infested with *Rhipicephalus microplus* and *Rhipicephalus decoloratus* ticks

by

Jacqueline Keena Marima

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Supervisor: **Prof Kennedy Dzama**

Co-supervisor: **Prof Nick Jonsson, Dr Bekezela Dube, Dr Munyaradzi Marufu**

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Declaration

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated) that reproduction and publication thereof by Stellenbosch University will not infringe any third-party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Summary

Tick resistance is a complex polygenic trait that is governed by immuno-genetic mechanisms that are currently not fully understood. It is, however, currently accepted that the *Bos indicus, Zebu* and *Sanga* breeds, which are better adapted to the harsh environmental conditions of the tropical and subtropical regions, often exhibit superior tick resistance as opposed to their *Bos taurus* counterparts. Breeding for natural host resistance presents a transformational alternative for tick control that will see cattle production industries move away from the excessive and often incorrect usage of chemical acaricides for tick control. However, selective breeding for this trait using tick count data, which varies according to the environment, is unreliable and often produces variable results. Marker-assisted selected breeding and the development of accurate prediction tests with practical feasibility in the field will enhance the accuracy of selection and increase genetic gains. Omics technologies, including transcriptomics, genomic and proteomics are tools that have been instrumental in uncovering putative genes, pathways, and potential biomarkers in cattle. Despite the progress made, there is still a lot that remains misunderstood about the tick resistance trait. Further studies are required.

While previous studies have investigated tick resistance in the different breeds and tick species in isolation of each other, in this study a comparison between two tick species (*Rhipicephalus microplus* and *R. decoloratus*) and three cattle breeds of different lineages was presented; the *Bos indicus* Brahman breed, the *Bos taurus* Angus breed and the indigenous *Bos taurus* africanus Nguni breed. The differentially expressed genes and their single nucleotide polymorphisms (SNPs) genotypes were of particular interest when studying the different host tick associations presented in this study. The study also included the transcriptome analysis of samples from the Santa Gertrudis, a composite breed recognised for its superior tick resistance while simultaneously boasting good reproductive and production efficiency and meat quality.

Using a 150K Bovine SNP chip to genotypes samples from the Angus, Brahman and Nguni breeds, the SNP genotypes, allele frequencies and dosages of SNPs of 37 candidate genes were determined. A total of 257 SNPs were discovered but the SABT2 gene produced a SNP (ARS-BFGL-NGS-94983) that showed a significant correlation with tick count and significantly different allele frequencies between breeds. No functional information regarding the role of the SATB2 gene in host resistance to tick, their further investigations are warranted.

The microarray analysis of blood samples from Santa Gertrudis cattle artificially infested with the invasive *R. microplus* tick species revealed variable levels of tick resistance accompanied by variable gene expression profiles across the tick-resistant and tick-susceptible phenotypes. It was evident

that upon long term exposure to the *R. microplus* the tick-resistant Santa Gertrudis cattle displayed an increased ability to develop and mount more robust adaptive responses against the tick infestations than the tick-susceptible animals

Lastly, the RNA sequencing study allowed the identification of several putative genes that have featured in previous studies of tick resistance in cattle. Using inter-breed and inter-tick species contrast across the Angus, Brahman, and Nguni cattle breeds as well as between the *R. microplus* and *R. decoloratus* tick species, variable gene expression profiles were observed. The CCL26 and MZB1 appeared as two of the factors to note in the inter-tick species comparisons, while the MMP12 gene was identified in the inter-breed comparison. The Nguni breed produces significantly different gene expression patterns than both the Angus and the Brahman breeds. The differential expression of the highlighted gene led to the conclusion that tick resistance is not only characterised by innate and adaptive immune responses but there are other crucial role players, presented in this study as components of the extracellular matrix.

The three studies included here inform further investigations into the roles of the highlighted genes and SNPs to further elucidate the complex phenotype of host resistance to ticks.

Dedications

This dissertation is a dedication to my late parents and grandmother, Lucas and Heather Marima and Stella Mphokane. Today I make you all proud. You invested so much in my education to get me to where I am now. Mama, I am deeply saddened that you had to leave before I even started my tertiary education journey, but you made sure to leave me in great hands. Papa did an immaculate job with me but sadly he had to come and join you before we had the chance to celebrate this milestone together. Gogo's prayer got me into varsity and pushing strong in times of difficulty and despair. Papa, I'm sorry I had to spend what could have been your 70th birthday (21st December 2021) glued to the screen, body aching, eye reddened and sleep-deprived trying to make this almost impossible deadline. I am here now, and I am bringing it home. I believe this is the best present I could have ever given you for your 70th birthday, completing the journey that we started together some many years ago. You both would have been proud because all your sacrifices have paid off. I promise to pay it forward and ensure that my nieces and nephews that you left in my care also get the opportunity to walk in my footsteps. This is the proudest moment of my life. Teary-eyed and fatigued and missing you both so much, I can finally say "I AM DONE…MAMA, PAPA, GOGO I MADE IT!!"

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Preface

This dissertation is presented as a compilation of six chapters.

Chapter 1 **General Introduction**

Chapter 2 **Literature review**
Applying 'omics technologies in the study of bovine host resistance to ticks.

Chapter 3 **Objective one:**
Compare SNP genotypes and frequencies, as well as describe the correlation between allele dosage and tick count, across breeds for a panel of 37 genes previously identified in genome-wide association studies.

Chapter 4 **Objective two:**
Analyze data from an Australian study that used DNA microarray analysis to investigate at a systems biology level, the extent to which immunity differentiates the tick-resistant phenotypes from the tick-susceptible phenotypes in the Santa Gertrudis breed artificially infested with R. microplus ticks.

Chapter 5 **Objective three:**
Profile the between-breed and between tick-species differential gene expression patterns and enriched pathways observed in the Angus, Brahman and Nguni cattle following artificial infestation with the R. microplus and R decoloratus ticks using RNA sequencing.

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Chapter 1: General Introduction

1.1 Background

The use of omics technologies; Genomics, Proteomics, Transcriptomics, and Metabolomics, has been instrumental in unpacking the system biology of tick bites and the host resistance phenomenon (Hill, 2017). Using omics technologies researchers have been able to determine host-pathogen interactions and observe alterations in gene expression and mutation through the identification of specific strand information, gene fusions, novel transcripts, and SNPs. These high throughput omics approaches have advanced to ensure fast turnaround time for large data generation. Although the data analysis associated with them might be daunting, newer and sophisticated but user-friendly algorithms and software are continuously being proposed to support the analysis of different data structures underlying different biological phenomena. RNA sequencing (RNA-Seq) in particular, haS created new platforms through which whole transcriptome annotation is possible. The information generated provides a global picture of the gene expression profiles underlying phenotypic, metabolic and physiological variability at the different developmental and disease stages as well as environmental influences at single-base resolution (Weikard et al., 2013). Several such studies involved reverse genomics, where candidate genes are first isolated followed by the identification of the origin and function of the respective genes (Fournier, 2017; Tabor et al., 2017). Be that as it may, the common recommendation that resonates across most studies is that further investigations need to be conducted before any inference of causality by any of the biomarkers or mechanisms can be made with confidence.

Recent genomic studies have focused on the development of accurate genomic prediction methods for tick resistance in cattle. Cardoso *et al.* (2015), who investigated the accuracy and bias of different methods for direct and blended genomic prediction in the select Braford and Hereford cattle populations resistant to cattle ticks, identified the single-step genomic BLUP prediction tool as yielding the highest accuracy with 84 to 93% improvement in prediction over the conventional pedigree BLUP (PBLUP) approach.

Animal breeding for host resistance is a proven effective strategy for reducing cattle tick infestations. However, the lack of a practically feasible test that can be applied in the field has hampered the effective use of this strategy for the improvement of beef breeds. The deleterious effects of cattle tick infestations on the susceptible taurine breeds in tropical and subtropical regions continue to preclude the use of British breeds in crossbreeding systems for beef breeds (Cardoso et al., 2015). The rapidly increasing economic losses experienced in the form of decreased cattle performance, hide devaluation, increased production costs due to constant vaccine and acaricide administration as well as the treatment of transmitted diseases have further exacerbated the preclusion of some

beef breeds from crossbreeding systems. However, the reported genetic variability in the heritability of the tick resistance trait has invigorated the well-funded effort towards the development of genetic resources for the improvement and genomic prediction of tick resistance in beef cattle (Mapholi et al., 2014; Cardoso et al., 2015).

Cattle develop differential immune responses to tick feeding that often inhibit subsequent feeding. Nevertheless, the genetic mechanisms involved in generating the appropriate immune responses continue to be misunderstood. The attachment and feeding dynamics in most host-tick associations remain poorly understood but progress is being made. More so, co-infection dynamics of two closely related tick species such as the *Rhipicephalus microplus* and the *R. decoloratus* are yet to be understood. There is evidence that British cattle breeds often acquired total immunity to ticks upon long-term exposure, however, immunity is not sustained leading to a subsequent relapse and increased susceptibility. It is yet to be investigated if tick resistance in the British breeds is a result of hyperallergic skin response while resistance in Indicine or Sanga breeds may be an immune-based response.

In Southern Africa, the spread of the introduced Asian cattle tick *R. microplus* has caused an emerging increase in bovine mortalities due to the transmission of anaplasmosis and babesiosis. This is in addition to challenges caused by the indigenous *R. decoloratus,* which has conversely been experiencing geographic displacement by the Asian relative (Nyangiwe et al., 2013). Moreover, tick bites induce an allergic reaction to red meat in humans (Cabezas-Cruz et al., 2017) which highlights the negative impact of ticks not only on animals but humans as well. Alternately, new anti-tick vaccines and acaricides for cattle are being developed with rapid progress being made.

Despite all the above mentioned, two questions remain to be answered: (1) Why should vaccines be developed rather than producing tick resistant cattle (Hue et al., 2017); (2) Which is the most economically sound and sustainable approach to stimulate appropriate protective and long-lasting immunity to ticks? Is it through the development of novel vaccines or could it be through the production of tick-resistant, chemical-free reared cattle through adjuvant selection programs or might a cocktail of the two approaches yield far better results to support a sustainable parasite protection strategy (Tabor et al., 2017)?

It is important to improve the understanding of the genetic basis of host resistance and lead to the development of practically feasible tick resistance prediction tests. The project builds on data from a related previous study on host resistance in cattle using candidate gene expression, proteomics, genome-wide association, and immunology assays. Using the deep RNA sequencing approach on parasitized and non-parasitized skin biopsy samples from three cattle breeds infested with tick species, the study describes the transcriptomes at the host-tick interface and isolate and annotate the novel coding and non-coding transcripts in the various ancient and modern host-tick associations.

1.2 Problem statement

The influx of ticks and tick-borne diseases (TTBDs) continues to pose a threat to the multi-million-dollar cattle production industry consequently endangering food supply. Chemical acaricides are the main source of tick control. However, cattle need multiple administrations of the acaricide before they can get relief. This practice is both costly and unsustainable. Current tick control methods are unsustainable for reasons including: 1) the rapid development of resistance by ticks to chemical control agents; 2) the cost and affordability of acaricides and insecticides particularly for smallholder farmers; 3) the contamination of the environment by chemical control methods resulting in the disturbance of terrestrial ecosystems; 4) the contamination of the animal products with chemical residues after use. One million cattle were estimated to have died due to East Coast Fever in South Africa in 1989 (Mukhebi et al., 1992). An estimated R500 million in South African and 30 billion dollars globally is spent annually on resources used to control TTBDs (Mapholi et al., 2014). Therefore, researchers have been tasked with finding cost-effective, environmentally sound, and sustainable tick control alternatives.

R. microplus and *R. decoloratus* tick species are important ectoparasites in South Africa. In addition to the deleterious effects associated with blood-sucking, these species transmit parasites that cause East Coast fever, babesiosis and anaplasmosis among others. Unfortunately, the wide variety of immune active proteins and lipocalins contained in the saliva of these species, which induce vasodilatory, anti-haemostatic and immunomodulatory activities to facilitate successful feeding and disease transmission to the host, have been poorly studied. There is a lack of in-depth analyses of the adaptive immune response and immune transcriptomes at the tick bite site which can be used to identify correlates of protection during tick infestations.

1.3 Justification

Gene expression studies produce snapshots of the active genes following infestation. Conversely, transcriptome analyses provide a global picture of the cell function following infestation by profiling coding and non-coding transcriptional activity and gene expression. Transcriptomic, involving next-generation sequencing technology, diverts from just profiling a panel of pre-selected candidate genes but profiles thousands of expressed genes simultaneously to fully characterize the myriad of genes at the host-tick interface in the modern and ancient tick-host associations. This transcriptomic study will not only map resistance to *R. microplus* and *R. decoloratus* in the key beef breeds used in the industry but will also identify the key candidate gene.

In gaining an understanding of how tick feeding induces the differential expression of tick-resistance related genes in the indicine cattle breeds as compared to taurine cattle breeds, the future development of efficacious vaccines or even chemical-free tick intervention strategies may be possible. The elucidation of the immunogenetic complex of bovine tick resistance lays the foundation

of the framework for the selection of tick resistant cattle. It is thus important to generate a good understanding of the causal mechanisms of tick resistance to develop tick resistant phenotypes through accurate genomic prediction tests for tick resistance with practical feasibility in the field that can be applied without having to muster the animals and conduct tick counts before selection.

To combat food insecurity, it is important to minimize the global material footprint through sustainable consumption and production patterns, as is outlined in SDG 12. Between the years 2000 and 2015 human-induced processes attributed towards the degradation of more than a fifth of the earth's total land area. Therefore, this project aligns with the Sustainable Development Goal (SDG) 15 which, in part, aims to promote sustainable agriculture and sustainable use of terrestrial ecosystems, respectively. By decreasing the use of chemical tick control approaches, which contaminate the environment and potentially alter the terrestrial ecosystems, the contribution of the beef production industry to biodiversity loss may be diminished.

1.4 Hypothesis

The central hypothesis for the study was that the presence of a unique array of tick-resistance conferring genes, unique to different cattle breeds, creates an immunologically disadvantageous micro-environment at the tick-host interface of the bovine host's skin that influences the level of tick resistance exhibited by the different cattle breeds. Therefore, variable tick burdens have been observed in the different breeds. It is further postulated that the activity of transcripts not yet annotated in the bovine genome may play a regulatory role in the expression of the complex phenotype of superior tick resistance displayed in some cattle breeds more than others. The following null hypotheses were outlined:

H_0 (1): The genotype of the animals does not affect the tick-burdens experienced by the animals.

H_0 (2): There are no between- or with-breed differences in gene expression patterns exhibited by the host following tick infestations.

H_0 (3): The are no differences in gene expression patterns displayed by the host following infestation with ticks of different species.

1.5 Research Aim

The aim of the research was to use SNP genotyping, transcriptome analysis technologies (RNA sequencing and DNA microarrays) to characterize the within- and between-breed genetic diversity of tick resistance in the Angus, Brahman, Nguni, and Santa Gertrudis cattle following artificial infestation with R. *microplus* and R. *decoloratus* larvae.

1.6 Objectives

1 Compare SNP genotypes and frequencies, as well as describe the correlation between allele dosage and tick count, across breeds for a panel of 37 genes previously identified in genome-wide association studies.

2 Analyze data from an Australian study that used DNA microarray analysis to investigate at a systems biology level, the extent to which immunity differentiates the tick-resistant phenotypes from the tick-susceptible phenotypes in the Santa Gertrudis breed artificially infested with R. microplus ticks.

3 Profile the differential gene expression profiles and enriched pathways observed in the Angus, Brahman and Nguni cattle following artificial infestation with the *R. microplus* and *R decoloratus* ticks and identify the potential biomarkers and pathways actively involved in bovine tick resistance using RNA sequencing.

1.7 Potential Outcomes

The results of this research will inform the development of more accurate selective breeding for high host resistance which may reduce mortalities and production losses associated with the *R. microplus* and *R. decoloratus*, and the pathogens that they transmit. Additionally, it will inform the transformation towards the reduced use of acaricide but rather the use of resistant breeds and selected resistant individual animals that experience lighter tick burdens. This research will contribute to the groundwork laid for the development of robust and effective vaccines for susceptible animals in the beef production industry.

1.8 References

Cabezas-Cruz, A., Mateos-Hernández, L., Villar, M., Alberdi, P., Gortazar, C., Agustín, E.-P., José, de la F., 2017. Immunity to α-Gal: implications for tick-induced allergy and the control of vector-borne pathogens, in: One Health - 9th Tick and Tick-Borne Pathogen Conference & 1st Asia Pacific Rickettsia Conference. Cairnes, Australia, p. 16.

Cardoso, F.F., Gomes, C.C.G., Sollero, B.P., Oliveira, M.M., Roso, V.M., Piccoli, M.L., Higa, R.H., Yokoo, M.J., Caetano, A.R., Aguilar, I., 2015. Genomic prediction for tick resistance in Braford and Hereford cattle. J. Anim. Sci. 93, 2693–2705. https://doi.org/10.2527/jas.2014-8832

Fournier, P.-E., 2017. Genomics of Rickettsia species, in: One Health 9th Tick and Tick-Borne Pathogen Conference & 1st Asia Pacific Rickettsia Conference. Cairnes, Australia, p. 6.

Hill, C., 2017. Combating tick-borne diseases on a global scale: a roadmap for tick research in the genomics

era, in: One Health 9th Tick and Tick-Borne Pathogen Conference & 1st Asia Pacific Rickettsia Conference. Cairnes, Australia, p. 7.

Hue, T., Mermoud, I., Rantoen, D., Lua, L., Munroe, C., Tony, V., Petermann, J., 2017. Prospecting the use of a novel Bm86-based vaccine for integrated Rhipicephalus australis management in New Caledonia, in: One Health - 9th Tick and Tick-Borne Pathogen Conference & 1st Asia Pacific Rickettsia Conference. Cairnes, Australia, p. 65.

Nyangiwe, N., Harrison, a., Horak, I.G., 2013. Displacement of Rhipicephalus decoloratus by Rhipicephalus microplus (Acari: Ixodidae) in the Eastern Cape Province, South Africa. Exp. Appl. Acarol. 61, 371–382. https://doi.org/10.1007/s10493-013-9705-7

Tabor, A., Bellgard, M., Guerrero, F., Valle, M.R., 2017. Reverse vaccinology for cattle ticks - a successful approach, in: One Health - 9th Tick and Tick-Borne Pathogen Conference & 1st Asia Pacific Rickettsia Conference. Cairnes, Australia, p. 95.

Weikard, R., Hadlich, F., Kuehn, C., 2013. Identification of novel transcripts and noncoding RNAs in bovine skin by deep next-generation sequencing. BMC Genomics 14, 789. https://doi.org/10.1186/1471-2164-14-789

Cabezas-Cruz, A., Mateos-Hernández, L., Villar, M., Alberdi, P., Gortazar, C., Agustín, E.-P., José, de la F., 2017. Immunity to α-Gal: implications for tick-induced allergy and th e control of vector-borne pathogens, in: One Health - 9th Tick and Tick-Borne Pathogen Conference & 1st Asia Pacific Rickettsia Conference. Cairnes, Australia, p. 16.

Cardoso, F.F., Gomes, C.C.G., Sollero, B.P., Oliveira, M.M., Roso, V.M., Piccoli, M.L., Higa, R.H., Yokoo, M.J., Caetano, A.R., Aguilar, I., 2015. Genomic prediction for tick resistance in Braford and Hereford cattle. J. Anim. Sci. 93, 2693–2705. https://doi.org/10.2527/jas.2014-8832

Fournier, P.-E., 2017. Genomics of Rickettsia species, in: One Health 9th Tick and Tick-Borne Pathogen Conference & 1st Asia Pacific Rickettsia Conference. Cairnes, Australia, p. 6.

Hill, C., 2017. Combating tick-borne diseases on a global scale: a roadmap for tick research in the genomics era, in: One Health 9th Tick and Tick-Borne Pathogen Conference & 1st Asia Pacific Rickettsia Conference. Cairnes, Australia, p. 7.

Hue, T., Mermoud, I., Rantoen, D., Lua, L., Munroe, C., Tony, V., Petermann, J., 2017. Prospecting the use of a novel Bm86-based vaccine for integrated Rhipicephalus australis management in New Caledonia, in: One Health - 9th Tick and Tick-Borne Pathogen Conference & 1st Asia Pacific Rickettsia Conference. Cairnes, Australia, p. 65.

Nyangiwe, N., Harrison, a., Horak, I.G., 2013. Displacement of Rhipicephalus decoloratus by Rhipicephalus microplus (Acari: Ixodidae) in the Eastern Cape Province, South Africa. Exp. Appl. Acarol. 61, 371–382.

https://doi.org/10.1007/s10493-013-9705-7

Tabor, A., Bellgard, M., Guerrero, F., Valle, M.R., 2017. Reverse vaccinology for cattle ticks - a successful approach, in: One Health - 9th Tick and Tick-Borne Pathogen Conference & 1st Asia Pacific Rickettsia Conference. Cairnes, Australia, p. 95.

Weikard, R., Hadlich, F., Kuehn, C., 2013. Identification of novel transcripts and noncoding RNAs in bovine skin by deep next-generation sequencing. BMC Genomics 14, 789. https://doi.org/10.1186/1471-2164-14-789

Chapter 2: Literature Review

2.1 Introduction

The complexity of host-tick associations in cattle is a phenomenon that is little understood. There is a dearth of literature on the differential immune responses of bovine hosts to tick challenge. Tick resistance from the parasite's perspective has been extensively studied in Brazil and Australia on the mechanisms by which ticks achieve and maintain feeding and disease transmission. However, the diverse microenvironments affiliated with different cattle breeds have posed a challenge in detailing the infection processes, as well as genetic, immunological, and histopathological components of tick resistance in the bovine species. The most well-studied examples of cellular responses to external stimuli include pathogen-induced phenotypic changes in host cells which are often directly correlated with marked modulation of associated genes (Jenner and Young, 2005). It is yet to be determined whether tick resistance in cattle is a result of a complex but coordinated innate immuno-genetic responses or merely an element of hyperallergic skin responses.

Some cattle breeds develop differential immune responses to tick feeding that often inhibit subsequent feeding. However, the attachment and feeding dynamics in most host-tick associations and the genetic mechanisms involved remain poorly understood. More so, studies on the co-infection dynamics of two closely related tick species such as the *Rhipicephalus microplus* and the *R. decoloratus* are limited. There is evidence that British cattle breeds often acquire total immunity to ticks upon long-term exposure, however, immunity is not sustained leading to a subsequent relapse and increased susceptibility. The costs associated with the relapse and treatment thereof are economically significant (Tabor et al., 2017a). These breeds exhibit sustained innate, immediate cell-mediated hypersensitivity-type or inflammatory reactions in response to an infestation (Piper et al., 2009), while the *B. indicus* and *Bos taurus africanus* hosts display less intense immediate immune reaction and employ delayed hypersensitivity reactions to acquire and sustain superior resistance (Piper et al., 2008; Marufu et al., 2013). The stimulus of infection may influence the maintenance of a solid immunity in the Nguni breed (Muchenje et al., 2008a). Purebred *Bos taurus* (*B. taurus* or British breeds) cattle rarely reach the superior levels of tick resistance exhibited by *B. indicus* and *Bos taurus africanus* breeds. The difference can be attributed to differences in the genetic composition; hence, applying breeding for host resistance can be an effective strategy for reducing tick loads on cattle. However, the lack of practically feasible breeding tests has hampered the effective use of this strategy.

To develop prediction tests for tick resistance in cattle that can be implemented in large scale agricultural breeding programs, it is necessary to employ low-cost technologies that can provide more information. Breeding for tick resistance requires accurate genetic determination, which can

be achieved by the use of genomic technologies. The genetic variability in the tick resistance trait has invigorated the well-funded effort towards the development of genetic resources for the improvement and genomic prediction of tick resistance in beef cattle (Frisch, 1999; Mapholi et al., 2014; Cardoso et al., 2015). For example, integrated tick control programs incorporating the application of gene editing technologies and tick-antigen derived vaccines could potentially produce environmentally sound, safe and effective tick and tick-borne diseases (TTBDs) control strategies (de la Fuente, 2018). Previously, transcriptome analyses lacked in both processing power and quality of results produced thus limiting the overall picture generated of the genetic profiles of tick resistance. The host immune mechanisms and the tick's feeding signatures should be understood to explore the more sustainable approach of harnessing the host's natural tick resistance mechanisms and developing novel vaccines.

Over the past decade, the profiling of tick resistance genes in cattle has transitioned from using PCR-based SNP assays to microarrays and now towards high-throughput genotyping by sequencing (Teng & Xiao, 2009). The introduction of RNA sequencing (RNA-seq) using next-generation sequencing (NGS) technology is a gradually advancing technique that streamlines the study of tick resistance while addressing the biological heterogeneity of different cattle breeds challenged with various cattle tick species (Colgan et al., 2017). The transcriptional outputs produced by RNA-seq may be instrumental in providing insights into the complex genetic networks mediating host-tick interactions in cattle. The information generated provides a global picture of the gene expression profiles underlying phenotypic, metabolic and physiological variability at the different developmental and disease stages as well as environmental influences at single-base resolution (Weikard et al., 2013). Several such studies involved reverse genomics, where candidate genes were first isolated followed by the description of the origin and function of the respective genes (Fournier, 2017; Tabor et al., 2017b).

Despite all the above mentioned, one major question remains to be answered: (1) Which is the most economically-sound and sustainable approach to stimulate appropriate protective and long-lasting immunity to ticks (Hue et al., 2017)? Is it through the development of novel vaccines or could it be through the production of tick-resistant, chemical-free reared cattle through adjuvant selection programs? Or might an integrated approach that involves the systematic application of two or more approaches yield far better results to support a sustainable parasite protection strategy (Tabor et al., 2017a; Rodriguez-Vivas et al., 2018)? Although an integrated approach would yield the best results for the control of TTBDs, the basic science behind TTBDs and their associated bovine hosts are still incomplete (de la Fuente, 2018). The application of the latest omics technologies could lend credibility to a better understanding of the evolution and function of host-tick interactions (de la Fuente, 2018).

2.2 Overview of beef production in South Africa

The beef industry contributes between 25 and 30% towards the annual agricultural outputs, making it the fastest-growing commodity in South African agriculture following the broiler sector (Musemwa et al., 2008). This industry consists of three co-existing major groups of farmers: the commercial farmers, the emerging farmers and the communal farmers (Nyangiwe, 2017). Commercial farmers use synthetic and/or crossbred animals resulting from the cross of *Bos indicus* or Sanga type breeds (DAFF, 2018). On the other hand, the emerging farmers use indigenous crossbred or exotic type breeds. The communal farmers use mostly indigenous breed types (DAFF, 2015; Nyangiwe, 2017). The beef cattle breeds used in the feedlot sector are fractioned into 29% Sanga types, 27% European types, 26% British types, 11% Zebu types and 7% diary or other breed types (DAFF, 2015). The benefits of using indigenous breeds in the beef production industry have been underestimated; hence their contribution to the formal national economy is limited, owing to their slow growth and low carcass yield (Chingala et al., 2017; Mapiye et al., 2019).

Beef production is practised all over South Africa. Eighty percent of viable agricultural land is suitable for stock farming, with 80% of the cattle production industry belonging to beef production (DAFF, 2015). Seventy percent of the beef production takes place in extensive production systems where the *R. decoloratus* and *R. microplus* tick species coexist (Figure 2.1) (Horak et al., 2009; Madder et al., 2014; Chingala et al., 2017). Of the 3.1 million beef cattle found in the Eastern Cape Province, 65 % are communally reared thus greatly exposing them to heavy tick infestations (Nyangiwe, 2017). The success of beef production, therefore, depends on the extent to which tick infestations can be controlled. Costs associated with tick control include administration of acaricides and vaccines, treatment of diseased animals and those the relapse. This influences the production costs which need to be minimised to improve profitability. Indiscriminate use of acaricides also leads to the accumulation of chemical residues in animal products and the environment. Together with increased consumer preference for organic, free-range, and chemical-free beef and beef products, the elevated cost of acaricidal control of TTBDs has forced the beef production industry to explore alternative chemical-free tick control approaches (Mapholi et al., 2014). The estimated annual cost of tick challenges and their control to the livestock industry in South Africa alone is between R70 – R550 million per annum (Mapholi et al., 2014; Nyangiwe, 2017). More recent estimates of economic losses from TTBDs range from US$20 to US$30 billion per annum (Lew-Tabor and Rodriguez Valle, 2016).

Securing the current and future values of SADC indigenous cattle breeds together with their genetic diversity and incorporating them into crossbreeding programs could be key in the production of reservoirs for genetic variation. This may result in cattle herds with superior tick resistance through selection (Frisch, 1999). The profitability and success of livestock production enterprises stand to be diminished through the losses incurred as a result of TTBDs should alternatively sustainable and cost-effective tick control methods not be developed (Grisi et al., 2014). It is essential to understand

the natural tick resistance trait exhibited by indigenous breeds as a means of enhancing tick resistance within beef herds without the use of chemical tick control strategies.

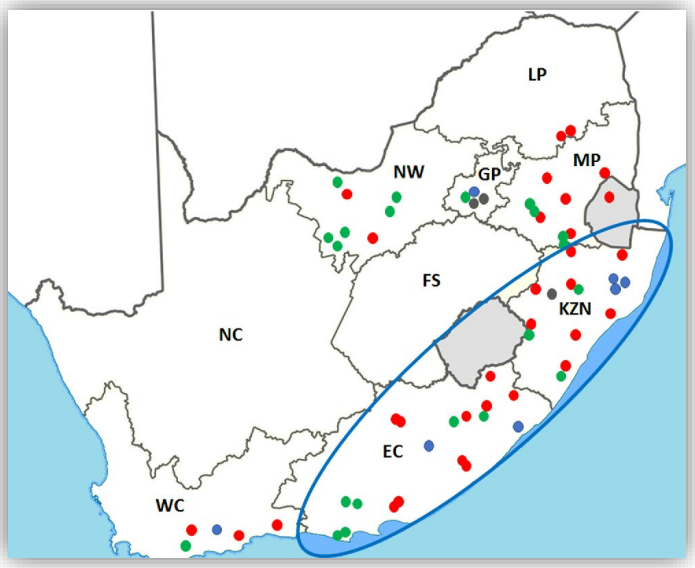


Figure 2.1: Distribution pattern of the *R. decoloratus* and *R. microplus* in South Africa. The green dots represent the *R. decoloratus*, the blue dots represent the *R. microplus*, the red dots represent areas where the two tick species co-exist, and the grey dots are where other cattle tick species could be found. The circled area represents the eastern coastal belt where both tick species are most abundant (Adapted from Baron, van der Merwe, *et al.*, 2018).

Tick and tick-borne diseases are considered the most important external parasites affecting approximately 80% of cattle populations globally (Nyangiwe, 2017; Burrow et al., 2019). Losses are experienced in the form of acaricide administration and the treatment of tick-borne disease. Furthermore, heavy infestations may lead to the formation of scar tissue (or granuloma) at the tick bite site which has been directly associated with hide devaluation and detrimental effects on cattle performance (Walker et al., 2003; Grisi et al., 2014).

2.3.1 African tick species diversity

The African continent supports over 650 tick species from seven genera (Mapholi, 2015). Of the seven genera, the *Rhipicephalus*, *Amblyomma*, *Hyalomma* and the subgenus *Rhipicephalus* (formerly Boophilus) prevail (Walker et al., 2003; Jongejan and Uilengberg, 2004). Figure 2.2 illustrates the distribution of the three major tick species in Africa, excluding the subgenus Boophilus. Eighty tick species have been identified in Southern Africa (Nyangiwe, 2017). Some of the tick species that are of importance from a veterinary perspective include the *Ambyomma hebraeum* (South African bont tick), the *Rhipicephalus appendiculus* (brown ear tick), the *R. evertsi evertsi* (red-

legged tick), the *R. microplus* (Asiatic blue tick) and the *R. decoloratus* (African blue tick) (Mapholi et al., 2014; Nyangiwe, 2017).

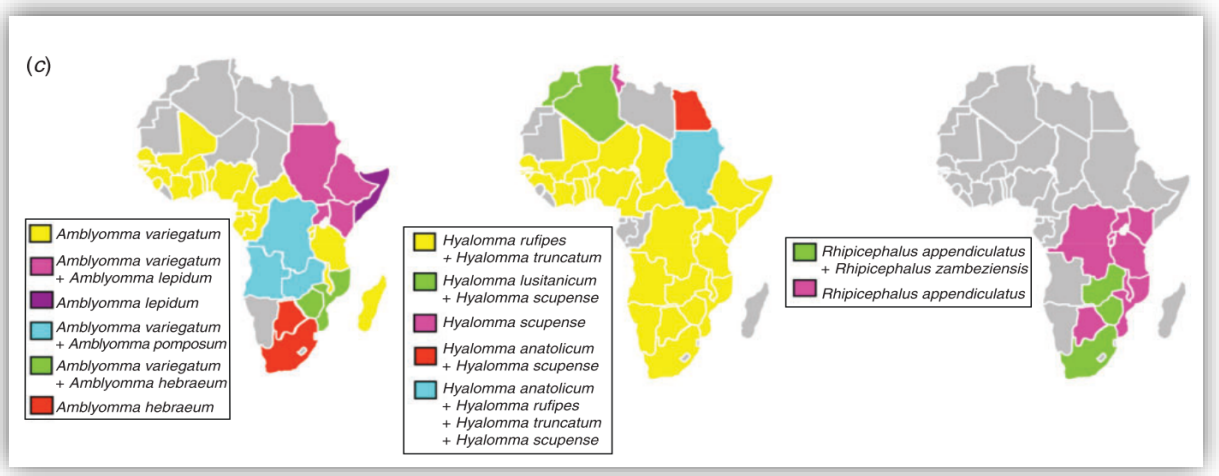


Figure 2.2: Distribution patterns of the major economically important cattle tick species in Africa, excluding subgenus Boophilus, as informed by data from studies by Walker and Olwage (1987) and Walker et al. (2003, 2000). The grey areas represent areas where ticks from that specific genus are absent. (Adapted from Burrow et al., 2019)

The genus *Rhipicephalus* subgenus *Boophilus* evolved in Africa. Arguably, some species from this genus evolved in the Middle East which is in contact with Africa (Barker and Walker, 2014). Figure 2.3 illustrates the distribution of the *R. microplus* and the *R. decoloratus* tick species in South Africa.

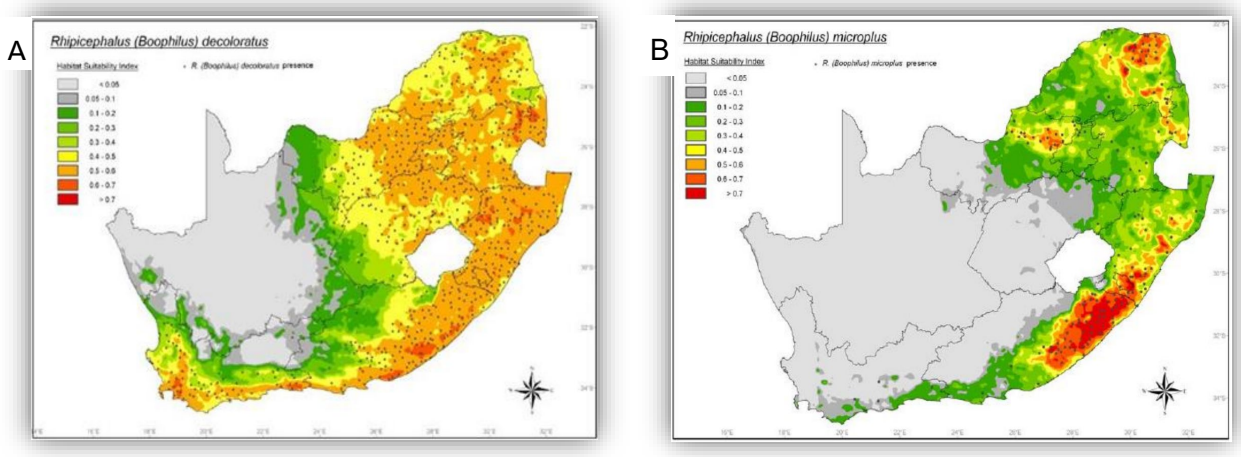


Figure 2.3: Distribution patterns of *R. microplus* (A) and *R. decoloratus* (B) mapped according to habitat suitability (Adapted from Nyangiwe, 2017). The black dots represent the tick populations (Adapted from Nyangiwe, 2017).

The South African indigenous *R. decoloratus*, commonly known as the African blue tick, is the most prevalent and widespread one-host tick species infesting cattle in Southern Africa (Walker et al., 2003). This tick species is considered less dangerous than the *R. microplus*. The *R. decoloratus*

transmits the less pathogenic *Babesia bigemina* and A*naplasma marginale* while its counterpart transmits both *B. bovis*, *B. bigemina*, and *A. marginale* protozoans (De Castro, 1997; Walker et al., 2003).

The Asiatic blue tick, *R. microplus*, evolved in China and other parts of Asia (Barker and Walker, 2014). The *R. microplus* shares numerous features with the *Rhipicephalus australis* (*R. australis*), with male ticks often showing features both the *R. microplus* and the *R. australis* (Berry et al., 2017). These two tick species transmit the same diseases and cause similar problems in the areas where they occur (Tabor et al., 2017b). As such, it would be expected that studies describing the feeding signature of the *R. australis* could potentially be aligned with research conducted on the *R. microplus* and vice versa. On the contrary, although morphological differentiation of the two tick species could not be achieved, subsequent studies identified the COX1-mitochondrial gene known to discriminate between the two tick species; thus placing *R. microplus* in a distinct clade from *R. australis* (Berry et al., 2017).

The anticipated climate-driven expansion of the distribution of some tick species to northern latitudes and higher latitudes (Dantas-Torres, 2015) warrants the study of variables and interacting factors which constitute the complex nature of host-tick interactions. Habit fragmentation can change host dynamics and influence risk to TTBDs (Nadolny et al., 2017). Changes in habitat type are ideal for introducing non-native tick species to an area, such as the *R. microplus* was introduced to South Africa. *R. microplus* has been one of the most successful invasive tick species in Southern Africa (Madder *et al.*, 2011). It has successfully established viable populations in certain regions of Southern Africa while displacing the indigenous *R. decoloratus* species in some regions (Horak, Nyangiwe *et al.*, 2009; Nyangiwe *et al.*, 2013). The *R. microplus* boasts a shorter life cycle and a higher reproductive potential compared to *R. decoloratus* (Walker et al., 2003; Horak et al., 2009). This has enabled the *R. microplus* to compete successfully against the *R. decoloratus* even displacing it in several areas in Southern Africa (Nyangiwe et al., 2013). The introduction of such invasive tick species as the *R. microplus* has motivated the need to optimize natural adaptability traits in locally used indigenous breeds and their European counterparts through gene introgression and selective breeding programs.

2.3.2 Species life cycle

The *R. microplus* and *R. decoloratus* tick species exhibit a monotropic behaviour with the maintenance host often being cattle. Illustrated in Figure 2.4 is the sequence of events during attachment, feeding, and moulting in the typical one-host life cycle of the *Boophilus* tick species. Whereas ticks rely on a constant supply of bloodmeal to complete their life cycles, the absence of DAP36 and SALP15 in the salivary glands of larval ticks is evidence that the larval stage of a tick's life cycle does not ingest blood (Franzin et al., 2017). Higher concentrations of transcripts encoding DAP36, SALP15, evasins, and matrix-degrading metalloproteases were observed in the salivary

glands of nymphs fed on tick susceptible hosts compared to those that had fed on resistant hosts (Franzin et al., 2017). On the contrary, ticks fed on resistant hosts presented more transcripts encoding basophil activating cysteine proteases. This could be associated with the diminished ability of ticks feeding on resistant hosts to evade host inflammatory responses. The different instars of the feeding tick stimulate different immune responses in the host animal (Franzin et al., 2017; Robbertse et al., 2018).

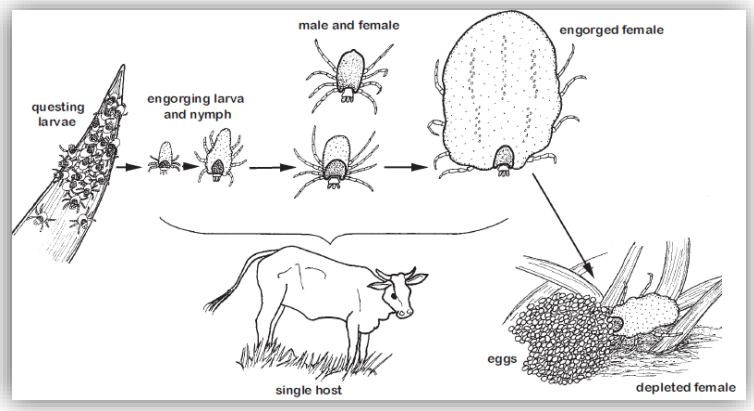


Figure 2.4: One-host tick life cycle (Adapted from Walker et al, 2003)

Similarly, the expression of different immunity constituents may also differ depending on the host tissue (i.e., skin, blood, lymphatic tissue) studied. These species can often be found feeding on the back, upper legs, neck, shoulder, dewlap and belly, in order of preference, of their cattle hosts (Walker et al., 2003). Previous artificial infestation studies have attempted to mimic the feeding preferences of the ticks by creating controlled, artificial microclimates on the animal's body, away from direct sunlight and potential predators, for tick attachment and feeding. It is thought that the act of mate-guarding ensures the propagation of the male's genes (Nuttall, 2017). This involves the male feeding on the host followed by mating with a female then continued feeding alongside the female.

2.3.3 Tick feeding mechanisms

Ticks are hard long-term feeders which access and maintain their bloodmeal by tearing the host epidermis and dermis with their mouthparts and secreting saliva concentrated with a cocktail of bioactive molecules into the host skin. In susceptible breeds, these salivary components may impair components of the extracellular matrix and neutralize numerous host defence components including coagulation, inflammation and wound repair components (Franzin et al., 2017). There is evidence that the different immune profiles of different hosts affect the expression of transcripts encoding components of salivary secreted inhibitors of host lymphocyte proliferation and signal transduction (DAP36 and SALP15, respectively), matrix-degrading proteases, lipocalins, cysteine proteases and chitinases and evasions (a class of chemokine-binding proteins).

Tick saliva is a complex xenobiotic substance that contains a wide array of immune-modulatory proteins that act to reduce T-lymphocyte proliferation, chemotaxis of the T_H1 cytokines interleukin-1 and interferon-γ, production of macrophage cytokines interleukin-1 and tumour necrosis factor and antibody responses at the tick bite site (Wikel, 1996; Francischetti et al., 2010; Mans et al., 2017). The complex cocktail of bioactive molecules in the saliva released at the host-tick interface are inclusive of wound healing counteractive molecules, vasoconstrictive molecules, and anticoagulants. These molecules facilitate blood-feeding and pathogen transmission by modulating host haemostasis and innate and adaptive immunity (Nuttall, 2017). The immunomodulatory and local immunosuppressive effects of tick saliva are achieved through the interference of leukocyte extravasation and migration to tick attachment site from blood vessels. The immunosuppressive effects are more intense in susceptible cattle breeds than in resistant breeds (Cardoso et al., 2015). However, it is yet to be understood if the salivary antimicrobial peptides (AMPs) can modulate skin microbiota in tick-infested cattle (Tabor et al., 2017a). Tick rejection has been associated with elevated histamine concentrations at the tick bite site (Tatchell, 1987). This concurs with Franzin et al. (2017), who observed higher concentrations on lipocalins, that bind small molecules like histamine, serotonin, leukotrienes ad volatile odorants, in the salivary glands of ticks fed on tick susceptible hosts. Therefore, ticks produce anti-inflammatory histamine binding lipocalins, which bind two molecules of histamine, as well as a tryptase inhibitor in susceptible cattle to prevent tissue remodelling as well as modulate itching and pain responses (Paesen et al., 1999; Mans, 2005; Nuttall, 2017). In tick resistant cattle blood histamine concentration spike to reach a peak following infestation with tick larvae (Riek, 1962).

Saliva activities during feeding include controlling the host's immune responses, establishing molecular individually, water balance, dynamic anti-chemokine activities and cement protein production to create a gasket and holdfast to keep the tick in place when feeding (Nuttall, 2017). Inflammatory responses surrounding the tick cement cone in the skin of the host often facilitate the tick attachment. However, in the genetically tick-resistant breed, the inflammatory responses destabilize the cone preventing normal engorgement by the tick and even resulting in death at the feeding site (Franzin et al., 2017). The salivary gland pairs differ among individual ticks therefore distinguishable species-related feeding signatures can be observed (Nuttall, 2017). Therefore, although the *R. microplus* and the *R. decoloratus* exhibit shared morphological characteristics, the host's immune responses to their attachment and feeding may differ.

Given the variation that exists in tick species, tick resistance by a particular breed may be species-specific. The variation is manifested in the characteristics of 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. Therefore, the tick species type, as well as the origin and diversity of the tick

population, may influence the degree of resistance by a breed type and ultimately the outcome of genetic selection for tick resistance in cattle (Burrow et al., 2019). Strains of tick species rapidly evolve to adapt to different host environments by altering their feeding signatures and mechanisms for successful feeding (Fournier, 2017). Consequently, ticks can transition between various feeding dynamics and microenvironments of different cattle breeds, ultimately achieving successful feeding endeavours on breeds with which no co-evolutionary history is shared.

2.4 The effect of breed on tick resistance

There are at least three distinct lineages of domestic cattle: *Bos taurus taurus* (European taurine); *Bos taurus indicus* (Indicine or Zebu); *Bos taurus africanus* (Sanga and Zenga). Taurine and indicine cattle are thought to have diverged 610,000-850,000 years ago (Machugh et al., 1997). Taurine breeds, most suited for milk and beef production, are best adapted to the temperate environmental conditions of Europe and the Near East. Alternately, Indicine breeds are better adapted to the tropical and subtropical environmental and socio-economic conditions of Asia and Africa (Machugh et al., 1997). These include breeds that evolved for specialised milk and beef production. To these two distinct groupings, a third group known as the “tropically adapted taurine breeds” was recently recognised. These are true taurine breeds that evolved in tropical environments to produce individuals with retained productive attributes of the taurine group but also the improved adaptability to the tropical environments of an indicine breed (Frisch et al., 1997). These include the African indigenous Afrikaner, Tuli and the South African indigenous Nguni breeds (Burrow et al., 2019). There are about 40 recognised indigenous cattle breeds in the SADC region, namely the Sanga (*Bos taurus africanus*: e.g., Nguni, Tuli and Tswana) and the Zebu breed types (*Bos indicus*: e.g., Malawi Zebu and Angoni) (Frisch et al., 1997). To these, a new breed class called the “Zenga”, a cross between the Zebu and Sanga (e.g., Bovines of Tete, Blukwa, Sukuma), has been added (Mwai et al., 2015; Zwane et al., 2016).

With the aid of many studies (Burrow et al., 2019), it is recognised that the Bos indicus breeds of cattle are particularly more resistant to external parasites than the Bos taurus breeds. Whereas the tropically adapted Bos taurus exhibit improved tick resistance than the European Bos taurus cattle. However, their degree of resistance remains inferior to that of the Bos indicus breeds (Spickett and Malan, 1978; Muchenje et al., 2008). Centuries of natural selection have enabled indigenous breeds to adapt to the local environment, producing individuals with improved tolerance and resistance to TTBDs, heat stress, disease and parasites, feed and water shortages in every successive century (Mwai et al., 2015; Lashmar et al., 2019). Therefore, it would greatly be beneficial to study these indigenous animals at a genetic level to elucidate the immunobiological mechanisms underlying their endemic resistance to environmental stressors such as ticks.

2.5 Immunobiological mechanisms of tick resistance

The immunobiological mechanisms of tick resistance in cattle affect ticks in several ways. The four currently known effects on the tick are by 1) interrupting the completion of the tick's life-cycle and disrupting the female tick's completion of its parasitic life-cycle; 2) minimizing the number of ticks that successfully attached and feed; 3) preventing full engorgement of adult female ticks and decreasing the mean weight of replete females; and lastly, by 4) impairing the tick's fertility, ova production and viability and egg-laying potential (Wikel, 1996). Currently, phenotypic aspects including the depression of the host's productive and reproductive traits, form the gold standard for distinguishing tick resistant cattle from tick susceptible cattle for selective breeding purposes (Burrow et al., 2019). Greater accuracy for selecting animals with superior resistance to ticks breeding may be achieved given a comprehensive understanding of heritable genetic components of tick resistance.

Studies that describe the immunobiological mechanisms of tick resistance in cattle date back to the 1960s. Despite the use of low throughput technologies, a few preliminary functions of the host's innate, adaptive, and cell-mediated immune responses mounted during tick infestations were elucidated. These studies were particularly instrumental in confirming the presence of breed-specific differences in the immunobiological mechanisms of tick resistance in cattle. The later advent of genome-wide association studies (GWAS) further expanded on the use of genomic selection to obtain the most promising improvement in tick resistance in cattle. A comprehensive knowledge of the genetic markers for tick resistance, based on NGS, SNP and QTL data, may aid the sustainability of low- and medium-input beef producers (Burrow et al., 2019). This section reviews the immuno-genetic mechanisms influencing the level of tick resistance in cattle.

2.5.1 Role of the adaptive and cell-mediated inflammatory responses

The skin serves as the key interface for the complex immunological activities arising from host-tick interactions (Wikel, 1996). Although host cutaneous reactions and defence mechanisms against tick infestations are currently little understood (Franzin et al., 2017), histological changes in the skin have formed the basis of immunohistopathological studies to characterise host-tick interactions. The gradual development of cutaneous responses in susceptible hosts facilitates successful tick feeding endeavours compared to resistant hosts. Atopic dermatitis and elevated self-grooming stimulations make up the adaptive immune responses expressed by resistant hosts. The skin of tick-susceptible cattle presents more transcripts that encode enzymes responsible for tissue detoxification. However, these enzymes simultaneously produce volatile semiochemicals that render tick-susceptible cattle more attractive to ticks (Franzin et al., 2017). On the contrary, the intense formation of epidermal lesions, spongiosis, microvesicles, vesicle and bullae is implicated in the mounting of robust tick resistance in genetically tick-resistant cattle (Franzin et al., 2017).

Early immunological responses at the host-tick interface are pro-inflammatory with a marked increase in the activation of regulatory and effector pathways leading to the saturation of the tick feeding foci with antigen-presenting cells, antibodies, T-lymphocytes and other bioactive molecules (Wikel, 1996; Marufu et al., 2014, 2013; Severo, 2013; Wikel, 1996). Resident skin cells which serve as the first line of defence against ticks upon contact with tick saliva or hypostomes include dendritic cells, Langerhans cells (epidermal dendritic cells), endothelial cells, T-cells, macrophages, fibroblasts, and keratinocytes (Kazimírová et al., 2017; Nuttall, 2017). The high infiltration of the larval attachment site with leukocyte phenotypes of a particular type has been associated with the development of more robust adaptive immune responses and subsequent protection against heavy tick burdens (Robbertse et al., 2018). Unfortunately, ticks produce saliva with a dense population of pharmacologically-active molecules, including dendritic cell modulators, to prevent the maturation of dendritic cells, and various other populations of immune cells, that can recognize and pickup tick antigens to promote innate and adaptive immune responses (Nuttall, 2017; Šimo et al., 2017). Figure 2.5 illustrates the host-tick interface and the various bioactive molecules secreted with tick saliva to modulate host immune responses and facilitate successful tick attachment and feeding.

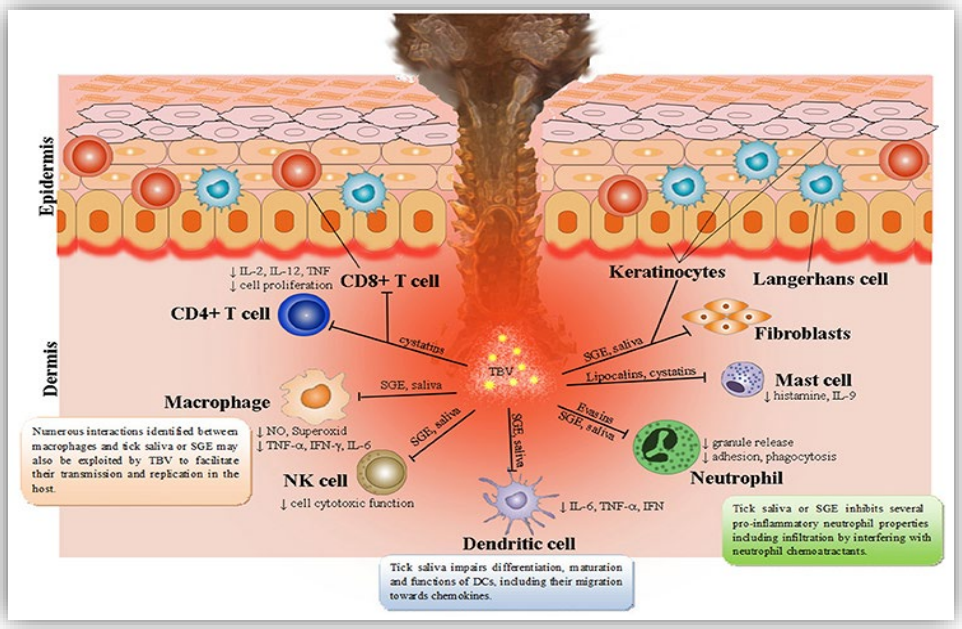


Figure 2.5: The tick-host interface following tick attachment. Upon attachment and insertion of the hypostome, the tick secretes saliva which is heavily concentrated with a diverse network of bioactive molecules with immunomodulatory and immunosuppressive roles to facilitate successful feeding. Lacerations in the host's skin bring the mouthparts of the tick in contact with a host of immune cells, including dendritic cells, neutrophils, B and T cells, mast cells, keratinocytes, fibroblasts, receptors of components of the innate immune system, anti-microbial peptides, chemokines, and cytokines as the first line of inflammatory defence and wound repair. This initial interaction between the tick and host results in responses that resemble allergic contact dermatitis (Kazimírová *et al.*, 2017).

Previous studies have highlighted the protective role of strong T-cell-mediated responses against the larval stage of tick infestations (Constantinoiu et al., 2010; Jonsson et al., 2014; Franzin et al., 2017; Robbertse et al., 2018). Franzin et al. (2017) reported significantly higher numbers of $CD3^+$ T

lymphocytes at the host-tick interface of nymphally-infested resistant cattle than that of susceptible cattle. This was in agreement with Robbertse et al. (2018), who observed significant increases in $CD3^+$ T-lymphocytes and tangible body macrophages in the lymph nodes of tick resistant (Brahman and Bonsmara) animals. The authors further noted a post-infestation increase in B-lymphocytes in susceptible (Holstein-Friesian) animals, whereas $CD20^+$ and $CD79a^+$ B-lymphocytes showed no fluctuation in the resistant animals. Franzin et al. (2017) further reported on the elevated populations of T γδ $WC1^+$ lymphocytes in resistant cattle. These could be significantly associated with the efficiency of resistant host immune systems to recognise nymphal tick salivary molecules and damaged host tissue, relative to a susceptible host. These results corresponded with those reported by Domingues et al. (2014), where a correlation was detected between T γδ cell activity and the immunological mechanisms of superior tick resistance in cattle following infestation with *R. microplus*. On the contrary, Robbertse et al. (2018) observed invariable increases in T γδ T-lymphocytes in the lymph nodes of susceptible (Holstein-Friesian) cattle in comparison to the more resistant Brahman and Bonsmara cattle infested with *R. microplus*.

Franzin et al. (2017) did not observe significant differences between tick resistant and tick-susceptible cattle for total cell counts of local cellular infiltrates and mononuclear cells present during inflammatory reactions to larval tick bites. This was also true for basophil counts. However, basophil counts were higher in tick-resistant cattle following nymphal infestation. There was also a significant increase in recruited cells (including $CD3^+$ T Lymphocytes) as the ticks matured from the larval to the nymphal stage in the resistant host. Eosinophil counts were higher in larvally-infested tick-susceptible cattle than the tick resistant cattle, with a reversed trend observed following nymphal infestation. Similar numbers of neutrophils were presented in both larvally- and nymphally-infested skin samples from both tick-resistant and tick-susceptible cattle. The mast cells were, however, greater in larvally-infested skin samples from susceptible cattle while counts were similar in nymphally-infested skin samples from resistant cattle relatives. In their study, Marima et al. (2020b) observed increased levels of lymphocytes and decreased levels of segmented neutrophils in tick-susceptible Angus cattle 12 hours post-infestation with larval ticks as opposed to the tick-resistant Brahman and Nguni cattle.

Adaptive immune responses are coupled with acute hypersensitivity reactions to the tick antigens (Burrow et al., 2019). These changes are typical of eosinophilic proliferation to the tick bite site (Riek, 1962). Given the tick's ability to modulate the host's local haemostatic reactions, resistant hosts may influence the downregulation of the expression of anti-haemostatics in tick salivary glands. However, genetically susceptible cattle do not have the same advantage. The local haemostatic reactions may be delayed in susceptible hosts thereby increasing blood clotting time at the tick bite site, consequently availing haemorrhagic pools for longer feeding events by the tick (Carvalho et al., 2010). The possibility of the host manipulating the expression of certain genes in the tick presents a potential criterion to explore in the selection of tick resistant cattle.

2.5.2 Role of the innate immune system

Riek (1962) presented the earliest evidence of the innate immune system's participation in modulating the tick resistance phenotype. Candidate systems and gene markers affecting tick resistance have previously been identified through GWAS studies, with the double amino-acid residue motif marker (glutamic acid serine) showing prominence. This marker is located on the bovine 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). In a study profiling 30 bovine Class-1 lymphocyte antigens, Stear et al. (1988) isolated antigens W6 and CA31 for their association with tick susceptibility in 75% Brahman x 25% Shorthorn crossbred calves. However, their effect on tick resistance was not definite given numerous unidentified factors that may influence the activity of both antigens (Burrow et al., 2019). In a follow-up study, Stear et al. (1990) reported on the diminished *R. microplus* tick burdens observed in cattle that possessed the bovine major histocompatibility (BoLA) antigens W6.1 and W7. Martinez et al. (2006) also concluded on the association of the BoLA marker DBR alleles with bovine resistance to *R. microplus* ticks. Morris (2007) later reported contradicting results that implicated the BoLA gene marker in 60% of the cattle exhibiting 96% tick susceptibility.

Transcriptomic data have connected the differential expression of chemokines, cytokines, and their receptors to the expression of differing levels of tick resistance observed within and between breeds. The expression of pro-inflammatory cytokines and chemokines (e.g. TNF, IL-1, IL-17 AND IFN-γ) (Franzin et al., 2017) leads to the chemotaxis of granulocytes and T-cells to the tick bite site and stimulates the release of histamine from basophils (Piper et al., 2008). Franzin et al. (2017) suggested that the strong upregulation (during larval infestation) of genes encoding neutrophil-recruiting cytokines and chemokines (IL-6 and CXCL-8 or IL-8) and basophil- and T lymphocyte-recruiting CCL-2, is responsible for the development of allergic contact-like dermatitis observed in tick-resistant cattle. Piper et al. (2010) reported a similar expression profile, although in tick susceptible Holstein-Friesian cattle. They proposed that the differential expression of IL-8 and CCL-2 worked in synergy with other well-known chemokines (regakine-1 included) to induce the chemo-attract activity of non-resident, inflammatory cell populations to the tick bite site. This trend strongly correlates with the cellular recruitment of neutrophils, $CD4^+$ and $CD8^+$ lymphocytes at the tick bite site. Lesions in the skin can be found in animals with some degree of resistance and are indicative of an allergic reaction. On the contrary, Piper et al. (2008) correlated the upregulation of chemokine ligands CCL-2 and CCL-26 and chemokine receptor CCR-1, observed in tick-susceptible Holstein-Friesian hosts, with cell-mediated inflammatory responses. Similarly, this trend was correlated with the transendothelial migration of monocytes and eosinophils to the dermal endothelial. Wang et al. (2007) reported downregulation of CCL-26 and CCR-1 in both tick-resistant and susceptible hosts, which contradicted the findings by Piper et al. (2008).

The significant upregulation of Claudin-11 (*Cldn 11*) was reported by Franzin et al. (2017). Although the expression and production of *Cldn-11* have not yet been described in the skin, the authors

assumed that the gene's products were involved in the ongoing cellular migration and tissue repair at the tick bite site in tick-resistant hosts. This was consistent with studies by Marima et al. (2020) and Wang et al. (2007). Wang et al. (2007) documented within breed differences in the expression of keratin/mitochondrial genes, odorant-binding protein (OBP) and *Bos taurus* major allergen BDA20, as well as the over-enrichment of extracellular matrix and immunoglobulin gene expression pathways. Marima et al. (2020) observed the significant upregulation of collagen-related genes *LUM*, *TBP*, *B2M* and *Traf-6* in high-resistance Brahman cattle in comparison to susceptible Angus cattle. These genes were not in core elements of the adaptive immune responses but encode components of the extracellular matrix. Of the four genes, *LUM* was upregulated at a much higher level in the Brahman than Angus cattle and was thus identified as a potential biomarker for tick resistance in cattle. *LUM* encodes products involved in regulating collagen fibril organization and circumferential growth, epithelial cell migration and cell repair. The results from the above studies were, however, not in agreement with those reported by Piper et al. (2008) found that none of the keratin- and collagen-coding transcripts were significantly differentially expressed between breeds. However, in a follow-up study Piper et al (2010), reported an upregulation of genes encoding constituents of the extracellular matrix (2 collagens, type 1 alpha and type 2 alpha 1, procollagen C-endopeptidase enhancer, Osteoglycin, and glutathione peroxidase-7) in Brahman cattle following infestation with *R. microplus* larvae.

The release of pro-inflammatory cytokines and chemokines leads to the chemotaxis of neutrophils, T cells, and B cells to the tick bite site (Kazimírová et al., 2017). Cell damage due to infection or injury is characterized by marked inflammatory responses orchestrated by cellular and plasma-derived factors. Consequently, circulating immune cells are recruited and mobilized to the affected tissue. To further resolve the inflammatory response, chemokines and cytokines expression may be upregulated mediating mounted immune cascade reactions to the invading organism (Qiagen, 2016). However, there is evidence that the production of anti-inflammatory molecules at the tick bite site, IL-10 in the case of Franzin et al. (2017), may be delayed in resistant hosts. This corresponds with Piper et al. (2008), where IL-10 was one of the 15 genes that were more highly expressed in tick susceptible Holstein-Friesian cattle as compared to the tick resistant Brahman cattle. Piper et al. (2008) further elaborated on the upregulation of transcripts of the toll-like receptor pathways (*TLR5, TLR7, TLR9, NFKBp50, MyD88, Traf-6, cd14 and IL-1β*) in conferring susceptibility in Holstein-Friesian hosts. Although these genes are associated with an innate inflammatory response specifically tasked with detecting invading agents and triggering inflammation, their diminished expression in Brahman hosts suggests that they might not contribute to the high level of resistance observed in tick resistant cattle biotypes.

The search for potential biomarkers for tick resistance may be as simple as identifying a phenotype that is consistently expressed in specific breed groups across a range of environmental and experimental conditions. However, the elucidation of the underlying mechanisms is very complex

due to the role of numerous interacting factors about adaptive and innate immune responses, pathology, and correlated responses with no direct functional relationship. Host resistance to ticks is a polygenic trait that is not simply explained by a handful of expressed genes. Using transcriptomics, genomics, and proteomics, could provide the multi-faceted need to unpack such a complex trait with potential incorporation into future breeding programs for improved resistance to ticks.

2.6 Conventional tick control strategies

In South Africa, the commercial beef sector is well developed and can afford the expensive and repeated application of chemical acaricides for tick control. This sector has concurrently realized the value of crossbreeding high production *B. taurus* breeds with the more adapted *B. indicus*/*Sanga*/*Zebu* breed types to optimize animal performance, adaptability, and product quality. Nonetheless, the environmental and monetary costs of controlling ticks are very high, and this affects both commercial and communal farmers. They require more cost-effective and sustainable tick control alternatives. While the latter two group makes use of indigenous breeds and indigenous knowledge systems, they have limited knowledge about animal health, disease control and policies regarding animal production, which has previously led to the malpractice of acaricide application and vaccination against TTBDs (DAFF, 2018).

2.6.1 Chemically- defined acaricides

The use of acaricides and macrocyclic lactones (MLs) for tick control is an unsustainable and expensive practice. Not only do the chemicals have short-lasting effects but ticks quickly become resistant to some of the major classes of active compounds in the acaricides and MLs due to the heightened intensity of their application for tick control (de la Fuente, 2018). Acaricide resistance, resulting from indiscriminate application practices, is common in one-host tick species than multi-host ticks, with the *R. microplus* often displaying the fastest development of acaricide resistance (Rodriguez-Vivas et al., 2011; Guerrero et al., 2012; Rodriguez-Vivas et al., 2018). The failure to reduce acaricides and MLs used in conventional beef production systems has been met by alarming consumer concerns relating to food and environmental safety issues. The apparent rise in the ability of some tick species to develop acaricide resistance has equally exacerbated the matter towards the development of new chemical and chemical-free alternative approaches (Rodriguez-Vivas et al., 2018). There is, however, no centralized information on acaricide resistance and South Africa, in particular, has no known information on acaricide resistance (Klafke et al., 2017). Reducing the frequency of acaricide application, modifications in the dosage, concentration or mixtures, use of synergistic formulations, rotation of acaricide classes and application of biosecurity protocols may help minimise the introduction of tick resistant strains (Rodriguez-Vivas et al., 2018).

Approximately all acaricides and MLs contain neurotoxins that affect the nervous system of the tick to discourage feeding (Mekonnen et al., 2002). Consequently, the ticks' direct damage and disease

epidemics can be controlled by maintaining populations below the critical threshold (Fouche et al., 2016). The major classes of commercially employed acaricides and MLs include the organochlorines, synthetic pyrethroids, organophosphates, amidines, phenylpyrazoles, formamidines, insect growth regulators and the MLs such as avermectins (doramectin, selamectin, abamectin, ivermectin, and eprinomectin), milbemycins (moxidectin and milbemycin omine) and Spinosyns (Spinosad) (Fouche et al., 2016; Rodriguez-Vivas et al., 2018). The non-specific nature of these chemicals bears ecological complications owing to the killing of non-targeted organisms and toxic residues in animal products aimed for human consumption (Fouche et al., 2016).

There is evidence that the decreased use of chemical tick control strategies could potentially result in financial benefits in the order of millions of Rands as well as reduce the negative effects of these chemicals on the environment. However, given those non-chemical alternatives for tick management have not yet been optimized, acaricide application continues to be the globally accepted method.

2.6.2 Botanically defined acaricides

The acaricidal activity of botanicals and essential oils has been explored to develop lower-cost, chemical-free and environmentally friendly natural alternatives for the control of both acaricides resistant and susceptible cattle tick species. Natives and locals have long relied on the repellent and acaricidal properties of indigenous plant species for the control of tick populations. Of the most commonly used plant species in Africa, Europe, Asia and the Americas, seven families (Asteraceae, Euphorbiaceae, Fabaceae, Lamiaceae, Meliaceae, Apocynaceae and Solanaceae) dominated (46%) the inventory (Pavela et al., 2016). The most popularly mentioned plant species across regions are the *Cissus quadrangularis*. Some research reports have listed two main modes through which botanical acaricides exert potency against ticks, namely: i) by causing neurotoxicity in the ticks and ii) by expelling highly concentrated polyphenols and limonoids which act as effective repellents (Pavela et al., 2016).

Essential oils from *Cymbopogon martinii*, *Cymbopogon citratus* and *Cedrus atlantica* were also studied and showed efficacy levels, to inhibit oviposition and hatchability, over 99% at various concentrations (Pazinato et al., 2016). Further *in vivo* tests are required to validate these results. Díaz et al. (2019) prosed phyto-formulations by combining individual oils to achieve increased efficacy. Their study reported 80%-100% acaricidal efficacy against *R. microplus* ticks from a mixture of essential oils composed of 66% *Cinnamomum zeylanicum* (cinnamon), 17% *Cuminum cyminum* (cumin) *and* 17% *Pimenta dioica* (allspice). Although these phyto-formulations may be useful in the control of ticks further biological tests are required for validation. Fouche et al. (2016) studied the acaricidal activity of 13 extracts from South African plants. Extracts of *Calpurnia aurea* (leaves, flowers) and the *Cissus quadrangularis* (stem) had the best activity against the *R. decoloratus* in comparison to the commercial chlorfenvinphos, with corrected mortality values of 83% and 80%, respectively. The same extracts, at 1% concentration, produced 100% corrected mortality estimates

against *R. microplus* when compared with chlorfenvinphos (Wellington et al., 2017). Furthermore, this study reported corrected mortality estimates of 97% using extracts from the *Senna italica* plant (roots, leaves and fruit) against the *R. microplus* tick species. Seed oils from the *Azadirachta indica*, commonly known as the neem tree, have also been identified for their acaricidal potential (100% mortality in 24-27h post-exposure) for the control of *R. decoloratus* (Choudhury, 2009).

All the above-mentioned botanicals present promising constituents for use in lower-cost eco-friendly integrated pest control systems. Plant-based acaricides, which are biodegradable and less toxic to the environment and non-targeted species, may mitigate the current shortfall of commercially recognised chemical acaricides. However, specific chemical and biological analyses are required to determine the environmental fate, species toxicity and skin toxicity of these botanical (Fouche et al., 2016). The mechanisms of action of components from plants like *Allium sativum*, *Azadirachta indica*, *Nicotiana tabacum*, *Chrysanthemum cinerariifolium*, *Capsicum* spp. (Pavela et al., 2016), have been thoroughly described and their components incorporated in the production of insecticides. However, some of these components require further scientific validation for value addition in the production of commercial acaricides for tick control.

2.6.3 Vaccination

Vaccinomics studies have yielded promising results that support the use of immunization as the most effective and environmentally sound TTBDs prevention strategy (de la Fuente et al., 2007). The use of “concealed” tick antigens in vaccine development may assist the host to counter the immune evasion mechanisms demonstrated by the tick during feeding (Kemp et al., 1989). This approach offers a green and sustainable alternative for tick control, consequently reducing reliance on pharmacological drugs and acaricides (Kuleš et al., 2016). The first tick antigen-derived vaccines were based on the highly variable gene transcripts of *R. microplus* BM86 or BM95 recombinant antigens (Willadsen et al., 1989). The BM86 “concealed” antigen is a membrane-bound glycoprotein presumably found on the plasma membrane of the midgut of partially engorged female ticks hidden away from the host immune system during tick feeding (Kemp et al., 1989; Willadsen et al., 1989; Manjunathachar et al., 2014). This vaccine, commercially known as tickGARD (Willadsen, 1997), has been applied in millions of animals to combat tick infestation, with an efficacy of 90% in reducing the prevalence of *R. microplus* tick-borne pathogens (TBPs) (de la Fuente, 2018; de la Fuente et al., 2007; Rodriguez-Valle et al., 2012). The mechanism of action of this vaccine encompasses lyses of the gut cells of the adult tick by inducing polyclonal antibody responses against the concealed antigens in the tick gut causing leakage of the gut content into the haemocoel of the tick (Rodriguez-Vivas et al., 2018). Consequently, this result in a reduction of the number, weight and reproductive/egg laying capacity of the engorging female ticks (Kemp et al., 1989; Willadsen et al., 1989). Sales of this vaccine were halted in 2010 due to its low efficacy in field trials and decreased popularity owing to the need to administer vaccination two to three times annually (Tabor et al., 2017). There is, however, a commercially available vaccine based on the above-mentioned tick

antigens (Gavac®) in various Latin American countries including Mexico, Argentina, and Colombia (Rodriguez-Valle, Vance, et al., 2012). Research towards the development of universal vaccines using newly identified targets with improved efficacy and delivery of exiting antigen-derived vaccines is greatly supported.

Reverse vaccinology is a new approach aimed at delivering next-generation vaccines with improved efficacy over the existing BM86-derived vaccines (Maritz-Olivier et al., 2012). Using high throughput *in silico* screening of the whole tick genome, genes that encode proteins with characteristics associated with vaccine-induced immunity can be identified and their immunogenicity systematically evaluated (Kuleš et al., 2016). Pan-genomic reverse vaccinology is an advancement in reverse vaccinology aimed at overcoming gene variability by identifying conserved vaccine candidates in antigenically diverse species (Serruto and Rappuoli, 2006). Lastly, there is subtractive reverse vaccinology. Developed from principles of subtractive comparative genome analysis, this approach compares the genome of a pathogenic isolate with that of a non-pathogenic or commensal isolate to potentially identify antigens critical in pathogenesis (Kuleš et al., 2016). This approach could potentially reduce the time of vaccine development. The use of omics technologies - functional genomics, transcriptomics, proteomics and immunomics - sophisticated bioinformatics and computational tools integrated with biophysical, structural and immunological evaluations is key in shifting the paradigm of vaccine development to optimize vaccine specificity with tailored immune responses (Kuleš et al., 2016).

2.6.4 Habitat and animal management

Pasture burning, grazing management and management of plant biodiversity in the pastures and animal nutrition are some of the less considered factors that may affect tick burdens of extensively reared cattle. Fully engorged adult female ticks fall from the host to lay their eggs in the layered vegetation for protection. Therefore, scheduled pasture burning will directly affect the tick population through the exposure of larvae, adults and eggs to high temperatures (Rodriguez-Vivas et al., 2018). South Africa, Zambia, Australia, the USA and Mexico are some of the countries that practice this method to control tick burdens (Abbas et al., 2014). In addition to this method, maintaining populations of certain plant species that are unfavourable to ticks has been shown to work. The *Stylosanthes scabra* and the *Acalypha fruticose* are some of the plant species with an ability to attract and trap between 12 and 27% tick larvae using a sticky exudate of glandular trichomes (Rodriguez-Vivas et al., 2018). An integrated approach to the two fore mention practices involves pasture rotation to interrupt the tick's life cycles (Abbas et al., 2014). This implies pastures spelling periods of between three and four months where the larval ticks are starved. However, this approach may be costly and could have adverse effects on pasture quality (Rodriguez-Vivas et al., 2018). Lastly, adequate energy and protein diet may facilitate maintained acquired resistance to ticks (Brossard and Wikel, 2016). Poor nutrition result in significant losses of tick resistance in *B. taurus* and *B. taurus* × *B. indicus* cattle (Sutherst et al., 1983).

2.6.5 Crossbreeding and marker-assisted selection

The complex breed structure of African cattle and their divergence from the European animals, on which most genomic data are based, has presented a challenge to genomic-based approaches to identifying quantitative trait loci (QTLs) for tick resistance and elucidating the mechanisms of host resistance in cattle. There is wide variability in the degrees of resistance expressed between and within cattle breeds. 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* tick species, while the Nguni and the Angus exhibited intermediate resistance and susceptibility, respectively port (Porto Neto et al., 2011; Manjunathachar et al., 2014; Marima, 2017;). Other studies have demonstrated an inverse resistance ranking order, with the Nguni displaying the highest level of resistance to various tick species in comparison to the Bonsmara and Angus breeds (Jonsson, 2006; Marufu et al., 2011; Muchenje et al., 2008b; Rechav and Kostrzewski, 1991). Host resistance to *R microplus* and *R. decoloratus* varies from 80% in typical taurine cattle to 99.9% in typical indicine cattle (Jonsson, 2006). This presents an opportunity to exploit the host's resistance to ticks in developing more sustainable and efficient tick control solutions without incurring additional costs (Burrow et al., 2019).

The ability to acquire tick resistance is heritable (Utech et al., 1978; Burrow et al., 2019). The tick resistance trait is moderate to highly heritable (h^2 = 0.34) in *Bos indicus* – cross cattle, which is sufficient to ensure successful selective breeding programs for the improvement of the tick resistance phenotype (Mackinnon et al., 1991; Porto Neto et al., 2011). Therefore, within-breed selection and crossbreeding for the direct improvement of tick resistance in cattle is possible. However, the lack of practically feasible tests that can be applied in the field hampers its use. Although knowledge of the mechanisms of tick resistance is suboptimal, this approach could provide a substantial solution for the reduction of acaricide usage (Rodriguez-Vivas et al., 2018). The adaptive traits of the indigenous breeds should be considered in the development of equitable sharing of genetic resources between breeds and within the beef industry for the development of sustainable alternative strategies for tick infestations (Mapiye et al., 2019). Crossbreeding and the replacement of taurine breed with indicine breeds presents an enticing option with rapid gains. However, molecular marker-assisted selection of taurine individuals within a specific breed with superior host resistance could yield better results, even from a production efficiency and product quality point of view (Porto Neto et al., 2011). Further developments, using the latest and high throughput molecular genetic technologies, are required to make this a viable option.

New anti-tick vaccines and acaricides are continuously being pursued with rapid progress being made, however, these are unlikely to confer total protection. The use of anti-tick vaccines and acaricides, topical and systemic, as the globally accepted tick intervention strategies in beef production systems has impeded the growth of the organic beef production industry. National and

global parasite control and resistance management approaches that are both sustainable and considerate to the public, environmental, and animal health are still lacking. The adoption of ethnobotanical information of the acaricidal and repellent activity of the different plant species in collaboration with the use of indigenous cattle breeds present promising strategies (Pavela et al., 2016). Therefore, the concept of naturally high host resistance to ticks is required to ensure a permanent solution. It is crucial to develop integrated tick intervention strategies not only to alleviate the real detrimental effects of tick infestation on cattle herds but also to minimise the potential annual economic losses incurred by beef producers.

2.7 The role of transcriptomic in the search for tick resistance genes in cattle

2.7.1 Overview of molecular biology technologies

Early attempts to understand the tick resistance phenotype were based on the potential of harnessing the host's natural ability to limit pathogen burdens as a more sustainable and cost-effective alternative to control tick infestations (Burrow et al., 2019). However, given the cost and time implications of compiling genomic data, greatly attributed to the large cattle resource populations and transcriptomic analyses needed to accurately record this trait, it still proves difficult to develop genomic-prediction equations for host resistance to ticks (Cardoso et al., 2015).

The use of molecular biology and omics technologies (genomics, proteomics, transcriptomics, metabolomics and lipomics) together with bioinformatics has been instrumental in the conception of principles about functional and/or structural alterations of cells, tissues, organs and whole organisms to tick infestations at a molecular level (Porto Neto et al., 2011; Schneider and Orchard, 2011). High throughput gene expression analysis technologies have aided in assembling snapshots of the diverse gene expression profiles and biomarkers with potential application in the development of prediction tests for host resistance to ticks (Wang et al., 2007; Kongsuwan et al., 2008; Franzin et al., 2017). There is a dearth of genomic studies that provide a global picture of the gene expression profiles conferred by the different host-tick associations. These comprehensive studies are central to the isolation of additional biomarkers for host resistance to ticks that may be useful in streamlining the selection of suitable individuals for large-scale agricultural breeding programs (Bustin, 2000; Wilhelm and Landry, 2009).

Over the past few decades, the principal methods for transcriptome analysis have evolved from Northern Blotting to Reverse Transcriptase Polymerase Chain Reaction (RT-qPCR) to DNA microarrays and now towards high-throughput RNA-Sequencing (RNA-seq) (Trayhurn, 1996; Schlamp et al., 2008; Wilhelm and Landry, 2009). The introduction of RNA-seq using next-generation sequencing (NGS) technology is a gradually advancing technique that streamlines the study of host resistance to ticks while addressing the biological heterogeneity of different cattle breeds challenged with various tick species (Colgan et al., 2017). The major constraints influencing a researcher's

choice of technologies to employ for sample processing and data analyses are often related to the budget, time, sample type and/or sample quality. In making the decision, it is imperative to consider the trade-offs in the quality, depth and relevance of the results reported attached to optimizing time and cost management. Although cost-effective, outdated technologies often prove laborious and time-consuming. They do, however, lend credibility to the newer technology employed by researchers seeking to maintain relevance.

Comparisons of the fundamental principles behind the different putative transcriptional technologies available are limited. This was evidenced by keyword searches that retrieved articles that only compared two technologies at a time (Baldwin et al., 1999; Taniguchi et al., 2001; Teng and Xiao, 2009; Soneson and Delorenzi, 2013), however, lacking details that compared the outputs, cost and time implications and the bioinformatics analyses associated with each technology. Two such examples of systematic comparisons were done by Git et al. (2010) and Morozova et al. (2009). In this conceptual review the origin, scientific contributions, cost implications and bioinformatic complexities in characterizing the mechanisms of cattle resistance to ticks, of each technology are outlined. This review aims to assist researchers in deciding on choosing the best molecular biology techniques to match strict time and budget constraints as well as the desired depth of data analysis.

2.7.2 Gene expression analyses technologies for host resistance to ticks in bovines

Genomics and transcriptomics are two of the most vastly used omics technologies in the search for candidate genes for host resistance to ticks in cattle. Genomics is a relatively new omics discipline typically based on DNA sequencing (Koboldt et al., 2013). This technology generated massive volumes of data that led to the development of computational tools with the capacity to mine information from large databases for biological data (Schneider and Orchard, 2011). Advances in this discipline have been paralleled with improved costs and scale of genome characterization over the past four decades (Koboldt et al., 2013). Transcriptomics incorporates the study of the complete set of transcripts and quantity within a cell as influenced by a specific physiological or developmental stage of an organism. It is through advances in omics technologies used in the study of gene expression patterns in cattle that biomarkers for host resistance to ticks can be identified with potential feasibility in the development of more robust vaccines and ultimately tick-resistant cattle populations.

Transcriptome sequencing is an approach that has been mastered over the past two decades through the construction of clonal colonies (Warren et al., 2011). However, the construction of the full-length gene structures of most species, human and livestock included, has spanned over several more decades and continues to date. The study of the systems biology of host resistance to ticks from all levels of complexity offers a possibility to explore the interaction of biological components that determine the properties of host immune responses to tick infestations. Coupled with advanced bioinformatics and computational tools for large data analyses, these technologies have the potential

to facilitate the development of predictive tests for tick resistance in cattle based on *in vivo* data (Schneider and Orchard, 2011). Among the most important bioinformatics tools is the Basic Local Alignment Search Tool (BLAST). This computational tool facilitates the comparison of gene sequences against many archived sequences on its databases. These can be used to establish regions of local similarity that can be used to infer functional and evolutionary relationships between a bovine host and the tick through the mapping of physiological and cellular responses to tick infestations (Schneider and Orchard, 2011).

2.7.3 Northern Blotting

The low-throughput approach of northern blotting resonated among some of the first candidate gene-based studies (Alwine et al., 1977). The northern blotting technique for RNA detection using radio-labelled DNA probes was developed in 1977 by J.C. Alwine and co-workers, with contributions from Gerhard Heinrich, at Stanford University (Alwine et al., 1977). This technique comprises the separation of cellular RNA by size using gel electrophoresis. This is followed by the transfer of the RNA to either a microcellulose or nylon membrane support where the presence and abundance of a specific RNA species of interest are inferred by hybridization of complementary radioactively labelled nucleic acid probe (Morozova et al., 2009; Wang and Yang, 2010). Although northern blotting has aged relative to high tech RT-qPCR, microarray and RNA-seq techniques, it still serves as the gold standard for gene expression studies based on mRNA detection and quantification (Streit et al., 2009; Wang and Yang, 2010).

2.7.3.1 Cost, limitations, and design pitfalls

Northern blotting analyses are inexpensive and relatively simple to perform with limited artefacts generated in comparison to the newer technologies (Drobyshev et al., 2003). The technique is, however, time-consuming and impractical for large clinical studies where the expression of hundreds of genes needs to be characterized simultaneously (Wang and Yang, 2010). Unlike microarray techniques, that facilitate the simultaneous visualization of thousands of genes, northern blotting is limited to the visualization of one or a small number of genes at a time, making the technique laborious (Baldwin et al., 1999; Taniguchi et al., 2001). Further limiting the use of this technique is the required large amounts (5-25 µg) of total RNA from each sample, which is not always attainable (Wang and Yang, 2010). This is especially true for gene expression studies aimed at characterizing host resistance to ticks that give preference to skin biopsy samples taken from the feeding site of actively engorging ticks. Moreover, as in many of the newer technologies, northern blotting is plagued with problems of sample degradation by RNases. However, proper sterilization of workstations and lab equipment combined with the use of diethylpyrocarbonate often immunizes samples from RNase degradation (Trayhurn, 1996).

2.7.3.2 Research impact and outputs in studying host resistance to ticks

Northern blotting is one of the first techniques which allowed researchers to observe differential gene expression profiles in various tissues and organs at different developmental, physiological, environmental and pathogen infection stages (Liang and Pardee, 1995; Baldwin et al., 1999). This technology has, however, been outcasted in the study of bovine resistance to ticks. The risk to researchers due to exposure to formaldehyde, radioactive material, ethidium bromide, diethylpyrocarbonate and UV light has further exacerbated the exclusion of northern blotting from present gene expression studies (Streit et al., 2009). Therefore, no gene expression studies using this technology are reported here. The inclusion of this technology in this review was merely to highlight the evolution of gene expression technologies to date.

2.7.4 Quantitative Reverse Transcription Polymerase Chain Reactions (qRT-PCR)

While Northern Blotting has formed the basis upon which some transcriptome analysis technologies were developed, RT-qPCR now forms the gold standard against which most of the newer technologies are validated. RT-qPCR is a method listed among the earliest and most extensively used techniques for gene expression analyses (Trayhurn, 1996). This technology provides a 1000-fold increase in sensitivity over the northern blotting technique and is useful in measuring lower levels of mRNA in tissues. RT-qPCR can be used to detect single positive copies of a specific transcript and quantify gene expression differences as small as 23% between samples while still maintaining lower coefficients of variation (Wong and Medrano, 2005). This can be added to its capacity to discriminate between almost identical mRNAs. However, with improved sensitivity comes a demand for precession in designing experiments and normalizing the data to avoid reporting false-positive and false-negative results. Unlike its predecessor, qRT-PCR assays require far less RNA template and can be high throughput given the availability of proper equipment and technical support. Provided the experiment has been well-designed with proper controls, qRT-PCR can be one of the most reproducible, efficient, fast and sensitive technologies for quantifying gene expression (Wong and Medrano, 2005).

2.7.4.1 Cost, limitations, and design pitfalls

RT-qPCR is a targeted technique that is instrumental in measuring the gene expression of a panel of candidate genes of interest from pathways and families known to infer certain physiological conditions. This confines the investigation to a limited number of genes; thus maximizing the chances of missing additional significant effects in the biological process which may be expressed in other metabolic and signalling pathways as well transcriptional networks across pathways (Malatji, 2017). Although considered robust in theory, the increased sensitivity of RT-qPCR assays exposes them to numerous experimental errors concerning reaction components, thermal cycling conditions, mispriming events and particularly cDNA synthesis (Wong and Medrano, 2005). If left undetected,

these variations may lead to the production of inaccurate data. RT-qPCR is especially prone to variation caused by human error due to pipetting errors. These cumulative errors can potentially be alleviated through precision pipetting, regular pipette calibration and running a standard curve during every reaction (Wong and Medrano, 2005).

2.7.4.2 Research impact and outputs in studying host resistance to ticks

RT-qPCR assays are used to validate gene expression outputs of modern RNA-seq, NGS and microarray technologies, producing results that are often found to mirror the expression profiles of the microarray experiment (Trayhurn, 1996; Wang et al., 2007). Table 2.1 lists gene expression studies that were conducted in the past two decades using RT-qPCR to profile candidate genes for bovine resistance to ticks. Therefore, these studies only retrieved snapshots of the genetic activity at the host-tick interface during tick attachment and feeding.

Table 2.1: Summary of gene expression studies using RT-qPCR to characterize host resistance to ticks in cattle.

Breed	Aim	Outcomes	Applications	Country
Angus, Brahman & Nguni	Profile differential expression of 17 genes in early (12h) immune responses to tick infestations	*LUM* as a potential biomarker; differential expression of components of the extracellular matrix may confer increase host resistance to ticks	Biomarker detection for selective breeding programs	South Africa (Marima et al., 2020)
Belmont Red	Characterize the molecular basis of host resistance	higher expression of the basal epidermal keratins KRT5 and KRT14, the lipid processing protein, lipocalin 9 (LCN9), the epidermal barrier catalysing enzyme transglutaminase 1 (TGM1), and the transcriptional regulator B lymphocyte-induced maturation protein 1 (Blimp1) in the skin of high tick-resistant animals; Epidermal barrier implicated in coffering increased tick resistance	Elucidating the genetic control of tick resistance	Australia (Kongsuwan et al., 2010)
Holstein-Friesian & Brahman	Analyze differential expression of 44 genes following 7 weekly infestations	Mechanisms of tick resistance in Bos indicus breeds differ from the innate, cell-mediated hypersensitivity-type reaction of Bos taurus breeds	Planning for further gene expression research to characterize host resistance to ticks	Australia (Piper et al., 2008)
Brahman & Holstein-Friesian	Identifying useful and selectable genes controlling cattle tick resistance	138 differentially expressed genes and key pathways were identified. These included immunological/host defence genes, extracellular matrix proteins and transcription factors, as well as genes involved in lipid metabolism	Development of a sustainable & environmentally sound tick control method through the breeding of tick resistant cattle	Australia (Kongsuwan et al., 2008)

2.7.4.3 Bioinformatics analyses

One of the first models for calculating fold changes between samples and amplification efficiencies of an experiment, without the use of standard curves, was designed by Gentle et al (Gentle et al., 2001; Wong and Medrano, 2005). Some of the leading suppliers of life science consumables and instrumentals have fine-tuned the process from data generation to data analyses by providing exclusive web-based software (SABiosciences – Qiagen) and handbooks for RT2 Profiler PCR Array Data Analysis. This simplifies pipelines for calculating differential expression patterns through the automated calculation of fold change, fold regulation and significance values following the comparative C_t method (Livak and Schmittgen, 2001). Combined with simple calculations, using SAS enterprise guide or XLSTAT, RT-qPCR data can be easily interpreted. Similarly, Q-Gene software can be used to simplify the process of experimental planning through to data representation (Wong and Medrano, 2005). The advantage of RT-qPCR over other assays comes from its production of quantitative data with an accurate dynamic range of 7 to 8 log order magnitude which does not require manipulation post-amplification (Wong and Medrano, 2005).

2.7.5 DNA Microarray technology

The technology most recently preceding RNA-seq is DNA microarray technology. The principles governing microarray technology were conceived approximately 30 years ago, in the late 1980s, emerging from Southern blotting (Jaluria et al., 2007). Reproducible results were only achieved a decade later following the conception of microarray technology, using high-quality slides with precision robots (Bumgarner, 2013). DNA microarrays came into widespread use in the 1990s and early 2000s and have since been used extensively to characterize and understand the molecular basis of phenotypic variations in tick resistance. The use of isolated DNA fragments affixed to a substrate and hybridization with a probe made from cellular RNA in DNA microarray analysis is akin to reverse northern blotting (Bumgarner, 2013). To date, DNA microarrays have received major facelifts in terms of quality, probe density and structural layout, making them superior to their predecessors (Jaluria et al., 2007). The deep and broad-scale coverage of DNA microarray technology expanded researchers' ability to monitor the effects of pathogens on host-cell gene expression programs in the abundance of transcripts (Jenner and Young, 2005). It is through the development of high throughput microarrays that genomics and proteomics research has advanced from a structural to a functional state (Schneider and Orchard, 2011).

2.7.5.1 Cost, limitations, and design pitfalls

Spotted microarrays (dual-channel) and oligonucleotide microarrays (single channel) are the most commonly used types of microarrays (Brown and Botstein, 1999). Oligonucleotide DNA microarrays have the advantage of producing precise measurements of gene expression while guaranteeing highly reproducible data (Jaluria et al., 2007). However, the cost attached to these microarrays is

quite high as opposed to that of spotted DNA microarrays, despite the technology's shortfall in detecting low abundance transcripts. Although cheaper than its counterpart, the use of spotted DNA microarrays is accompanied by issues relating to the labelling efficiency of the dyes and their limited capacity to only determine relative expression levels (Bumgarner, 2013). On the contrary, oligonucleotide microarrays are not only able to generate absolute expression levels but are also capable of detecting alternative splice variants (Jaluria et al., 2007).

As opposed to qRT-PCR approaches, microarray technology still has numerous limitations and design pitfalls (Eisen and Brown, 1996; Schena et al., 1995). The major pitfall is that DNA microarray technology requires prior knowledge of the sequence (Eisen and Brown, 1996). Consequently, limited information can be generated for genes without expression in the reference/control sample. The resulting transcriptome is often unable to account for post-transcriptional events like splicing (Malatji, 2017). This technology requires high-quality control and clone access although chances of cross-hybridization of probes across gene families are often inevitable (Malatji, 2017). As a result, the quality of expression profiling data is highly dependent on the design and quality, target preparation and probe construction, of the arrays themselves (Drobyshev et al., 2003).

2.7.5.2 Research impact and outputs *in studying host resistance to ticks*

Microarray technology was extensively used in toxicology, evolutionary biology, drug development and production, disease characterization, diagnostics development, cellular physiology and stress responses and forensics (Jaluria et al., 2007). Table 2.2 shows some of the research outputs achieved using microarray technology to characterize host resistance to ticks.

Table 2.2: Summary of gene expression studies using DNA microarrays to characterize host resistance to ticks in cattle

Breed	Aim	Outcomes	Applications	Country
Holstein & Nelore	Comparison of transcriptional and inflammatory response profiles in cutaneous reactions to tick bites	1131 DEGs were identified, allergic contact-like dermatitis implicated in the production of IL-6, CXCL-8 and CCL-2 leading to expression of pro-inflammatory chemokines and cytokines that recruit granulocytes and T lymphocytes.	Understanding the molecular basis of differences in tick-resistant and susceptible cattle for anti-tick vaccine development	Brazil (Franzin et al., 2017)
F2 (Gir × Holstein) of contrasting phenotypes	Characterization of early (0, 12h & 48h) immune responses triggered by R. microplus ticks	Lipid metabolism genes & impaired acute phase responses	Improvement & development of novel tick control tools using immunomodulation tools	Brazil (Carvalho et al., 2014)
Brahman & Holstein-Friesian	Describe processes responsible for superior tick resistance in Brahman compared to low-resistance Holstein–Friesian cattle	Gene involved in inflammatory processes and immune responsiveness were upregulated only in the Holstein-Friesian hosts; genes encoding constituents of the extracellular matrix were upregulated in Brahmans	Predictive biomarker development for use in selective breeding programs and anti-tick vaccine development	Australia (Piper et al., 2010)
Hereford Shorthorn	Define the nature of Bos taurus tick resistance (0 & 24h post-infestation)	Expression of keratin genes or mitochondrial genes, odorant-binding protein & major allergen BDA20 were significantly altered post-infestation	Marker-assisted selection strategies for cattle breeding	Queensland (Wang et al., 2007)

2.7.5.3 Bioinformatics analyses

Methods for microarray data analyses were well established over a decade since the introduction of the technology. Standard quality control measures are carried out to assess the quality of the array on the probe level data and the summarized data. These may include intensity correlations between the arrays, pseudo-images of the arrays, MA scatter plots of arrays versus a pseudo-median reference chip, boxplots and histograms of raw log intensities, boxplots of relative log intensities, boxplots of normalized unscaled standard errors (NUSE), plots of principal component analysis (PCA) for Person perfect match (PM) and Mismatch (MM) raw probes, as well as the summary and plots of RNA degradation per chip (Piper et al., 2017, 2009). Numerous summarization methods are recognized, with MAS 5.0, RMA, GCRMA, PLIER and DCHIP listed among the topmost used methods. MAS parameters include background correction for both PM and MM. The lowest 2% of the probe intensities are used as a background value for each of the 16 grids. Each probe is adjusted based upon a weighted average of the background of each grid. Scale normalization is performed on all arrays using the same mean value (200). The arrays are then summarized using an adjusted $\log_2$ scale average using one-step Tukey biweight (Welsh et al., 2013). RMA parameters include convolution background correction, quantile normalization and median polish summarization.

GCRMA parameters involve background correction using an affinity model based on probe sequences and MM intensities. This is followed by quantile normalization and median polish summarization. PLIER accounts for experimentally observed patterns for feature behaviour and handling error at the appropriately low and high signal values. The parameters include quantile normalization and both PM and MM background correction. DCHIP parameters include PM-MM background correction, pair-wise rank invariant normalization and MBEI model summarization (Welsh et al., 2013).

The statistical computing language R and Bioconductor packages are vastly used for most standard quality control measures, pre-processing, and differential expression profiling. *Limma* software is one of the proposed tools for bioinformatic analysis of microarray data (Soneson and Delorenzi, 2013). Carvalho et al. (2014) measured chip quality in R language using *affy*, *simpleaffy* and *affQRe port* from the Bioconductor package. The data were subjected to linear regression analyses in the Bioconductor Limma package. This facilitated the analyses of the differentially expressed genes in the context of pathways, networks, and maps using MetaCore® software. The availability of web-based gene annotation gene-annotation enrichment analysis tools has simplified the functional interpretation of microarray data. These platforms include the Affymetrix online platform (http://www.affymetrix.com/products/index.affx) and NCBI Blast for gene annotation of differentially expressed probe sets as well as the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/tools.jsp) for gene-annotation enrichment analysis of the differentially expressed genes and pathway over-representation analysis.

2.7.6 RNA sequencing using Next Generation Sequencing Technology

The advent of NGS technologies transformed the field of transcriptome analyses by providing a platform for shotgun whole transcriptome coverage (Wang et al., 2009). A powerful competitor to DNA microarray technology emerged in the form of RNA-seq technology that uses NGS methods to sequence cDNA derived from RNA (Soneson and Delorenzi, 2013). Consequently, former technologies such as DNA microarray and northern blotting analyses are gradually being phased out, except for RT-qPCR that continues to be used to validate sequencing results. RNA-seq technology has provided a new horizon where the unannotated transcriptional activity can be deciphered through the isolation of protein-coding and non-coding transcripts as well as alternative splice variants of known annotated transcripts (Weikard et al., 2013). RNA sequencing has evolved from the construction of individual cDNA clones into the more comprehensive construction of cDNA-sequencing libraries representative of the species transcriptome (Motoaki Seki et al., 2002).

As an emerging tool of choice for gene expression profiling, RNA-Seq bears the unprecedented potential to outline transcriptional response in cattle to various tick species (Colgan et al., 2017). High-throughput transcriptome analyses, which facilitate quantitative measurement of even poorly expressed genes within a specific sample, have been made possible through direct cDNA fragment

sequencing that requires no cloning (Malatji, 2017; Marioni et al., 2008). Setting RNA-seq apart from its predecessors is its capacity to decipher the complex architecture of host-pathogen interactions by determining transcript boundaries, promoter sequencing, antisense transcripts, riboregulators and identifying RNA-binding proteins without pre-established oligonucleotide arrays or DNA sequencing primers (Colgan et al., 2017). This makes RNA-Seq especially ideal for investigating transcriptional responses in host-tick interactions.

2.7.6.1 Cost, *limitations, and design pitfalls*

Besides being associated with elevated costs upon introduction into the fields of genetics, NGS approaches have been met with rich appreciation. This has led to a decline in the cost of generating NGS data (Lister et al., 2009). Furthermore, the cost of bulk RNA-seq, as opposed to single-cell RNA-seq, is significantly reduced. The exponentially decreasing cost of NGS technologies has promoted an increase in large-scale investigations of rare variations of complex disease genetics (Koboldt et al., 2013).

Like its counterparts, RNA-seq data is only as good as the experimental design, quality of samples used, and computational resources used to mine the biological information. A major challenge impeding the use of RNA-seq lies in the standard of RNA quality required. While blood samples may yield an increased concentration of intact RNA, the genomic data they produce is often less informative in genes expression studies. Skin biopsy samples from the host-tick interface are favoured as they constitute more informative biological data about actively expressed genes. Sadly, the texture and size of bovine skin biopsies often pose great difficulty in initially preserving the RNA within the samples and eventually extracting acceptable quality RNA of the desired volume.

In addition, scientists in the computational biology community differ on the number of replicates over the depth of sequencing coupled with the choice of statistical tool to ensure valid biological interpretation of results, high statistical power and efficient use of sequencing resources (Liu et al., 2013; Schurch et al., 2016). One school of thought argues that more than six replicates must be used. They support increasing the replicates to increase the likelihood of identifying significantly differentially expressed genes (DEG) for all fold changes. The other motivates towards achieving appropriate sequencing read coverage through deeper sequencing depth (Koboldt et al., 2013). Studies have revealed that increased sequencing depth beyond 10 million reads diminishes the returns on power to detect DEGs whereas increased biological replicates improve power irrespective of sequencing depth (Liu et al., 2013). With decisions influenced by budget, researchers may often select the approach that optimizes cost-effectiveness.

2.7.6.2 Research impact and outputs in studying host resistance to ticks

RNA-seq promises to eliminate the shortcomings of microarray technology by facilitating the identification and quantification of rare transcripts without prior knowledge of the particular gene thereby providing information regarding alternative splicing and sequence variations in those genes (Wang et al., 2009; Malatji, 2017). The development of third-generation sequencers promises to revolutionize transcriptome analysis by addressing the technological shortcomings (short read length and high error rate) of most new generations sequencers (Morozova et al., 2009). The advantage of sequencing lies in its capacity to discover common and rare variants using the appropriate sequencing read coverage, algorithmic methods and precise orthogonal validation (qRT-PCR) to confirm true from false-positive readings (Koboldt et al., 2013).

The earliest record of RNA-seq studies was published in 2008 (Pepke et al., 2009). RNA-Seq has been used extensively to create a multidimensional understanding of the molecular aberrations underlying variable complex diseases in humans (Morozova et al., 2009). Currently, single-cell transcriptome sequencing and cell sorting approaches are being explored to correlate chemokine and cytokine production to the specific cells producing them as a means of isolating candidate cells to target in vaccine designs (de la Fuente et al., 2016). There is, however, a dearth of literature documenting studies conducted using RNA-seq technology to characterize bovine resistance to ticks. Modifications are required preceding the detailed resolution of genetic mechanisms of bovine resistance to ticks. The most significant application of the RNA-seq technology platform in the study of pathogen-vector-host has been in the field of vaccinology. NGS studies conducted to understand the tick's physiology and gene expression profiles following interaction with a specific host have been instrumental in the progress made to develop vaccines that offer cross-protection against multiple tick species and tick-borne diseases simultaneously (see Table 2.3). While the popularity of RNA-seq may increase and its associated running costs decreasing, the discipline of parasite and vector research is yet to harness its benefits.

Table 2.3: Summary of gene expression studies using RNA sequencing through Next generation sequencing to characterize host resistance to ticks in cattle

Breed/Tick species	Aim	Outcomes	Applications	Country
Haemaphysalis longicornis	Investigate the physiological response of the tick to cypermethrin	Detoxification metabolism genes were upregulated in the cypermethrin-tolerant group coupled with the heightened expression of genes encoding CYP450, GST, and ABC transporters	Tick control strategies	South Korea (Moon et al., 2021)
R. microplus	To identify candidates for novel tick control approaches by characterizing the tick salivary gland gene expression in tick-susceptible and tick resistant hosts	Putative targets for infestation control included genes involved in stress response during blood-feeding; Coordinated regulation of a small heat shock protein and negative elongation factor B-like genes along with upregulation of a 26S proteasome subunit and calnexin were identified in ticks fed on tick-susceptible cattle	Tick control strategies	Brazil (Giachetto et al., 2020)
Braford	Identify genetic pathways and transcription factors affecting host resistance to ticks	Members of the Ap1 and NF-kB families were the most relevant transcription factors predicted in both resistant and susceptible animals; Leucocyte chemotaxis was over-represented in both phenotypes and skin degradation and remodeling was over-represented in resistant hosts; activation of the WNT-signaling pathway was identified as a potential candidate mechanism of resistance	Search for candidate genes for tick resistance in bovine	Brazil (Moré et al., 2019)
R. decoloratus	Elucidate the complex mechanism underlying amitraz resistance	A potential relationship exists between the α-adrenergic-like octopamine receptor and ionotropic glutamate receptors in establishing amitraz resistance; it is proposed that in amitraz-susceptible ticks Ca2+ entry into cells is inhibited followed by membrane hyperpolarization which prevents the release of neurotransmitters; Ionotropic glutamate receptors (NMDA and AMPA) prevent this action in amitraz-resistant ticks	Drug development for tick control	South Africa (Baron et al., 2018)
R. microplus fed on Nelore and Holstein calves	Examine the expression profiles of genes encoding secreted tick proteins that mediate parasitism in larvae and nymphs feeding of the two breeds	Transcripts encoding immunomodulatory molecules secreted in the tick saliva were more abundant in ticks fed on tick-susceptible hosts; Resistant hosts expose ticks to an earlier inflammatory response that results in significantly lower expression of genes encoding salivary proteins that suppress host immunity, coagulation, and inflammation	Elucidation of the molecular basis of the differences between tick-susceptible and tick-resistant bovine hosts and the associated modulation of the tick secretome	Brazil (Franzin et al., 2017)

2.7.6.3 Bioinformatics analyses

RNA-seq generates large volumes of data. Despite the availability of numerous web-based data analysis platforms for RNA-seq data, the inherent difficulties associated with operating the software packages and interpreting the data while controlling non-uniformities between samples may be intimidating (Soneson and Delorenzi, 2013). Consequently, more complex schemes and approaches are required to support standard analysis tools. There are distinct differences in the data obtained from RNA-seq compared to microarray that prevents the transfer of methods used in one technology to another. These differences are listed in detail in a comparison with Soneson and Delorenzi (2013).

There is currently no principal analytical approach for RNA-seq data analyses. A combination of a variety of bioinformatics tools and algorithms, optimized to the researcher's requirement has worked in the past (Mutz et al., 2013). The challenges and limitations of these combinations have been reviewed in detail by Treangen and Salzberg (2012), Garber et al. (2011) and Pepke et al. (2009). Computational pipelines such as the R programming language, Partek flow Genomics Analysis software and the Galaxy web-based platform have been successfully employed in the analysis of RNA-seq data. Four fundamental analysis steps are recognized for organisms with existing reference genomes or transcriptomes (Mutz et al., 2013). In summary, these steps involve 1) the conversion of raw image data into short-read sequences; 2) alignment of reads to reference genome or transcriptome (the most computationally intensive step); 3) quantification of mapped reads and gene expression levels determined using peak calling algorithms; 4) quantification of differential gene expression using statistical tests. A popular start to finish pipeline used for RNA-seq data analysis is the Tuxedo suite. This is comprised of the Tophat spliced read mapper for read alignment, Bowtie or Bowtie 2 short read aligners, Cufflinks tools to assemble transcripts and estimate their abundance as well as Cuffdiff to identify differentially expressed genes. The Tuxedo suite has recently been upgraded to a complete analysis protocol intended to correspond to many users' designs (Pertea et al., 2016). It consists of three software tools; HISAT (http://ccb.jhu.edu/software/hisat2 or http://github.com/infphilo/hisat2) for mapping sample reads to the reference genome, Stringtie and Stringtie merge (http://ccb.jhu.edu/software/stringtie or https://github.com/gpertea/stringtie) for transcript assembly, estimation of expression levels of genes and merging of gene structures found in each sample, and lastly Ballgown for quantification of transcript and gene differential expression between samples (Pertea et al., 2016). Using these pipelines, computational packages such as *edgeR*, *DESeq* and HTSeq can be applied to analyse samples with replicates below 12 and where more replicates are available, respectively (Schurch et al., 2016). However, before applying this software and algorithms the raw sequence data undergoes pre-processing to ensure high-quality data. Computational protocols such as Trimmomatic, FASTX toolkit (http://hannonlab.cshl.edu/fastx_toolkit) and FastQC (http://www.bioinformatics.babraham.ac. uk/projects/fastqc) are recommended for quality control, read trimming for quality and adaptor sequences and ultimately quality control for RNA-seq alignment data (Pertea et al., 2016).

The pace of innovations of these analysis pipelines and algorithms continues to excite and engage the computational biology community. Newer and more sophisticated algorithms and software are continuously being proposed to support the analysis of different aspects of data structures underlying different biological phenomena (Pepke et al., 2009; Koboldt et al., 2013).

The integration of outputs from multiple transcriptome analysis technologies is required to create a multidimensional understanding of the molecular features underlying the tick resistance phenotype. Multiple options exist that cater to different aspects and needs for transcriptome analyses. Therefore, it may prove difficult to determine the correct technology, detection chemistry, quantitative method and bioinformatic tools to suit the study's budget and time constraints. To maintain relevance in the research domain, increased accessibility to the latest and more relevant technology is necessary. This is achievable through modifications to improve cost, labour and data analysis factors attached to the latest technologies while maintaining the elevated standards of data quality. Given the constraints attached to genomics studies, the high throughput nature of RNA-seq provides the most data-rich and rewarding option for gene expression profiling than its counterparts. The decreasing cost of RNA-seq and the advancing computational tools that support large data analyses threaten to phase out microarray and PCR-qPCR technologies.

2.8 Summary

The transcriptomic, quantitative-trait mapping, immunology, pathology, and vaccinology studies reviewed above have demonstrated the divergent and heritable nature of the tick resistance phenotypes. These studies have been instrumental in identifying and quantifying new vaccine candidates and unpacking numerous chromosome segments, SNPs, gene profiles, and cellular profiles associated with tick resistance in cattle. However, they have equally established that the effector mechanisms modulating host resistance to ticks consist of complex, interacting factors that are often tick species- and breed-specific. Therefore, co-selection for acquired resistance to different tick species may be a complex phenomenon. Although much remains to be explained, compilations of candidate causal variants for tick resistance/ susceptibility exist. The variation in the innate and adaptive immune responses to tick infestations are mostly marked between *B. taurus* and *B. indicus* breeds. Therefore, there remains a lot of work to be done before comprehensive panels of multiple markers for tick resistance in Zebu and Taurine breeds can be developed for future selection and breeding programs. It is indisputable that the use of genetically resistant animals and vaccines that offer cross-protection are the most sustainable approaches to control ticks. Currently, vaccines derived from antigens from one tick species are ineffective in providing cross-protection against other tick species. This warrant increased accessibility to the latest and most relevant transcriptome analyses technologies, detection chemistry, quantitative methods and bioinformatic tools to aid in accelerating the process of new antigen and biomarker identification and the development of

prediction tools for application in selective breeding programs. On the basis that co-selection for acquired resistance to different tick species may not be possible, further studies are needed to determine the genetic mechanisms of tick resistance correlated to tick-resistant phenotypes. In the interim, new vaccine candidates and botanically based acaricides, that offer cross-protection against various tick species, should be explored.

2.9 References

Abbas, R.Z., Zaman, M.A., Colwell, D.D., Gilleard, J., Iqbal, Z., 2014. Acaricide resistance in cattle ticks and approaches to its management: The state of play. Vet. Parasitol. 203, 6–20. https://doi.org/10.1016/j.vetpar.2014.03.006

Alwine, J.C., Kemp, D.J., Stark, G.R., 1977. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. Proc. Natl. Acad. Sci. U. S. A. 74, 5350–5354. https://doi.org/10.1073/pnas.74.12.5350

Baldwin, D., Crane, V., Rice, D., 1999. A comparison of gel-based, nylon filter and microarray techniques to detect differential RNA expression in plants. Curr. Opin. Plant Biol. 2, 96–103. https://doi.org/10.1016/S1369-5266(99)80020-X

Barker, S.C., Walker, A.R., 2014. Ticks of Australia. The species that infest domestic animals and humans, Zootaxa. https://doi.org/10.11646/zootaxa.3816.1.1

Baron, S., van der Merwe, N.A., Maritz-Olivier, C., 2018. The genetic relationship between R. microplus and R. decoloratus ticks in South Africa and their population structure. Mol. Phylogenet. Evol. 129, 60–69. https://doi.org/10.1016/j.ympev.2018.08.003

Berry, C., Jonsson, N., Constantinoiu, C., Klafke, G., Miller, R., Nyangiwe, N., Koh-Tan, C., Tidwell, J., Jittapalapong, S., 2017. Examination of the taxonomic status of Rhipicephalus (Boophilus) microplus, in: One Health - 9th Tick and Tick-Borne Pathogen Conference & 1st Asia Pacific Rickettsia Conference. Cairnes, Australia, p. 94.

Brossard, M., Wikel, S.K., 2016. Tick immunobiology. https://doi.org/10.1017/S0031182004004834

Brown, P.O., Botstein, D., 1999. Exploring the new world of the genome with DNA microarrays. Nat. Genet. 21, 33–37.

Bumgarner, R., 2013. Overview of DNA microarrays: Types, applications, and their future. Curr. Protoc. Mol. Biol. 1–11. https://doi.org/10.1002/0471142727.mb2201s101

Burrow, H.M., Mans, B.J., Cardoso, F.F., Birkett, M.A., Kotze, A.C., Hayes, B.J., Mapholi, N.O., Dzama, K., Marufu, M.C., Githaka, N.W., Djikeng, A., 2019. Towards a new phenotype for tick resistance in beef and dairy cattle: a review. Anim. Prod. Sci. 59, 1401–1427. https://doi.org/10.1071/AN18487

Bustin, S.A., 2000. Absolute quantification of mRNA using real-time reverse transcription-polymerase chain

reaction assays. J. Mol. Endocrinol. 25, 169–193. https://doi.org/JME00927 [pii]

Cardoso, F.F., Gomes, C.C.G., Sollero, B.P., Oliveira, M.M., Roso, V.M., Piccoli, M.L., Higa, R.H., Yokoo, M.J., Caetano, A.R., Aguilar, I., 2015. Genomic prediction for tick resistance in Braford and Hereford cattle. J. Anim. Sci. 93, 2693–2705. https://doi.org/10.2527/jas.2014-8832

Carvalho, W.A., Domingues, R., de Azevedo Prata, M.C., da Silva, M.V.G.B., de Oliveira, G.C., Guimarães, S.E.F., Machado, M.A., 2014. Microarray analysis of tick-infested skin in resistant and susceptible cattle confirms the role of inflammatory pathways in immune activation and larval rejection. Vet. Parasitol. 205, 307–317. https://doi.org/10.1016/j.vetpar.2014.07.018

Carvalho, W.A., Franzin, A.M., Abatepaulo, A.R.R., de Oliveira, C.J.F., Mor??, D.D., da Silva, J.S., Ferreira, B.R., de Miranda Santos, I.K.F., 2010. Modulation of cutaneous inflammation induced by ticks in contrasting phenotypes of infestation in bovines. Vet. Parasitol. 167, 260–273. https://doi.org/10.1016/j.vetpar.2009.09.028

Chingala, G., Raffrenato, E., Dzama, K., Hoffman, L.C., Mapiye, C., 2017. Towards a regional beef carcass classification system for Southern Africa. South African J. Anim. Sci. 47, 408–423. https://doi.org/10.4314/sajas.v47i4.1

Choudhury, M.K., 2009. Toxicity of neem seed oil against the larvae of Boophilus decoloratus, a one-host tick in cattle. Indian J. Pharm. Sci. 71, 562–563. https://doi.org/10.4103/0250-474X.58191

Colgan, A.M., Cameron, A.D., Kröger, C., 2017. If it transcribes, we can sequence it: mining the complexities of host-pathogen–environment interactions using RNA-seq. Curr. Opin. Microbiol. 36, 37–46. https://doi.org/10.1016/j.mib.2017.01.010

Constantinoiu, C.C., Jackson, L. a., Jorgensen, W.K., Lew-Tabor, a. E., Piper, E.K., Mayer, D.G., Venus, B., Jonsson, N.N., 2010. Local immune response against larvae of Rhipicephalus (Boophilus) microplus in Bos taurus indicus and Bos taurus taurus cattle. Int. J. Parasitol. 40, 865–875. https://doi.org/10.1016/j.ijpara.2010.01.004

DAFF, 2018. A PROFILE OF THE SOUTH AFRICAN BEEF MARKET VALUE CHAIN. Arcadia.

DAFF, 2015. A PROFILE OF THE SOUTH AFRICAN BEEF MARKET VALUE CHAIN.

Dantas-Torres, F., 2015. Climate change, biodiversity, ticks and tick-borne diseases: The butterfly effect. Int. J. Parasitol. Parasites Wildl. 4, 452–461. https://doi.org/10.1016/j.ijppaw.2015.07.001

De Castro, J.J., 1997. Sustainable tick and tick-borne disease control in livestock improvement in developing countries. Vet. Parasitol. 71, 77–97.

de la Fuente, J., 2018. Controlling ticks and tick-borne diseases…looking forward. Ticks Tick. Borne. Dis. https://doi.org/10.1016/j.ttbdis.2018.04.001

de la Fuente, J., Almazán, C., Canales, M., Pérez de la Lastra, J.M., Kocan, K.M., Willadsen, P., 2007. A ten-

year review of commercial vaccine performance for control of tick infestations on cattle. Anim. Health Res. Rev. 8, 23–8. https://doi.org/10.1017/S1466252307001193

de la Fuente, J., Kopáček, P., Lew-Tabor, A., Maritz-Olivier, C., 2016. Strategies for new and improved vaccines against ticks and tick-borne diseases. Parasite Immunol. https://doi.org/10.1111/pim.12339

Domingues, R., Wohlres-Viana, S., Reis, D.R.L., Teixeira, H.C., Ferreira, A.P., Guimarães, S.E.F., Prata, M.C.A., Furlong, J., Verneque, R.S., Machado, M.A., 2014. Expression of immune response genes in peripheral blood of cattle infested with Rhipicephalus microplus. Genet. Mol. Res. 13, 4013–4021. https://doi.org/10.4238/2014.May.23.12

Drobyshev, A.L., Angelis, M.H.., Beckers, J., 2003. Artefacts and Reliability of DNA Microarray Expression Profiling Data, in: Christian Néri (Ed.), Current Genomics. Bentham Science Publishers, pp. 615–621. https://doi.org/https://doi.org/10.2174/1389202033490204

Eisen, M.B., Brown, P.O., 1996. [12] DNA Arrays for Analysis of Gene Expression. Methods Enzymol. 303, 179–205.

Fouche, G., Ramafuthula, M., Maselela, V., Mokoena, M., Senabe, J., Leboho, T., Sakong, B.M., Adenubi, O.T., Eloff, J.N., Wellington, K.W., 2016. Acaricidal activity of the organic extracts of thirteen South African plants against Rhipicephalus (Boophilus) decoloratus (Acari: Ixodidae). Vet. Parasitol. 224, 39–43. https://doi.org/10.1016/j.vetpar.2016.05.011

Fournier, P.-E., 2017. Genomics of Rickettsia species, in: One Health 9th Tick and Tick-Borne Pathogen Conference & 1st Asia Pacific Rickettsia Conference. Cairnes, Australia, p. 6.

Francischetti, I.M.B., Sá-Nunes, A., Mans, B.J., Santos, I.M., Ribeiro, J.M.C., 2010. THE ROLE OF SALIVA IN TICK FEEDING. Front Biosci. 14, 2051–2088.

Franzin, A.M., Maruyama, S.R., Garcia, G.R., Oliveira, R.P., Ribeiro, J.M.C., Bishop, R., Maia, A.A.M., Moré, D.D., Ferreira, B.R., De Miranda Santos, I.K.F., 2017. Immune and biochemical responses in skin differ between bovine hosts genetically susceptible and resistant to the cattle tick Rhipicephalus microplus. Parasites and Vectors 10, 1–24. https://doi.org/10.1186/s13071-016-1945-z

Frisch, J.E., 1999. Towards a permanent solution for controlling cattle ticks. Int. J. Parasitol. 29, 57–71.

Frisch, J.E., Drinkwater, R., Harrison, B., Johnson, S., 1997. Classification of the southern African sanga and East African short-horned zebu. Anim. Genet. 28, 77–83. https://doi.org/10.1111/j.1365-2052.1997.00088.x

Gentle, A., Anastasopoulus, F., McBrien, N.A., 2001. High-resolution semi-quantitative real-time PCR without the use of a standard curve. Biotechniques 31, 502–507.

Git, A., Dvinge, G., Salmon-Divon, M., Osborne, M., Kutter, C., Hadfield, J., Bertone, P., Caldas, C., 2010. Systematic comparison of microarray profiling, real-time PCR, and next-generation sequencing technologies for measuring differential microRNA expression. RNA Soc. 16, 991–1006.

Grisi, L., Leite, R.C., Martins, J.R. de S., Barros, A.T.M. de, Andreotti, R., Cançado, P.H.D., León, A.A.P. de, Pereira, J.B., Villela, H.S., 2014. Reassessment of the potential economic impact of cattle parasites in Brazil. Rev. Bras. Parasitol. Veterinária 23, 150–156. https://doi.org/10.1590/S1984-29612014042

Guerrero, F.D., Miller, R.J., Pérez de León, A. a., 2012. Cattle tick vaccines: Many candidate antigens, but will a commercially viable product emerge? Int. J. Parasitol. 42, 421–427. https://doi.org/10.1016/j.ijpara.2012.04.003

Horak, I.G., Nyangiwe, N., De Matos, C., Neves, L., 2009. Species composition and geographic distribution of ticks infesting cattle, goats and dogs in a temperate and in a subtropical region of south-east Africa. Onderstepoort J. Vet. Res. 76, 263–276. https://doi.org/10.4102/ojvr.v76i3.28

Hue, T., Mermoud, I., Rantoen, D., Lua, L., Munroe, C., Tony, V., Petermann, J., 2017. Prospecting the use of a novel Bm86-based vaccine for integrated Rhipicephalus australis management in New Caledonia, in: One Health - 9th Tick and Tick-Borne Pathogen Conference & 1st Asia Pacific Rickettsia Conference. Cairnes, Australia, p. 65.

Jaluria, P., Konstantopoulos, K., Betenbaugh, M., Shiloach, J., 2007. A perspective on microarrays: Current applications, pitfalls, and potential uses. Microb. Cell Fact. 6, 1–14. https://doi.org/10.1186/1475-2859-6-4

Jenner, R.G., Young, R.A., 2005. Insights into host responses against pathogens from transcriptional profiling. Nat. Rev. Microbiol. 3, 281–294. https://doi.org/10.1038/nrmicro1126

Jongejan, F., Uilengberg, G., 2004. The global importance of ticks. Parasitology 129, S3–S14. https://doi.org/10.1017/S0031182004005967

Jonsson, N.N., 2006. The productivity effects of cattle tick (Boophilus microplus) infestation on cattle, with particular reference to Bos indicus cattle and their crosses. Vet. Parasitol. 137, 1–10. https://doi.org/10.1016/j.vetpar.2006.01.010

Jonsson, N.N., Piper, E.K., Constantinoiu, C.C., 2014. Host resistance in cattle to infestation with the cattle tick Rhipicephalus microplus. Parasite Immunol. 36, 553–559. https://doi.org/10.1111/pim.12140

Kazimírová, M., Thangamani, S., Bartíková, P., Hermance, M., Holíková, V., Nuttall, Š.I., 2017. Tick-Borne Viruses and Biological Processes at the Tick-Host-Virus Interface 7. https://doi.org/10.3389/fcimb.2017.00339

Kemp, D., Pearson, R., Gough, J., Willadsen, P., 1989. Vaccination against Boophilus microplus: localization of antigens on tick gut cells and their interaction with the host immune system.https://www.ncbi.nlm.nih.gov/pubmed/?term=Kemp%20DH%5BAuthor%5D&cauthor=true&cauthor_uid=2667918. Exp. Appl. Acarol. 7, 43–58.

Klafke, G., Tidwell, J., Miller, R., de León, P.A., 2017. High-resolution melt (HRM) analysis for detection of SNPs associated with acaricide resistance in the cattle tick, Rhipicephalus microplus., in: One Health -

9th Tick and Tick-Borne Pathogen Conference & 1st Asia Pacific Rickettsia Conference. Cairnes, Australia, p. 114.

Koboldt, D.C., Steinberg, K.M., Larson, D.E., Wilson, R.K., Mardis, E.R., 2013. The next-generation sequencing revolution and its impact on genomics. Cell 155, 27–38. https://doi.org/10.1016/j.cell.2013.09.006

Kongsuwan, K., Piper, E.K., Bagnall, N.H., Ryan, K., Moolhuijzen, P., Bellgard, M., Lew, A., Jackson, L., Jonsson, N.N., 2008. Identification of genes involved with tick infestation in Bos taurus and Bos indicus. Anim. Genomics Anim. Heal. 132, 77–88.

Kuleš, J., Horvatić, A., Guillemin, N., Galan, A., Mrljak, V., Bhide, M., 2016. New approaches and omics tools for mining of vaccine candidates against vector-borne diseases. Mol. Biosyst. 12, 2680–2694. https://doi.org/10.1039/c6mb00268d

Lashmar, S., Muchadeyi, F., Visser, C., 2019. Genotype imputation as a cost-saving genomic strategy for South African Sanga cattle: A review. S. Afr. J. Anim. Sci. 2019, 262–280. https://doi.org/10.4314/sajas.v49i2.7

Lazcano Díaz, E., Padilla Camberos, E., Castillo Herrera, G.A., Estarrón Espinosa, M., Espinosa Andrews, H., Paniagua Buelnas, N.A., Gutiérrez Ortega, A., Martínez Velázquez, M., 2019. Development of essential oil-based phyto-formulations to control the cattle tick Rhipicephalus microplus using a mixture design approach. Exp. Parasitol. 201, 26–33. https://doi.org/10.1016/j.exppara.2019.04.008

Lew-Tabor, A.E., Rodriguez Valle, M., 2016. A review of reverse vaccinology approaches for the development of vaccines against ticks and tick-borne diseases. Ticks Tick. Borne. Dis. https://doi.org/10.1016/j.ttbdis.2015.12.012

Liang, P., Pardee, A.B., 1995. Recent advances in differential display. Curr. Opin. Immunol. 7, 274–280. https://doi.org/10.1016/0952-7915(95)80015-8

Lister, R., Pelizzola, M., Dowen, R.H., Hawkins, R.D., Hon, G., Tonti-Filippini, J., Nery, J.R., Lee, L., Ye, Z., Ngo, Q.M., Edsall, L., Antosiewicz-Bourget, J., Stewart, R., Ruotti, V., Millar, A.H., Thomson, J.A., Ren, B., Ecker, J.R., 2009. Human DNA methylomes at base resolution show widespread epigenomic differences. Nature 462, 315–322. https://doi.org/10.1038/nature08514

Liu, Y., Zhou, J., White, K.P., 2013. RNA-seq differential expression studies: More sequence or more replication? Bioinformatics 30, 301–304. https://doi.org/10.1093/bioinformatics/btt688

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262

Machugh, D.E., Shriver, M.D., Loftus, R.T., Cunningham, P., Bradley, D.G., 1997. Domestication and Phylogeography of Taurine and Zebu Cattle (BOS tuum and Bos indicus).

Mackinnon, M.., Meyer, K., Hetzel, D.J.., 1991. Genetic variation and covariation for growth, parasite

resistance and heat tolerance in tropical cattle. Livest. Prod. Sci. 27, 105–122.

Madder, M., Horak, I., Stoltsz, H., 2014. Ticks: Tick importance - disease transmission [WWW Document]. URL http://www.afrivip.org/sites/default/files/Ticks-importance/rhipicephalus.html (accessed 11.2.15).

Madder, M., Thys, E., Achi, L., Touré, A., De Deken, R., 2011. Rhipicephalus (Boophilus) microplus: a most successful invasive tick species in West Africa. Exp. Appl. Acarol. 53, 139–145. https://doi.org/10.1007/s10493-010-9390-8

Malatji, D.P., 2017. A genomic insight into the diversity and pathogenesis of village chicken in an Ascaridia Galli infested environment. The University of Pretoria.

Manjunathachar, H.V., Saravanan, B.C., Kesavan, M., Karthik, K., Rathod, P., Gopi, M., Tamilmahan, P., Balaraju, B.L., 2014. Economic importance of ticks and their effective control strategies. Asian Pacific J. Trop. Dis. 4, S770–S779. https://doi.org/10.1016/S2222-1808(14)60725-8

Mans, B., Pienaar, R., de Klerk, D., de Castro, M., Latif, A., 2017. Evolution of blood-feeding behaviour in Argasids: perspectives from salivary gland transcriptomes, in: One Health - 9th Tick and Tick-Borne Pathogen Conference & 1st Asia Pacific Rickettsia Conference. Cairnes, Australia, p. 46.

Mans, B.J., 2005. Tick histamine-binding proteins and related lipocalins: potential as therapeutic agents. Curr. Opin. Investig. Drugs 6, 1131–5.

Mapholi, Ntanganedzeni O, Marufu, M.C., Maiwashe, A., Banga, C.B., Muchenje, V., Macneil, M.D., Chimonyo, M., Dzama, K., 2014. Ticks and Tick-borne Diseases Towards a genomics approach to tick (Acari : Ixodidae) control in cattle : A review. Ticks Tick. Borne. Dis. 5, 475–483. https://doi.org/10.1016/j.ttbdis.2014.04.006

Mapholi, Ntanganedzeni O., Marufu, M.C., Maiwashe, A., Banga, C.B., Muchenje, V., MacNeil, M.D., Chimonyo, M., Dzama, K., 2014. Towards a genomics approach to tick (Acari: Ixodidae) control in cattle: A review. Ticks Tick. Borne. Dis. 5, 475–483. https://doi.org/10.1016/j.ttbdis.2014.04.006

Mapiye, C., Chikwanha, O.C., Chimonyo, M., Dzama, K., 2019. Strategies for sustainable use of indigenous cattle genetic resources in southern Africa. Diversity 11, 214. https://doi.org/10.3390/d11110214

Marima, J.K., 2017. GENE EXPRESSION PROFILES ASSOCIATED WITH BEEF CATTLE RESISTANCE TO RHIPICEPHALUS TICKS By Jacqueline Keena Marima Thesis submitted in partial fulfilment of the requirements for the degree of Master of Science (Agriculture) in the Faculty of Agrisciences.

Marima, J.K., Nel, C.L., Marufu, M.C., Jonsson, N.N., Dube, B., Dzama, K., 2020. A genetic and immunological comparison of tick-resistance in beef cattle following artificial infestation with Rhipicephalus ticks. Exp. Appl. Acarol. 80, 569–590. https://doi.org/10.1007/s10493-020-00480-8

Marioni, J.C., Mason, C.E., Mane, S.M., Stephens, M., Gilad, Y., 2008. RNA-seq: An assessment of technical reproducibility and comparison with gene expression arrays. Genome Res. 18, 1509–1517. https://doi.org/10.1101/gr.079558.108

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

Martinez, M.L., Machado, M. a, Nascimento, C.S., Silva, M.V.G.B., Teodoro, R.L., Furlong, J., Prata, M.C. a, Campos, a L., Guimarães, M.F.M., Azevedo, a L.S., Pires, M.F. a, Verneque, R.S., 2006. Association of BoLA-DRB3.2 alleles with tick (Boophilus microplus) resistance in cattle. Genet. Mol. Res. 5, 513–24.

Marufu, M.C., Chimonyo, M., Mans, B.J., Dzama, K., 2013. Cutaneous hypersensitivity responses to Rhipicephalus tick larval antigens in pre-sensitized cattle. Ticks Tick. Borne. Dis. 4, 311–316. https://doi.org/10.1016/j.ttbdis.2012.12.001

Marufu, M.C., Dzama, K., Chimonyo, M., 2014. Cellular responses to Rhipicephalus microplus infestations in pre-sensitised cattle with differing phenotypes of infestation. Exp. Appl. Acarol. 62, 241–252. https://doi.org/10.1007/s10493-013-9723-5

Marufu, M.C., Qokweni, L., Chimonyo, M., Dzama, K., 2011. Relationships between tick counts and coat characteristics in Nguni and Bonsmara cattle reared on semiarid rangelands in South Africa. Ticks Tick. Borne. Dis. 2, 172–177. https://doi.org/10.1016/j.ttbdis.2011.07.001

Mekonnen, S., Bryson, N.., Fourie, L.., Peter, R.., Spickett, A.., Taylor, R.., Strydom, T., Horak, I.G., 2002. Acaricide resistance profiles of single- and multi-host ticks from communal and commercial farming areas in the Eastern Cape and North-West Provinces of South Africa. Onderstepoort J. Vet. Res. 69, 99–105.

Morozova, O., Hirst, M., Marra, M.A., 2009. Applications of new sequencing technologies for transcriptome analysis. Annu. Rev. Genomics Hum. Genet. 10, 135–151. https://doi.org/10.1146/annurev-genom-082908-145957

Morris, C. a., 2007. A review of genetic resistance to disease in Bos taurus cattle. Vet. J. 174, 481–491. https://doi.org/10.1016/j.tvjl.2006.09.006

Motoaki Seki, Mari Narusaka, Asako Kamiya, Junko Ishida, Masakazu Satou, Tetsuya Sakurai, Maiko Nakajima, Akiko Enju, Kenji Akiyama, Youko Oono, Masami Muramatsu, Yoshihide Hayashizaki, Jun Kawai, Piero Carninci, Masayoshi Itoh, Yoshiyuki Ishii, Takahiro Arakawa, Kazuhiro Shibata, Akira Shinagawa, Kazuo Shinozaki, 2002. Functional Annotation of a Full-Length Arabidopsis cDNA Collection. Science (80-.). 296, 141–145. https://doi.org/10.1126/science.290.5499.2105

Muchenje, V., Dzama, K., Chimonyo, M., Raats, J.G., Strydom, P.E., 2008a. Tick susceptibility and its effects on growth performance and carcass characteristics of Nguni, Bonsmara and Angus steers raised on natural pasture. animal 2, 298–304. https://doi.org/10.1017/S1751731107001036

Muchenje, V., Dzama, K., Chimonyo, M., Raats, J.G., Strydom, P.E., 2008b. Tick susceptibility and its effects on growth performance and carcass characteristics of Nguni, Bonsmara and Angus steers raised on natural pasture. Animal 2, 298–304. https://doi.org/10.1017/S1751731107001036

Musemwa, L., Mushunje, A., Chimonyo, M., Fraser, G., Mapiye, C., Muchenje, V., 2008. Nguni cattle marketing constraints and opportunities in the communal areas of South Africa: Review, African Journal of Agricultural Research.

Mwai, O., Hanotte, O., Kwon, Y.J., Cho, S., 2015. - Invited review - African indigenous cattle: Unique genetic resources in a rapidly changing world. Asian-Australasian J. Anim. Sci. 28, 911–921. https://doi.org/10.5713/ajas.15.0002R

Nadolny, R., Walters, E., Gaff, H., 2017. Disturbance events facilitate tick invasions and increase disease risk, in: One Health - 9th Tick and Tick-Borne Pathogen Conference & 1st Asia Pacific Rickettsia Conference. Cairnes, Australia, p. 108.

Nuttall, P.A., 2017. The wonders of tick saliva, in: One Health 9th Tick and Tick-Borne Pathogen Conference & 1st Asia Pacific Rickettsia Conference. Cairnes, Australia, p. 13.

Nyangiwe, N., 2017. Distribution and ecology of economically important ticks on cattle, with special reference to the Eastern Cape Province, South Africa and Namibia.

Nyangiwe, N., Harrison, a., Horak, I.G., 2013. Displacement of Rhipicephalus decoloratus by Rhipicephalus microplus (Acari: Ixodidae) in the Eastern Cape Province, South Africa. Exp. Appl. Acarol. 61, 371–382. https://doi.org/10.1007/s10493-013-9705-7

Olivia, N., 2015. Exploring Genetic Architecture of Tick Resistance in South African Nguni Cattle By Ntanganedzeni Olivia Mapholi Dissertation presented in fulfilment of the requirements for the degree Doctor of Philosophy at the Department of Animal Sciences, Stellenbosc. University of Stellenbosch.

Paesen, G.C., Adams, P.L., Harlos, K., Nuttall, P.A., Stuart, D.I., 1999. Tick histamine-binding proteins: isolation, cloning, and three-dimensional structure. Mol. Cell 3, 661–71. https://doi.org/10.1016/s1097-2765(00)80359-7

Pavela, R., Canale, A., Mehlhorn, H., Benelli, G., 2016. Application of ethnobotanical repellents and acaricides in prevention, control and management of livestock ticks: A review. Res. Vet. Sci. https://doi.org/10.1016/j.rvsc.2016.09.001

Pazinato, R., Volpato, A., Baldissera, M.D., Santos, R.C.V., Baretta, D., Vaucher, R.A., Giongo, J.L., Boligon, A.A., Stefani, L.M., Da Silva, A.S., 2016. In vitro effect of seven essential oils on the reproduction of the cattle tick Rhipicephalus microplus. J. Adv. Res. 7, 1029–1034. https://doi.org/10.1016/j.jare.2016.05.003

Piper, E.K., Jackson, L. a., Bagnall, N.H., Kongsuwan, K.K., Lew, A.E., Jonsson, N.N., 2008. Gene expression in the skin of Bos taurus and Bos indicus cattle infested with the cattle tick, Rhipicephalus (Boophilus) microplus. Vet. Immunol. Immunopathol. 126, 110–119. https://doi.org/10.1016/j.vetimm.2008.06.011

Piper, E.K., Jackson, L. a., Bielefeldt-Ohmann, H., Gondro, C., Lew-Tabor, A.E., Jonsson, N.N., 2010. Tick-susceptible Bos taurus cattle display an increased cellular response at the site of larval Rhipicephalus (Boophilus) microplus attachment, compared with tick-resistant Bos indicus cattle. Int. J. Parasitol. 40,

431–441. https://doi.org/10.1016/j.ijpara.2009.09.009

Piper, E.K., Jonsson, N.N., Gondro, C., Lew-Tabor, A.E., Moolhuijzen, P., Vance, M.E., Jackson, L. a, 2009. Immunological profiles of Bos taurus and Bos indicus cattle infested with the cattle tick, Rhipicephalus (Boophilus) microplus. Clin. Vaccine Immunol. 16, 1074–86. https://doi.org/10.1128/CVI.00157-09

Porto Neto, L.R., Jonsson, N.N., D'Occhio, M.J., Barendse, W., 2011. Molecular genetic approaches for identifying the basis of variation in resistance to tick infestation in cattle. Vet. Parasitol. 180, 165–172. https://doi.org/10.1016/j.vetpar.2011.05.048

Qiagen, 2016. Cow inflammatory cytokines and Receptors PCR Arrays [WWW Document]. URL http://www.sabiosciences.com/rt_pcr_product/HTML/PABT-011Z.html (accessed 4.20.16).

Rechav, Y., Kostrzewski, M.W., 1991. Relative resistance of six cattle breeds to the tick Boophilus decoloratus in South Africa. Onderstepoort J. Vet. Res. 58, 181–186.

Riek, R., 1962. Studies on the reactions of animals to infestation with ticks. VI. Resistance of cattle to infestation with the tick *Boophilus microplus* (Canestrini). Aust. J. Agric. Res. 13, 532. https://doi.org/10.1071/AR9620532

Robbertse, L., Richards, S.A., Clift, S.J., Barnard, A.C., Leisewitz, A., Crafford, J.E., Maritz-Olivier, C., 2018. Comparison of the differential regulation of T and B-lymphocyte subsets in the skin and lymph nodes amongst three cattle breeds as potential mediators of immune-resistance to Rhipicephalus microplus. Ticks Tick. Borne. Dis. 9, 976–987. https://doi.org/10.1016/j.ttbdis.2018.03.034

Rodriguez-Valle, M., Vance, M., Moolhuijzen, P.M., Tao, X., Lew-Tabor, A.E., 2012. Differential recognition by tick-resistant cattle of the recombinantly expressed Rhipicephalus microplus serine protease inhibitor-3 (RMS-3). Ticks Tick. Borne. Dis. 3, 159–169. https://doi.org/10.1016/j.ttbdis.2012.03.002

Rodriguez-Vivas, R.I., Jonsson, N.N., Bhushan, C., 2018. Strategies for the control of Rhipicephalus microplus ticks in a world of conventional acaricide and macrocyclic lactone resistance. Parasitol. Res. https://doi.org/10.1007/s00436-017-5677-6

Rodriguez-vivas, R.I., Trees, A.J., Rosado-aguilar, J.A., Villegas-perez, S.L., Hodgkinson, J.E., 2011. Evolution of acaricide resistance : Phenotypic and genotypic changes in field populations of Rhipicephalus (Boophilus) microplus in response to pyrethroid selection pressure. Int. J. Parasitol. 41, 895–903. https://doi.org/10.1016/j.ijpara.2011.03.012

Schena, M., Shalon, D., Davis, R., Brown, P., 1995. Quantitative Monitoring of Gene Expression Patterns with a Complementary DNA Microarray. Adv. Sci. 270, 467–470.

Schlamp, K., Weinmann, A., Krupp, M., Maass, T., Galle, P., Teufel, A., 2008. BlotBase: A northern blot database. Gene 427, 47–50. https://doi.org/10.1016/j.gene.2008.08.026

Schneider, M. V., Orchard, S., 2011. Omics technologies, data and bioinformatics principles. Methods Mol. Biol. https://doi.org/10.1007/978-1-61779-027-0_1

Schurch, N.J., Schofield, P., Gierliński, M., Cole, C., Sherstnev, A., Singh, V., Wrobel, N., Gharbi, K., Simpson, G., Owen-Hughes, T., Blaxter, M., Barton, G.J., 2016. How many biological replicates are needed in an RNA-seq experiment and which differential expression tool should you use? Rna 22, 1641-. https://doi.org/10.1261/rna.053959.115.

Serruto, D., Rappuoli, R., 2006. Post-genomic vaccine development. FEBS Lett. 580, 2985–2992. https://doi.org/10.1016/j.febslet.2006.04.084

Severo, M.S., 2013. UC Riverside UC Riverside Electronic Theses and Dissertations Author.

Šimo, L., Kazimirova, M., Richardson, J., Bonnet, S.I., 2017. The Essential Role of Tick Salivary Glands and Saliva in Tick Feeding and Pathogen Transmission. Front. Cell. Infect. Microbiol. https://doi.org/10.3389/fcimb.2017.00281

Soneson, C., Delorenzi, M., 2013. A comparison of methods for differential expression analysis of RNA-seq data. BMC Bioinformatics 14. https://doi.org/10.1186/1471-2105-14-91

Spickett, A.M., Malan, J.R., 1978. Genetic incompatibility between Boophilus decoloratus (Koch, 1844) and Boophilus microplus (Canestrini, 1888) and hybrid sterility of Australian and South African Boophilus microplus (Acarina: Ixodidae). Onderstepoort J. Vet. Res. 45, 149–53.

Stear, M.J., Hetzel, D.J.S., Brown, S.C., Gershwin, L.J., Mackinnon, M.J., Nicholas, F.W., 1990. The relationships among ecto- and endoparasite levels, class I antigens of the bovine major histocompatibility system, immunoglobulin E levels and weight gain. Vet. Parasitol. 34, 303–321. https://doi.org/10.1016/0304-4017(90)90077-O

STEAR, M.J., TIERNEY, T.J., BALDOCK, F.C., BROWN, S.C., NICHOLAS, F.W., RUDDER, T.H., 1988. Class I antigens of the bovine major histocompatibility system are weakly associated with variation in faecal worm egg counts in naturally infected cattle. Anim. Genet. 19, 115–122. https://doi.org/10.1111/j.1365-2052.1988.tb00797.x

Streit, S., Michalski, C.W., Erkan, M., Kleeff, J., Friess, H., 2009. Northern blot analysis for detection and quantification of RNA in pancreatic cancer cells and tissues. Nat. Protoc. 4, 37–43. https://doi.org/10.1038/nprot.2008.216

Sutherst, R.W., Kerr, J.D., Maywald, G.F., Stegeman, D.A., 1983. The effect of season and nutrition on the resistance of cattle to the tick Boophilus microplus. Aust. J. Agric. Res. 34, 329–339. https://doi.org/10.1071/AR9830329

Tabor, A., Ali, A., Rehman, G., Rocha Garcia, G., Zangirolamo, A.F., Malardo, T., Jonsson, N.N., 2017a. Cattle Tick Rhipicephalus microplus-Host Interface: A Review of Resistant and Susceptible Host Responses. Front. Cell. Infect. Microbiol. https://doi.org/10.3389/fcimb.2017.00506

Tabor, A., Bellgard, M., Guerrero, F., Valle, M.R., 2017b. Reverse vaccinology for cattle ticks - a successful approach, in: One Health - 9th Tick and Tick-Borne Pathogen Conference & 1st Asia Pacific Rickettsia

Conference. Cairnes, Australia, p. 95.

Taniguchi, M., Miura, K., Iwao, H., Yamanaka, S., 2001. Quantitative assessment of DNA microarrays - Comparison with Northern blot analyses. Genomics 71, 34–39. https://doi.org/10.1006/geno.2000.6427

Tatchell, R.J., 1987. Interactions between ticks and their hosts. Int. J. Parasitol. 17, 597–606. https://doi.org/10.1016/0020-7519(87)90136-6

Teng, X., Xiao, H., 2009. Perspectives of DNA microarray and next-generation DNA sequencing technologies. Sci. China, Ser. C Life Sci. 52, 7–16. https://doi.org/10.1007/s11427-009-0012-9

Trayhurn, P., 1996. Northern blotting. Proc. Nutr. Soc. 55, 583–589. https://doi.org/10.1079/PNS19960051

Utech, K.B., Wharton, R.H., Kerr, J.D., 1978. Resistance to Boophilus microplus (Canestrini) in different breeds of cattle. Aust. J. Agric. Res. 29, 885–895. https://doi.org/10.1071/AR9780885

Walker, a. ., Bouattour, A., Camicas, J.., Estrada-peña, A., Horak, I.., Latif, A.., Pegram, R.., Preston, P.., 2003. Ticks of domestic animals in Africa: a guide to identification of species, The University of Edinburgh.

Walker, J.B., Keirans, J.E., Horak, I.G., 2000. The Genus Rhipicephalus (Acari, Ixodidae): A Guide to the Brown Ticks of the tick of the world. Cambridge University Press, United States of America.

Walker, J.B., Olwage, A., 1987. The tick vectors of Cowdria ruminantium (Ixodoidea, Ixodidae, genus Amblyomma) and their distribution. Onderstepoort J. Vet. Res. 54, 353–79.

Wang, Y.H., Reverter, A., Kemp, D., McWilliam, S., Ingham, A., Davis, C., Moore, R., Lehnert, S., 2007. Gene expression profiling of Hereford Shorthorn cattle following challenge with Boophilus microplus tick larvae. Aust. J. Exp. Agric. 47, 1397–1407. https://doi.org/10.1071/EA07012

Wang, Z., Gerstein, M., Snyder, M., 2009. RNA-Seq: a revolutionary tool for transcriptomics. Nat. Rev. Genet. 57–63. https://doi.org/10.1038/nrg2484

Wang, Z., Yang, B., 2010. Northern Blotting and Its Variants for Detecting Expression and Analyzing Tissue Distribution of miRNAs, in: MicroRNA Expression Detection Methods. Springer, Berlin, Heidelberg, pp. 83–100. https://doi.org/https://doi.org/10.1007/978-3-642-04928-6_3

Warren, W.C., Clayton, D.F., Ellegren, H., Arnold, A.P., Ladeana, W., Künstner, A., Searle, S., White, S., Vilella, A.J., Fairley, S., Heger, A., Kong, L., Ponting, C.P., Jarvis, E.D., Mello, C. V, Lovell, P., Velho, T. a F., Ferris, M., Balakrishnan, C.N., Sinha, S., Blatti, C., London, S.E., Li, Y., Lin, Y., George, J., 2011. The genome of a songbird. Nature 464, 757–762. https://doi.org/10.1038/nature08819.The

Weikard, R., Hadlich, F., Kuehn, C., 2013. Identification of novel transcripts and noncoding RNAs in bovine skin by deep next-generation sequencing. BMC Genomics 14, 789. https://doi.org/10.1186/1471-2164-14-789

Wellington, K.W., Leboho, T., Sakong, B.M., Adenubi, O.T., Eloff, J.N., Fouche, G., 2017. Further studies on

South African plants: Acaricidal activity of organic plant extracts against Rhipicephalus (Boophilus) microplus (Acari: Ixodidae). Vet. Parasitol. 234, 10–12. https://doi.org/10.1016/j.vetpar.2016.12.014

Wikel, S.K., 1996. HOST IMMUNITY TO TICKS. Annu. Rev. Entomol. 41, 1–22.

Wilhelm, B.T., Landry, J.R., 2009. RNA-Seq-quantitative measurement of expression through massively parallel RNA-sequencing. Methods 48, 249–257. https://doi.org/10.1016/j.ymeth.2009.03.016

Willadsen, P., 1997. Novel vaccines for ectoparasites. Vet. Parasitol. 71, 209–22.

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

Wong, M.L., Medrano, J.F., 2005. Real-time PCR FOR mRNA quantitation. Biotechniques 39, 1–11.

Zwane, A.A., Maiwashe, A., Makgahlela, M.L., Choudhury, A., Taylor, J.F., van Marle-Köster, E., 2016. Genome-wide identification of breed-informative single-nucleotide polymorphisms in three South African indigenous cattle breeds. South African J. Anim. Sci. 46, 302–312. https://doi.org/10.4314/sajas. v46i3.10

Chapter 3:
Determining the genotypes of SNPs from previously identified candidate genes associated with host resistance to ticks in Angus, Brahman and Nguni cattle populations

Abstract

The capacity of an animal to resist the tick challenge is influenced by the animal's genotype. *Bos indicus*, Sanga and Zebu type breeds are thought to possess a superior level of resistance to most cattle tick species compared to their European counterparts. This may either be attributed to their co-evolutionary history with some of the tick species or maybe a consequence of centuries of natural selections for certain adaptation traits, given the harsh environment in which most of these breeds evolved. Using a 150K Bovine SNP chip the SNPs genotypes of 37 genes, previously identified in GWA studies, were profiled in the Angus, Brahman and Nguni breeds using tick count data from an artificial infestation with *R. microplus* as the correlating phenotype. A total of 257 SNPs were retrieved from 28 of the candidate genes. Five SNPs showed significantly different genotype frequencies across breeds and three SNPs showed a significant correlation between allele dosage and tick count. The SNP genotype frequencies of the Nguni differed significantly from those of the Angus but did not differ from those of the Brahman. A similar trend was observed with the tick counts. SNP ARS-BFGL-NGS-94983 of the *SATB2* gene presented significant genotype frequencies and a slightly positive correlation (0.182) between allele dosage and tick count. The SNP had a minor allele frequency of 0.64 in the Nguni. The *SATB2* gene currently only features in human cancer and brain development studies and would need to be investigated further for its effect on the bovine host resistance to ticks.

3.1 Introduction

The Taurine and Zebu domestic cattle lineages diverged from each other approximately 330 000 years back (Takeshima et al., 2018). From these, a third distinct lineage arose, known as the "tropically adapted taurine breeds" (Machugh et al., 1997). These included true taurine breeds which evolved in tropical environments to produce individuals with retained productive attributes of the taurine group but also the improved adaptability to the tropical and sub-tropical environments where the *Rhipicephalus microplus* (*R. microplus*) tick species are endemic (Frisch et al., 1997). Listed within this group are the African indigenous Afrikaner, Tuli and the South indigenous Nguni breeds (Burrow et al., 2019). With the divergence in lineages came an accumulation of many genetic variations. Centuries of natural selection have erased genotypes deemed susceptible to parasites and disease, thereby enabling indigenous breeds to adapt to the local environment, producing individuals with improved tolerance and resistance to tick and tick-borne diseases (TTBDs), heat

stress, diseases and parasites, feed and water shortages in every successive century (Mwai et al., 2015; Lashmar et al., 2019). The *Bos indicus* and *Bos taurus africanus* breeds, including the Brahman and Nguni breeds, respectively, are recognised for their superior resistance to TTBDs and their superior adaptation to tropical and sub-tropical regions (Ibelli et al., 2012; Otto et al., 2018). European breeds of cattle, including the Angus breed (*B taurus*), are commonly used for beef production in tropical and sub-tropical regions due to their superior meat quality. However, their susceptibility to TTBDs presents challenges with significant financial implications. The repeated need to administer chemical acaricides and vaccines to combat heavy tick burdens that the cattle may suffer as well as the associated tick-borne diseases are costly and unsustainable. This warrants the transformational use of naturally tick resistant breeds and individuals within a population.

Breeding for increased host resistance to ticks is possible (Rodriguez-Valle et al., 2013; Mapholi et al., 2016). Crossbreeding to produce hybrids with both superior meat quality and increased resistance to ticks is desired, however, there may be trade-offs in meat quality traits as the survival traits improve (Piper et al., 2017). Historically, the selection of breeding animals was conducted using estimated breeding values based on pedigree and phenotypic information (Mapholi et al., 2016). However, this approach did not offer much understanding of the genetic mechanisms underlying the tick resistance phenotype to produce consistent results with every selection cycle (Ashton et al., 1968). The genomic selection of cattle with superior tick resistance, using genetic markers to estimate the merit of the animals, presents an opportunity for great genetic improvement of tick resistance (Burrow et al., 2021). This provides an alternative to the selection of resistant animals based on repetitive tick counts that are prone to inaccuracies and uncontrolled environmental effects (Mukhund et al., 2020). The rapidly decreasing cost of whole-genome sequencing and single nucleotide polymorphism (SNP) analysis in conjunction with the vast computational capacity available for data processing and storage, supports the opportunity to isolate potential biomarkers for use in the identification of between- and within-breed selection (Burrow et al., 2021). Therefore, marker-assisted selective breeding presents a more attainable alternative. It is important to identify markers or SNPs that are associated with increased host resistance to ticks to aid the development of genomic tests that can be used for the selection of tick resistant individuals within a herd. This type of selection may be especially useful in improving the sustainability of low- and medium-input beef producers (Burrow et al., 2019).

In the context of a complex, polygenic trait such as tick resistance in cattle it is important to understand the multiple distinct biological and genetic processes (these are discussed in depth in chapters 4 and 5). Several earlier studies identified candidate SNPs and quantitative trait loci (QTLs) associated with tick counts (Francis and Ashton, 1967; Mapholi et al., 2016; L. R. Porto Neto et al., 2010c; Porto Neto et al., 2012; Turner et al., 2010a), thereby guiding the present study. Among the first identified biomarkers for tick resistance in cattle were serum amylase (Ashton et al., 1968) and the Bovine Leukocyte Antigen (BoLA) gene marker known as the glutamic acid serine (Stear et al.,

1990, 1989, 1984). Serum amylase is encoded by a locus on chromosome 3 while the BoLA marker was detected on the bovine major histocompatibility complex (MHC) of axon class II BoLA-DBR3 gene (Mapholi et al., 2014; Martinez et al., 2006). Morris (2007) reported the presence of the BoLA gene marker in 60% of the cattle that were suspected to be susceptible to ticks. Therewithin, it induced 96% susceptibility to tick infestations. Similarly, in their study on dairy cattle in Brazil, Martinez et al. (2006) observed an association between bovine host resistance to the *R. microplus* ticks species and the BoLA marker DBR alleles 3.2, 18, 20 and 27. Inconsistencies appear in the way the BoLA allele was previously associated with the level of tick resistance exhibited (Porto Neto et al., 2011). Furthermore, there are poor characterizations of the BoLA gene polymorphisms in beef breeds, especial the Zebu type breeds.

The genetic architecture that makes the Brahman and Nguni breed naturally more resistant to ticks has sparked great interest in the search for more sustainable and environmentally sound alternative tick control measures. Porto Neto et al. (2010a; 2010b;2010c) detected SNPs in the regions of candidate genes *ITGA11, ADGRL4, LAPTM5, ALPL, MFSD2A, DHHRS7, GNPNAT1, SERINC5, SIRPA, CA2*, and *AAD3* in the Brahman breed. Additionally, Mapholi et al. (2016) detected SNPs in the regions of genes *SMIM12, SMIM12, KCNQ4, TRPM8, CSN1S2, FER, LINGO2, CACFD1, LRBA, SLC38A7, GPR142* and *PRKG1* in the Nguni breed. Several other genes have presented SNPs found to be in association with the tick resistance trait, however, very few are functional mutations that can be used as potential biomarkers for tick resistance in cattle.

This study aimed to identify SNPs for a panel of genes that were previously identified in genome-wide association studies (GWAS) that are associated with bovine host resistance to ticks. Furthermore, the study aimed to preliminarily determine the association between the identified SNPs genotypes, allele dosages, and frequencies, with the tick resistance phenotype across the Angus, Brahman, and Nguni breeds using post artificial infestation tick count data.

3.2 Materials and Methods

3.2.1 Research animals and sample collection

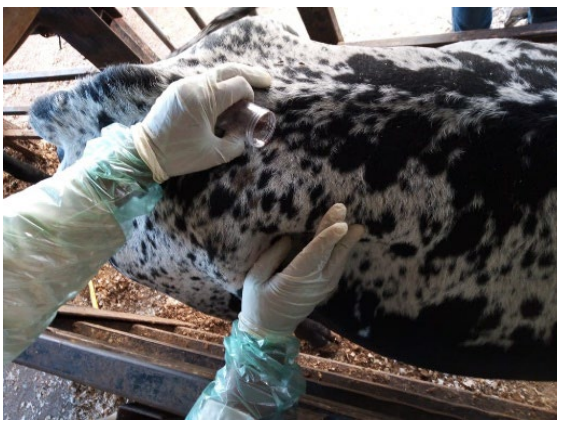
Hair root samples were collected from the tails of 10 Angus (*B. taurus*), 10 Brahman (*B. indicus*) and 10 Nguni (*B. taurus africanus*) cattle. The animals were between the ages of 12 and 15 months with prior exposure to both *R. microplus* and *R. decoloratus* tick infestations. Despite the divergent ages of the three breeds, the cattle were deemed to be at a similar developmental stage (Constantinoiu et al., 2010). The three breeds were chosen for this study because they represent beef cattle breeds commonly used in *R. microplus* endemic areas in South Africa. The three breed groups were especially considered based on their diverse production potential and environmental adaptive capacity.

The study was conducted at the Mariendal experimental farm of Stellenbosch University, located 33.8452599419S 18.834721521E in the Western Cape Province of South Africa. This coastal region received winter rainfall with an average of 159.14 mm per annum over the past 20 years. Temperatures during the duration of the trial were on average 18.65: maximum temp = 38.17 ^{0}C and minimum temp = 8.13 ^{0}C. The average humidity of the region for the duration of the trial was reported at 74.31% on average with a maximum of 100.00% and a minimum of 21.72% (Meijers, 2019).

All animals used in the study underwent procedures approved by the Stellenbosch University Research Ethics Committee: Animal Care and Use (REC: ACU Reference #: ACU-2018-1719) and that complied with internationally accepted standards for animal welfare and ethics (Austin et al., 2005). Furthermore, the South African Department of Agriculture, Forestry and Fisheries (DAFF) granted a Section 20 permit for the use of *R. microplus* on Angus, Brahman and Nguni cattle.

3.2.2 Artificial infestation and tick count data collection

Freshly hatched unfed tick larvae (UFL) of the *R. microplus* species, harvested from the aseptic colonies of Clinvet International Laboratories, were used for all the artificial infestations. Upon delivery to the Stellenbosch University laboratories, the larvae were stored in a salt-water humidity container (400g coarse salt: 1 litre distilled water), with approximately 75% humidity, at 20 -24^{0}C for 24 hours ahead of the artificial infestation. All the animals were subjected to a full-body artificial infestation with approximately 10 000 tick larvae. Aliquots of 0.5g of tick larvae, equivalent to 10 000 larvae, were placed into small vials and used for the infestation. The larvae were applied equally to parts of the body highlighted in Figure 3.1.



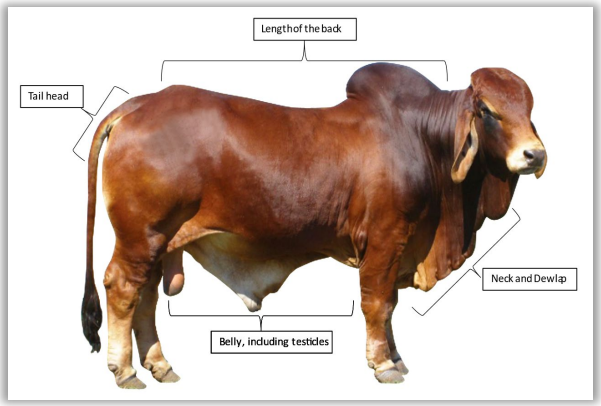


Figure 3.1: Areas of the animals that were directly exposed to tick challenge

Two trained enumerators were used to count semi- and fully engorged ticks ranging from 4 to 8 mm in length on the whole body of the animal. Tick count data were collected from day 16 to day 23 post-infestation, resulting in a total of 8 counts per animal.

3.2.3 DNA extraction

Genomic DNA was extracted from all 30 samples following the proteinase-K digestion and phenol: chloroform: isoamyl alcohol extraction and ethanol precipitation protocol (Mapholi et al., 2016). The concentration and purity/quality of the extracted DNA was checked using the Qubit® 2.0 Fluorometer and the Nanodrop 200 spectrophotometer, respectively. The minimum desired DNA concentration was 64 ng/ul intend to achieve 500ng total high-quality DNA. Furthermore, minimum optical density ratios of 1.9 (260/230) and 2.0 (260/280) were also preferred.

3.2.4 Genotyping and data pre-processing

Genotyping of samples from all 30 animals was performed at the South African Agricultural Research Council Biotechnology Platform using the GGP Bovine 150K array (Illumina, Inc. San Diego, California) according to the manufacturer's instructions. The GGP Bovine 150K chips were scanned using Illumina iScan and the data was analysed using the GenomeStudio® Genotyping Module v2.0 software (Illumina, Inc. San Diego, California).

Quality control was performed on the SNP data using Plink v1.9 to exclude samples with suboptimal genotyping quality. A strict criterion was applied to exclude samples with low minor allele frequencies (MAF < 0.01), missing genotype data (Geno > 0.10 or Mind < 0.10) and a SNP call rate greater than 90% as well as markers that significantly deviated from Hardy Weinberg proportion (HWE < 0.0000001). Of the 142, 716 SNPs on the chip, only 133 628 SNPs passed the filters and QC for downstream testing.

3.2.5 Candidate SNP discovery

A list of candidate genes was compiled based on previous GWA studies within which candidate SNPs associated with host resistance to ticks in cattle of various breeds were spotted. Summarized in Table 3.1 are the details of 37 genes that were identified. Using a code scripted using R v4.1.1 (2021-08-010) in RStudio v1.4.1717 the genotypes and minor alleles frequencies of the retained SNPs associated with the candidate genes were determined for each of the breed groups. Furthermore, the relationships between the SNP genotypes and breed type as well as each of the breed's levels of resistance to the *R. microplus* infestations was determined. Figure 3.2 illustrates the code applied to generate the results for the abovementioned objectives.

Table 3.1: List of candidate SNPs previously detected and associated with tick resistance in cattle of various breeds

Reference	GWA method	Cattle breed	Chr. no	Variant Object	Gene Name (inferred if not genetic)	Gene Stable ID	Gene Stable version	Gene Start	Gene End
Francis & Ashton (1967)	Protein Electrophoresis	Droughtmaster	1	TF	Serum transferrin	ENSBTAG00000007273	ENSBTAG00000007273.6	135579069	135618070
			6	ALB	Post albumin	ENSBTAG00000017121	ENSBTAG00000017121.6	88484915	88503341
L. R. Porto Neto et al. (2010b)	DNA-SNP	N/A	10	ITGA11	integrin alpha 11	ENSBTAG00000008380	ENSBTAG00000008380.5	15129322	15262276
L. R. Porto Neto et al., (2010a)		Taurine cattle, Brahman & Tropical Composite beef cattle	3	ELTD1 or ADGRL4	EGF, latrophilin and seven transmembrane domain containing 1	ENSBTAG00000019733	ENSBTAG00000019733.5	65667175	65807163
Porto Neto et al. (2012)		Dairy cattle (*Bos taurus L.)*	14	RIPK2	receptor-interacting serine-threonine kinase 2	ENSBTAG00000015271	ENSBTAG00000015271.4	73895603	73936735
L. R. Porto Neto et al., (2010c)	DNA-SNP-meta-cand	Dairy cattle of various breeds and Brahman	2	LAPTM5	lysosomal protein transmembrane 5	ENSBTAG00000005477	ENSBTAG00000005477.6	122807359	122833480
			2	ALPL	alkaline phosphatase, liver/bone/kidney	ENSBTAG00000008951	ENSBTAG00000008951.6	131181416	131245100
			3	MFSD2A	major facilitator superfamily domain containing 2A	ENSBTAG00000013054	ENSBTAG00000013054.6	106122567	106136408
			10	DHRS7	dehydrogenase/reductase (SDR family) member 7	ENSBTAG00000020729	ENSBTAG00000020729.6	72362347	72385747
			10	GNPNAT1	glucosamine-phosphate N-acetyltransferase 1	ENSBTAG00000005344	ENSBTAG00000005344.6	11392564	11404461
			10	SERINC5	serine incorporator 5	ENSBTAG00000001619	ENSBTAG00000001619.6	11111559	11216841
			13	SIRPA	signal regulatory protein alpha	ENSBTAG00000007213	ENSBTAG00000007213.6	53196957	53240508
			14	CA2	carbonic anhydrase II	ENSBTAG00000017733	ENSBTAG00000017733.4	76994568	77012297
			26	ADD3	adducin 3 (gamma)	ENSBTAG00000003265	ENSBTAG00000003265.6	30579476	30711953
Turner et al., (2010b)	DNA-SNP	Dairy cattle (*Bos taurus L.*)	1	NAALADL2	N-acetylated alpha-linked acidic dipeptidase like 2	ENSBTAG00000048493	ENSBTAG00000048493.1	91436009	92436553
			2	SATB2	SATB homeobox 2	ENSBTAG00000016334	ENSBTAG00000016334.5	87843246	88046645
			4	GLCCI1	glucocorticoid induced 1	ENSBTAG00000008533	ENSBTAG00000008533.6	16051521	16156180
			7	MAN2A1	mannosidase alpha class 2A member 1	ENSBTAG00000025029	ENSBTAG00000025029.5	108794483	109024141

			8	TNFSF8	TNF superfamily member 8	ENSBTAG00000025782	ENSBTAG00000025782.3	104084879	104113971
			10	SAMD4A	sterile alpha motif domain containing 4A	ENSBTAG00000001468	ENSBTAG00000001468.5	67062902	67289712
			13	FLRT3	fibronectin leucine rich transmembrane protein 3	ENSBTAG00000003319	ENSBTAG00000003319.6	7894630	7899756
			13	SIRPA	signal-regulatory protein alpha	ENSBTAG00000007213	ENSBTAG00000007213.6	53196957	53240508
			13	VAPB	VAMP associated protein B and C	ENSBTAG00000017424	ENSBTAG00000017424.5	57923612	57971092
			14	RALYL	RALY RNA binding protein like	ENSBTAG00000017717	ENSBTAG00000017717.6	77668311	77921529
			19	CNP	2',3'-cyclic nucleotide 3' phosphodiesterase	ENSBTAG00000025762	ENSBTAG00000025762.4	42130921	42138136
			19	ABCA9	ATP binding cassette subfamily A member 9	ENSBTAG00000006896	ENSBTAG00000006896.6	61493353	61539725
			20	PARP8	poly(ADP-ribose) polymerase family member 8	ENSBTAG00000004066	ENSBTAG00000004066.6	28302955	28495429
Mapholi et al. (2016)	DNA-SNP	Nguni	3	SMIM12	Small integral membrane protein 12	ENSBTAG00000014235	ENSBTAG00000014235.4	110835276	110839536
			3	KCNQ4	Potassium voltage-gated channel, KQT-like subfamily, member 4	ENSBTAG00000002644	ENSBTAG00000002644.4	105375258	105433489
			3	TRPM8	Transient receptor potential cation channel, subfamily M, member 8	ENSBTAG00000014652	ENSBTAG00000014652.5	113586057	113672155
			6	CSN1S2	Casein alpha-S2	ENSBTAG00000005005	ENSBTAG00000005005.6	85529905	85548556
			7	FER	Fer (fps/fes related) tyrosine kinase	ENSBTAG00000003051	ENSBTAG00000003051.7	107827212	108280766
			8	LINGO2	Leucine rich repeat and Ig domain containing 2	ENSBTAG00000016070	ENSBTAG00000016070.6	16299032	16300852
			11	CACFD1	Calcium channel flower domain containing 1	ENSBTAG00000015097	ENSBTAG00000015097.5	104352204	104361244
			17	LRBA	LPS-responsive vesicle trafficking, beach and anchor containing	ENSBTAG00000018546	ENSBTAG00000018546.7	6801065	7555668
			18	SLC38A7	Solute carrier family 38, member 7	ENSBTAG00000007170	ENSBTAG00000007170.5	26429234	26440641
			19	GPR142	G protein-coupled receptor 142	ENSBTAG00000048415	ENSBTAG00000048415.1	57036059	57041099
			26	PRKG1	Protein kinase, cGMP-dependent, type I	ENSBTAG00000018404	ENSBTAG00000018404.6	6899619	8313722

```
# Load the relevant libraries
library(tidyverse)
install.packages("caroline") - need this to write proper tab delimited files
library(caroline)
install.packages("xlsx") – need this to write the excel files containing the SNP genotype information
library("xlsx")

# Read file containing gene location (file can be downloaded from Biomart -Ensembl) should be based on the most recent version of the bovine genome (ARS-UCD-1.2)
Genome <- read.csv("ARS_UCD1_2_Unique_Genes_20210323.csv")
# Obtain a list of genes of interest for the study
GWASgenes <- read.csv("Candidate_SNPs_List.csv")
# Extract the relevant rows from the genome file - i.e., those that correspond with the genes in the gene variant file
locations <-Genome[Genome$GeneName %in% GWASgenes$GeneID,]
# Check if the number of genes in location file corresponds with the list of candidate genes
setdiff(GWASgenes$GeneID,locations$GeneName)
# Write to a BED4 format (tab-delimited file - chromosome, int start, int end, feature)
bed4.locations <- locations[,c("Chromosome","GeneStart","GeneEnd")]
write.delim(bed4.locations, "bed4.locations.keena.bed", quote = FALSE, row.names = FALSE, sep = "\t")
# Read in the SNP data
options(stringsAsFactors = FALSE)
SNPs<- read.delim("Keena_Bov150K_FinalReport.txt", skip=9,header = TRUE)
Map1 <- read.delim("SNP_Map.txt", header=TRUE)
# Filter out SNPs with GC scores <0.6
SNPs.f1 <- SNPs[SNPs$GC.Score >= 0.6,]
# Need to get SNP location and animal ID properly into SNP files - will use a join
# Check duplicates
which(duplicated(SNPs)==TRUE)
Map1$SNP.Name <- Map1$Name
# Set up a BED file for the SNP data
# Take SNPs that are within 1000 bp of the begin or end of a gene
SNPs.f1$start <- SNPs.f1$Position-1000
SNPs.f1$end <- SNPs.f1$Position+1000
# Eliminate negative locations
SNPs.f1$start[which(SNPs.f1$start < 0)] <- 0
# Write this to a tab delimited BED file
bed4.snps.keena <- SNPs.f1[,c("Chr","start","end")]
write.delim(bed4.snps.keena,"snps_f1.keena.bed", quote = FALSE, row.names = FALSE, sep = "\t")

# Get a merged file with the SNPs and locations from map file (but with lots of duplicate information)
SNPmerge <- merge(SNPs.f1,Map1, by="SNP.Name",no.dups = TRUE)
# Check this for one animal
summary(SNPmerge[which(SNPmerge$Sample.ID=="AD1"),])
# Check that there are SNPs on all chromosomes as expected
unique(SNPmerge$Chr)
```

```
# Get the intersections from using Bedtools in Galaxy after submitting the 2 bed files - one file has all the SNP locations with 1000 bp up and down while the other file has all the intervals for the genes of interest.
# The bedtools function was "Intersect intervals find overlapping intervals in various ways (Galaxy Version 2.27.0.1)"
# Generates an intersection file: "Galaxy22_snp_names_intersect_intervals.bed"
# Read in SNPs with intersections
intersects <- read.delim("Galaxy25_keena-intersect_intervals_on_data_17_and_data_18.bed")
#Output is a dataframe with no names - need to add these
names(intersects) <- c("Chromosome","Location1","Location2","SNP.Name")
#Check the total number of unique SNPs
snp.intersect <- unique(intersects$SNP.Name)
length(snp.intersect)

# Extract the rows that correspond with these SNPs from SNPmerge
snp.lists <- SNPmerge[SNPmerge$SNP.Name %in% snp.intersect,]
# snp.list file contains a list of all the SNP data (i.e., all genotype information) for all the cattle for regions of interest
# Check for each SNP, try some examples of filtering
snp.lists[snp.lists$SNP.Name=="ARS-BFGL-BAC-34198",c(1:4,18,19,22,33,34)][4:10,]
snp.lists[snp.lists$Sample.ID=="NM3",c(1:4,18,19,22,33,34)][4:10,]
snp.lists[15,c(1:4,18,19,22,33,34)]

#Remove extra rows caused by distinct Affy probes - easiest to remove the column
locations <- locations[,c(1:6,8:9)]
dim(locations)
locations <- unique(locations)
dim locations
table(locations$GeneName,locations$Chromosome)

# As example, find RIPK2
locations$Chromosome[which(locations$GeneName == "RIPK2")]
# Output > [1] "14"
locations$GeneStart[locations$GeneName == "RIPK2"]
# Output > [1] 73895603
locations$GeneEnd[locations$GeneName == "RIPK2"]
# Output > [1] 73936735
# Print the SNPs in this region
snp.lists[snp.lists$Chromosome=="14",c(1:4,18,19,22,33,34)]
# Print the genotypes
RIPK2 <- snp.lists[snp.lists$SNP.Name=="ARS-BFGL-NGS-60058",c(1:4,33,34)]

#Or alternatively do the following which is much easier and more accurate:
RIPK2 <- snp.lists$SNP.Name[which(snp.lists$Position.x<locations[37,4]&snp.lists$Position.x>locations[37,3]&
snp.lists$Chromosome==locations[37,8])]
unique(RIPK)
# Write the output files for each of the SNPs
Write.xlsx(ARS_BFGL_NGS_86536, “C:/Users/KeenaMarima/Desktop/SNPanalysis_2021_09_22/snp_genotype/
RIPK2/ ARS-BFGL-NGS-86536.xlsx”,row.name=FALSE)
```

Figure 3.2: Images of the R code that was used to determine the relationship between previously identified candidate SNPs for host resistance to ticks and each of the animals from the Angus, Brahman and Nguni cattle breed populations included in the above study. The code was used to also generate the genotypes of the animals of each breed for each of the known SNPs linked with host resistance to ticks.

3.2.6 Statistical analysis

A Pearson's chi-square (χ^2) statistic was performed to test the hypothesis that there are no differences in the genotypic frequencies of the different SNPs in the Angus, Brahman, and Nguni cattle breeds. Additionally, the relationship between the allele dosage of the different SNPs and the average tick count on each of the breed groups was analysed using simple linear regression analysis. Only SNPs that presented p-values lesser than 0.05 were considered significant for either of the analyses conducted. The tick count data were log10 transformed to achieve a normal distribution before applying the linear regression analysis. Pairwise comparison of the differences in tick count between each of the three breeds was conducted using the Tukey (HSD) analysis, to establish which breeds differed in terms of tick count for the SNPs that were identified by the linear regression analysis

3.3 Results

3.3.1 Genotype and population structure analysis

A total of 1680 SNPs were removed from the group of genotyped samples due to missing genotype data (--Mind and –Geno, respectively). An additional 6408 SNPs were removed due to the MAF threshold. The SNPs were further filtered to retain only those with a GC score greater than or equal to 0.6. This resulted in a further diminish in the number of retained SNPs, with some samples retaining as little as ±86 000 SNPs from the original ±140 000 SNPs. The samples from each of the breed groups clustered together into three distinct cohorts, except for one sample from the Nguni breed group that display partial (± 23%) association with the Brahman population.

Using the 150K SNP chip, no SNPs were retrieved for nine out of the 37 total genes included in the current study. These included *ADGRL4, KCNQ4, MFSD2A, CNP, GPR142, VAPB, LINGO2, ALB* and *TF*. The remaining 28 genes could each be associated with SNP lists ranging from 1 to 60 SNPs.

3.3.2 SNP discovery

A total of 257 SNPs were identified across the panel of 28 candidate genes remaining from the original gene list. Genes PRKG1 and *NAALADL2* and *FE*R had the largest number of SNPs found in their regions, with 60, 48 and 32 SNPs, respectively. A total of five were found to have genotype frequencies that differed significantly ($p < 0.05$) among the Angus, Brahman, and Nguni breeds. Summarised in Table 3.2 are the results of the χ^2 test and the associated p-values for the five significant SNPs in association with each breed. Overall, the SNPs were found to have variable SNP

dosages and allele frequencies across breeds. Table 3.3 shows that the ARS-BFGL-BAC-34198 SNP presented genotypes CC, CG, and GG, while the remaining four SNPs all presented the AA, AG, and GG genotypes. The CC and GG genotypes were absent in the Angus and Brahman animals, for SNPs ARS-BFGL-BAC-34198 and ARS-BFGL-NGS-94983, respectively. The AA genotype was absent in the Nguni animals for the latter SNP. Similarly, the AG and GG genotypes were absent in the Brahman and Nguni animals for SNPs ARS-BFGL-NGS-119397 and BovineHD2600001806, respectively, while the Angus animals presented an absence of the GG for SNP BTB-01748272.

Table 3.2: Genotype frequencies for SNPs that differ significantly among the Angus, Brahman and Nguni cattle

Chr no.	Gene ID	SNP Name	SNP Genotype	Expected	Observed	χ^2 (df=2)	p-value
1	*NAALADL2*	BTB-01748272	AA	0.357	0	11.162	0.025
			AG	3.929	1		
			GG	5.714	9		
2	*SATB2*	ARS-BFGL-NGS-94983	AA	2.880	1	10.831	0.029
			AG	1.440	4		
			GG	4.680	4		
10	*SERINC5*	ARS-BFGL-NGS-119397	AA	3.667	5	10.630	0.031
			AG	3.667	3		
			GG	0.667	0		
18	*SLC38A7*	ARS-BFGL-BAC-34198	CC	5.586	4	11.976	0.018
			CG	3.103	4		
			GG	0.310	1		
26	*PRKG1*	BovineHD2600001806	AA	2.423	2	11.371	0.023
			AG	4.500	7		
			GG	2.077	0		

df – degrees of freedom

significant at 5% level

Table 3.3: Genotype and allele frequencies of the χ^2 significant SNPs

Gene ID	SNP Name	Breed	No. of animals per genotype					Minor allele	Allele Frequency		
			AA	AG	CC	CG	GG		A	C	G
NAALADL2	BTB-01748272	Angus	2	7	-	-	0	G	0.61	-	0.39
		Brahman	3	1			5		0.41		**0.59**
		Nguni	2	5			1		0.60		0.40
SATB2	ARS-BFGL-NGS-94983	Angus	5	3	-	-	0	G	0.78	-	0.22
		Brahman	6	3			0		0.82		0.18
		Nguni	0	5			2		0.36		**0.64**
SERINC5	ARS-BFGL-NGS-119397	Angus	1	4	-	-	4	G	0.55	-	0.45
		Brahman	5	0			4		0.81		0.19
		Nguni	2	0			5		0.51		0.49
SLC38A7	ARS-BFGL-BAC-34198	Angus	-	-	0	1	9	C	-	0.06	0.94
		Brahman			0	4	6			0.20	0.80
		Nguni			1	6	1			**0.51**	0.49
PRKG1	BovineHD2600001806	Angus	4	4	-	-	1	G	0.65	-	0.35
		Brahman	10	0			0		**1**		0
		Nguni	4	6			0		0.71		0.29

The proportions of the minor allele frequencies (MAFs) were highest for SNP BTB-01748272 SNP in the Brahman breed as well as for SNPs ARS-BFGL-NGS-94983 and ARS-BFGL-BAC-34198 in the Nguni breed. The MAFs for both SNPs were reduced in the Angus breed. 100% A and 94% G major allele frequencies were observed for the BovineHD2600001806 and ARS-BFGL-BAC-34198 SNPs, respectively (see Table 3.3).

Three SNPs presented significant correlations ($p < 0.05$) between SNP dosage and tick count (Table 3.4). SNPs ARS-BFGL-NGS-94983 and BovineHD1000005009 showed a slight positive correlation ($r = 0.182$ and $r = 0.0161$, respectively) of each allele dosage on the transformed tick count, while a slightly negative correlation was observed for the BovineHD1000003812 SNPs ($r = -0.135$). Therefore, there may be a significant decrease in tick count by decreasing the dose of the A allele for the ARS-BFGL-NGS-94983 SNP and by decreasing the C allele dosage for the BovineHD1000005009 SNP. Similarly, increasing the dosage of the G allele in the BovineHD1000003812 may result in a slight decrease in tick count in the Angus animals while the opposite increase in the A allele dosage may result in the same effect in the Brahman and Nguni animals

Table 3.4: SNPs with allele dosages that significantly influence tick count

Chromosome	Gene ID	SNP Name	Estimate	Std error	T value	p-value
2	*SATB2*	ARS-BFGL-NGS-94983	0.182	0.064	2.828	0.009
10	*SERINC5*	BovineHD1000003812	-0.135	0.063	-2.156	0.041
10	*ITGA11*	BovineHD1000005009	0.161	0.076	2.113	0.045

The Nguni animals presented different allele dosages than Angus cattle. Presenting the absence of the GG genotype for the BovineHD1000003812 SNP and the presence of the GG and AA genotypes for the SNPs ARS-BFGL-NGS-94983 and BovineHD1000005009, respectively, the Nguni animals carried fewer ($p < 0.05$) ticks than the Angus animals. The corresponding tick counts of the Brahman animals similarly differed significantly from those of the Angus, however, did not differ significantly from those of the Nguni (Table 3.5).

Table 3.5: Mean tick count for SNP genotypes where allele dosage influences the tick count

SNP Name	Breed	Tick count per genotype per SNP					p-value (Breed differences)		
		AA	AC	AG	CC	GG	Angus vs Brahman	Angus vs Nguni	Nguni vs Brahman
ARS-BFGL-NGS-94983	Angus	402.98	-	374.44	-	-	0.000*	0.001*	0.203
	Brahman	266.85	-	205.40	-	-			
	Nguni	-	-	193.49	-	162.50			
BovineHD1000003812	Angus	429.57	-	353.23	-	408.79	0.001*	0.001*	0.323
	Brahman	248.28	-	272.98	-	-			
	Nguni	179.82	-	256.43	-	-			
BovineHD1000005009	Angus	-	333.83	-	397.64	-	0.005*	0.003*	0.397
	Brahman	-	223.75	-	263.67	-			
	Nguni	56.36	-	-	227.35	-			

3.4 Discussion

The Illumina GGP Bovine 150K SNP array is a rich source of SNPs. It features more than 134 000 SNPs for both *B. taurus* and *B. indicus* breeds with an average marker spacing of approximately 20 kb and higher concentrations in telomeric regions. The array content includes more than 100 SNPs with causative functions for various cattle breeds, making it ideal for genomic selection and imputation studies for different applications in livestock (Dadi et al., 2012). The use of similar arrays has assisted the investigation of complex traits in livestock, including dairy cattle breeds, to develop marker-assisted selection programs for greater genetic gains.

Genetic variation between breeds is explainable using allele dosages and frequencies. In this study, considerable variation was apparent in the number of individuals presenting a specific genotype in the different breeds. Of the 37 genes selected for this study, seven genes presented a total of seven SNPs that displayed either significant differences in genotype frequencies among breeds or significant correlations with tick count in the different breeds. The SNP discovery analysis also revealed the significance of the ARS-BFGL-NGS-94983 SNP which is found in the region of the *SATB2* gene. The SNP was identified in both analyses as presenting allele frequencies that differed significantly between breeds as well as possessing allele dosages that had a slight positive correlation with tick count. Presenting a 64% MAF in the Nguni breed, this SNP bears potential as a factor differentiating the Nguni from both the Brahman and Angus breeds. An additional two SNPs, found in the region of the *SERINC5,* were also identified as displaying allele frequencies that differed significantly among breeds (ARS-BFGL-NGS-119397) and possessing an allele dosage that slightly negatively correlated with tick count (BovineHD1000003812).

SATB2 has been identified in carcinogenesis as an oncogene that can be targeted for cancer treatment and prevention. *SATB2* is the mammalian ortholog of *DVE-1*, a gene expressed in worms, that is involved in the regulation of innate immune responses and mitochondrial unfolded protein responses to mediate mitochondrial homeostasis (Shao et al., 2020). Similar to *DVE-1*, which forms part of the transcription regulator complex, *SATB2* orchestrates a myriad of physiological and pathological processes by regulating gene transcription and expression through the organization of chromatin architecture (Huang et al., 2021; Shao et al., 2020). In the GWAS conducted by Turner et al. (2010b), a location (position 92 Mb) on the *SATB2* gene was one of 25 identified for their significance in association to tick burden in cattle.

Alternately, *SERINC5* codes for proteins that act as restriction factors that inhibit the early step of the viral infection thus greatly decreasing the viral infectivity (Shi et al., 2018). *SIRINC5* proteins are localized to the plasma membrane and were previously detected on the surface of T lymphoid cells using flow cytometry (Usami et al., 2015). Although the primary function and physiology of *SERINC5* proteins are not yet fully understood in the bovine species, the gene has been successfully linked with pathways of the innate immune response in humans. In their study, Porto Neto et al., (2004) listed *SERINC5* as one of the top ten genes that were in the overlapping regions of significantly differentially expressed genes in the tick naïve and tick-infested cattle comparisons using the Brahman and other dairy breeds.

Polymorphic genotypes were observed for each of the five SNPs that displayed significant differences in genotype frequencies across breeds. This greatly distinguished the Angus from the Brahman and Nguni individuals. The significant SNPs detected in this study shared alleles albeit the frequencies thereof differing between the Angus and Brahmana/Nguni. While the MAFs across all SNPs and breeds were greater than 5%, notably 100% of the Brahman and 90% of the Angus presented the AA and GG genotypes for SNPs BovineHD2600001806 and ARS-BFGL-BAC-34198, respectively. IT may be inferred that selective advantage favoured the homozygous AA and GG genotypes of these SNPs. Additionally, this may imply that the major alleles for these SNPs were conserved in the Brahman and Angus populations. These SNPs, however, did not show any significant correlation to tick count. On average, the major allele was conserved at a rate of 60% across the five significant SNPs and breeds.

Comparing the expected and observed genotype frequency statistics presented in Table 3.2, it was evident that all three breeds presented SNPs that significantly deviated from the Hardy-Weinberg equilibrium of selective neutrality. Numerous factors may contribute towards the deviation from the HW equilibrium, including genetic drift, population size, non-random mating, mutation, ancient divergence, and the presence of null alleles, among other factors (Mukhund et al., 2020; Zwane et al., 2016). In the case of the present study, the small population sizes studied in each of the breeds

may have resulted in somewhat deceiving $\chi 2$ statistics. However, the preliminary detection of the listed SNPs provides insight worth exploring further using greater population sizes.

3.5 Conclusions

The tick resistance phenotype is complex and greatly variable. The current SNP discovery study made it possible to distinguish certain SNP genotype frequencies expressed in the Nguni and Brahman cattle, breeds that have been classified as resistant to ticks, as opposed to the tick susceptible Angus cattle. However, the results were not sufficient to support the speculation that the SNPs identified would affect tick burdens in the greater populations. The study further identified SATB2, which upon further investigation may be found to elicit interesting immune responses in the bovine host following tick attachment. Although studies detailing the roles of *SATB2* and *SERINC5* are currently limited to cancer, human brain development and immunodeficiency studies, the detection of significant SNPs within regions of these genes in association with the tick resistance phenotype renders these genes as potential biomarkers to explore in the elucidation of the complex tick resistance trait in cattle. Further research on these two genes, in conjunction with the other four mentioned in the results of this study, may help unpack the functional and systems biology of those genes in the bovine species. Larger populations of the Angus, Brahman and Nguni breed with an increased tick count database may increase the accuracy of identifying the large effects of the SNP genotypes, dosages, and frequencies on the tick resistance phenotype. This study also highlighted the need for a more accurate method of phenotyping the tick resistance trait to support the reliable inference of correlation between tick count and SNP genotype and dosage.

3.6 References

Ashton, B.G.C., Seifert, G.W., Francis, J., 1968. An association between serum amylase phenotype 303–308.

Austin, E.J.., Deary, I.J.., Edwards-Jones, G., Arey, D., 2005. Attitudes to Farm Animal Welfare: Factor Structure and Personality Correlates in Farmers and Agriculture Students. J. Individ. Differ. 26, 107–120.

Burrow, H.M., Mans, B.J., Cardoso, F.F., Birkett, M.A., Kotze, A.C., Hayes, B.J., Mapholi, N.O., Dzama, K., Marufu, M.C., Githaka, N.W., Djikeng, A., 2019. Towards a new phenotype for tick resistance in beef and dairy cattle: a review. Anim. Prod. Sci. 59, 1401–1427. https://doi.org/10.1071/AN18487

Burrow, H.M., Mrode, R., Mwai, A.O., Coffey, M.P., Hayes, B.J., 2021. Challenges and Opportunities in Applying Genomic Selection to Ruminants Owned by Smallholder Farmers. Agriculture 11, 1172. https://doi.org/10.3390/agriculture11111172

Constantinoiu, C.C., Jackson, L. a., Jorgensen, W.K., Lew-Tabor, a. E., Piper, E.K., Mayer, D.G., Venus, B., Jonsson, N.N., 2010. Local immune response against larvae of Rhipicephalus (Boophilus) microplus in Bos taurus indicus and Bos taurus taurus cattle. Int. J. Parasitol. 40, 865–875.

https://doi.org/10.1016/j.ijpara.2010.01.004

Dadi, H., Kim, J.J., Yoon, D., Kim, K.S., 2012. Evaluation of single nucleotide polymorphisms (SNPs) genotyped by the Illumina Bovine SNP50K in cattle focusing on Hanwoo breed. Asian-Australasian J. Anim. Sci. 25, 28–32. https://doi.org/10.5713/ajas.2011.11232

Francis, J., Ashton, G., 1967. Tick resistance in cattle : its stability and correlation with various genetic characteristics. Aust. J. exp. Biol. med. Sci 45, 131–140. https://doi.org/10.1038/icb.1967.10

Frisch, J.E., Drinkwater, R., Harrison, B., Johnson, S., 1997. Classification of the southern African sanga and East African shorthorned zebu. Anim. Genet. 28, 77–83. https://doi.org/10.1111/j.1365-2052.1997.00088.x

Ibelli, A.M.G., Ribeiro, A.R.B., Giglioti, R., Regitano, L.C.A., Alencar, M.M., Chagas, A.C.S., Paço, A.L., Oliveira, H.N., Duarte, J.M.S., Oliveira, M.C.S., 2012. Resistance of cattle of various genetic groups to the tick Rhipicephalus microplus and the relationship with coat traits. Vet. Parasitol. 186, 425–430. https://doi.org/10.1016/j.vetpar.2011.11.019

Lashmar, S., Muchadeyi, F., Visser, C., 2019. Genotype imputation as a cost-saving genomic strategy for South African Sanga cattle: A review. S. Afr. J. Anim. Sci. 2019, 262–280. https://doi.org/10.4314/sajas.v49i2.7

Machugh, D.E., Shriver, M.D., Loftus, R.T., Cunningham, P., Bradley, D.G., 1997. hDomestication and Phylogeography of Taurine and Zebu Cattle.

Mapholi, N.O., Maiwashe, A., Matika, O., Riggio, V., Bishop, S.C., MacNeil, M.D., Banga, C., Taylor, J.F., Dzama, K., 2016. Genome-wide association study of tick resistance in South African Nguni cattle. Ticks Tick. Borne. Dis. 7, 487–497. https://doi.org/10.1016/j.ttbdis.2016.02.005

Mapholi, N.O., Marufu, M.C., Maiwashe, A., Banga, C.B., Muchenje, V., MacNeil, M.D., Chimonyo, M., Dzama, K., 2014. Towards a genomics approach to tick (Acari: Ixodidae) control in cattle: A review. Ticks Tick. Borne. Dis. 5, 475–483. https://doi.org/10.1016/j.ttbdis.2014.04.006

Martinez, M.L., Machado, M.A., Nascimento, C.S., Silva, M.V.G.B., Teodoro, R.L., Furlong, J., Prata, M.C.A., Campos1, A.., Guimarães, M.F.., Azevedo, A.L.., Pires, M.. F.A., Verneque, R.S., 2006. Association of BoLA-DRB3 . 2 alleles with tick (Boophilus microplus) resistance in cattle. Genet. Mol. Res. 5, 513–524.

Meijers, J.., 2019. Stellenbosch Weather [WWW Document]. A JPMeijers.com Creat. URL weather.sun.ac.za (accessed 5.14.19).

Morris, C. a., 2007. A review of genetic resistance to disease in Bos taurus cattle. Vet. J. 174, 481–491. https://doi.org/10.1016/j.tvjl.2006.09.006

Mukhund, B.K., Dinesh, C.N., Rojan, P.M., Ravindran, R., Bindu, K.A., Asaf, V.N.M., Sreeshma, P.R., Deepna, T., 2020. Analysis of Single Nucleotide Polymorphisms in the QTLS Associated with Tick Resistance/Susceptibility in the Native Vechur and Cross red Cattle of Kerala State. Int. J. Curr. Microbiol.

Appl. Sci. 9, 3078–3087. https://doi.org/10.20546/ijcmas.2020.909.380

Mwai, O., Hanotte, O., Kwon, Y.J., Cho, S., 2015. - Invited review - African indigenous cattle: Unique genetic resources in a rapidly changing world. Asian-Australasian J. Anim. Sci. 28, 911–921. https://doi.org/10.5713/ajas.15.0002R

Otto, P.I., Guimarães, S.E.F., Verardo, L.L., Azevedo, A.L.S., Vandenplas, J., Soares, A.C.C., Sevillano, C.A., Veroneze, R., de Fatima A. Pires, M., de Freitas, C., Prata, M.C.A., Furlong, J., Verneque, R.S., Martins, M.F., Panetto, J.C.C., Carvalho, W.A., Gobo, D.O.R., da Silva, M.V.G.B., Machado, M.A., 2018. Genome-wide association studies for tick resistance in Bos taurus × Bos indicus crossbred cattle: A deeper look into this intricate mechanism. J. Dairy Sci. 101, 11020–11032. https://doi.org/10.3168/jds.2017-14223

Piper, E.K., Jonsson, N.N., Gondro, C., Vance, M.E., Lew-Tabor, A., Jackson, L.A., 2017. Peripheral cellular and humoral responses to infestation with the cattle tick Rhipicephalus microplus in Santa Gertrudis cattle. Parasite Immunol. 39, 1–12. https://doi.org/10.1111/pim.12402

Porto Neto, L.R., Bunch, R.J., Harrison, B.E., Barendse, W., 2010a. DNA variation in the gene ELTD1 is associated with tick burden in cattle. Anim. Genet. 42, 50–55. https://doi.org/10.1111/j.1365-2052.2010.02120.x

Porto Neto, L.R., Bunch, R.J., Harrison, B.E., Prayaga, K.C., Barendse, W., 2010b. Haplotypes that include the integrin alpha 11 gene are associated with tick burden in cattle. BMC Genet. 2010 111 11, S3–S14. https://doi.org/10.1186/1471-2156-11-55

Porto Neto, L.R., Jonsson, N.N., D'Occhio, M.J., Barendse, W., 2011. Molecular genetic approaches for identifying the basis of variation in resistance to tick infestation in cattle. Vet. Parasitol. 180, 165–172. https://doi.org/10.1016/j.vetpar.2011.05.048

Porto Neto, L.R., Jonsson, N.N., Ingham, A., Bunch, R.J., Harrison, B.E., Barendse, W., 2012. The RIPK2 gene: A positional candidate for tick burden supported by genetic associations in cattle and immunological response of knockout mouse. Immunogenetics 64, 379–388. https://doi.org/10.1007/s00251-012-0601-9

Porto Neto, L.R., Piper, E.K., Jonsson, N.N., Barendse, W., Gondro, C., 2010c. Meta-analysis of Genome Wide Association and Gene Expression Studies to Identify Candidate Genes for Tick Burden in Cattle, in: 9th World Congres of Genetics Applied to Livestock Production, Leipzig, Germany. p. 0664.

Porto Neto, L.R., Piper, E.K., Jonsson, N.N., Barendse, W., Gondro, C., 2004. Meta-analysis of Genome Wide Association and Gene Expression Studies to Identify Candidate Genes for Tick Burden in Cattle. 9th World Congr. Genet. Appl. to Livest. Prod. Leipzig, Ger. 0664.

Porto Neto, L.R.P., Bunch, R.J., Harrison, B.E., Prayaga, K.C., Barendse, W., 2010. Haplotypes that include the integrin alpha 11 gene are associated with tick burden in cattle. BMC Genet. 2010 111 11, S3–S14. https://doi.org/10.1186/1471-2156-11-55

Rodriguez-Valle, M., Moolhuijzen, P., Piper, E.K., Weiss, O., Vance, M., Bellgard, M., Lew-Tabor, A., 2013. Rhipicephalus microplus lipocalins (LRMs): Genomic identification and analysis of the bovine immune response using in silico predicted B and T cell epitopes. Int. J. Parasitol. 43, 739–752. https://doi.org/10.1016/j.ijpara.2013.04.005

Stear, M.J., Hetzel, D.J.S., Brown, S.C., Gershwin, L.J., Mackinnon, M.J., Nicholas, F.W., 1990. The relationships among ecto- and endoparasite levels, class I antigens of the bovine major histocompatibility system, immunoglobulin E levels and weight gain. Vet. Parasitol. 34, 303–321. https://doi.org/10.1016/0304-4017(90)90077-O

Stear, M.J., Newman, M.J., Nicholas, F.W., Brown, S.C., Holroyd, R.G., 1984. Tick resistance and the major histocompatibility system. Aust. J. Exp. Biol. Med. Sci. 62, 47–52. https://doi.org/10.1038/icb.1984.4

Stear, M.J., Nicholas, F.W., Brown, S.C., Holroyd, R.G., 1989. Class I antigens of the bovine major histocompatibility system and resistance to the cattle tick (Boophilus microplus) assessed in three different seasons. Vet. Parasitol. 31, 303–315. https://doi.org/10.1016/0304-4017(89)90080-0

Takeshima, S. nosuke, Corbi-Botto, C., Giovambattista, G., Aida, Y., 2018. Genetic diversity of BoLA-DRB3 in South American Zebu cattle populations. BMC Genet. 19, 1–13. https://doi.org/10.1186/s12863-018-0618-7

Turner, L.B., Harrison, B.E., Bunch, R.J., Neto, L.R.P., Li, Y., Barendse, W., 2010a. A genome-wide association study of tick burden and milk composition in cattle. Anim. Prod. Sci. 50, 235–245. https://doi.org/10.1071/AN09135

Turner, L.B., Harrison, B.E., Bunch, R.J., Porto Neto, L.R., Li, Y., Barendse, W., 2010b. A genome-wide association study of tick burden and milk composition in cattle. Anim. Prod. Sci. 235–245.

Zwane, A.A., Maiwashe, A., Makgahlela, M.L., Choudhury, A., Taylor, J.F., van Marle-Köster, E., 2016. Genome-wide identification of breed-informative single-nucleotide polymorphisms in three South African indigenous cattle breeds. South African J. Anim. Sci. 46, 302–312. https://doi.org/10.4314/sajas. v46i3.10

Chapter 4:
Microarray analysis to characterize immune responses to *Rhipicephalus microplus* infestations in Santa Gertrudis cattle displaying varying tick-resistance phenotypes

Abstract

Acaricide resistance has rendered the chemical methods applied in the control of *Rhipicephalus microplus* ineffective. While selective breeding offers opportunities for sustainable tick control, the lack of practically feasible methods to quantify tick resistance traits prevent its adoption commercially. As such, the objective of this study was to understand, at a systems biology level, the extent to which immunity differentiates the tick-resistant phenotypes from the tick-susceptible phenotype. This was achieved by examining the differential gene expression and relevant pathways enrichment between resistant and susceptible phenotypes in Santa Gertrudis cattle, pre- and post-tick infestation at different time points. The genetic modulation of host immunity induced by *R. microplus* ticks was mapped using microarray technology. The study showed that at pre-infestation, tick resistant animals showed significant differential expression of genes involved in response initiations and pro-inflammatory immune responses. Furthermore, the critical role of both the innate and the adaptive immune system responses that may function collaboratively with non-immune responses in determining the various levels of host resistance to tick infestation was highlighted. Nevertheless, it was evident that upon long term exposure to the *R. microplus* the tick-resistant Santa Gertrudis animals displayed an increased ability to develop and mount more robust adaptive responses against the tick infestations than the tick-susceptible animals.

4.1 Introduction

Chemical acaricides have been the principal control measure against the Asian blue cattle tick *Rhipicephalus microplus*. However, the approach is both ineffective and unsustainable as ticks often develop acaricide resistance. Selective breeding to achieve higher levels of resistance presents an attractive alternative for tick control (Frisch, 1999; Machado et al., 2010; Porto Neto et al., 2011). The contrasting heritable phenotypes presented by cattle within a breed encourage the use of selective breeding to improve host resistance to ticks within a herd. Moderate heritability estimates ranging from 0.34 ± 0.06 to 0.41 ± 0.08 have been reported for the tick resistance trait in cattle (Mackinnon et al., 1991; Morris, 2007). This entails that the trait can undergo selection. However, the inability to accurately quantify the trait with absolute reliance on the currently used tick-count-data driven approach of selecting tick-resistant animals within a herd is not commercially viable (Mackinnon et al., 1991; Morris, 2007; Regitano and Prayaga, 2011). Therefore, transcriptomic

analyses that identify biomarkers for host resistance to ticks are required to support the development of genomic tests with practical feasibility in the field.

Microarray technology is an esteemed transcriptome analysis approach that has been instrumental in elucidating the systems biology of host resistance to ticks and the discovery of biomarkers for the tick resistance trait in cattle. It did, however, take two decades since the advent of the technology in the 1980s (Jenner and Young, 2005) before microarray technology was employed in the characterization of host resistance to ticks. Table 4.1 shows some of the research outputs that were achieved using microarray technology to characterize host resistance to ticks.

Table 4.1: Summary of transcriptome analysis studies conducted using microarray technology to characterize host resistance to ticks in cattle

Breed	Aim	Outcomes	Applications	Country
Holstein & Nelore	Comparison of transcriptional and inflammatory response profiles in cutaneous reactions to tick bites	1131 DEGs were identified, allergic contact-like dermatitis implicated in the production of *IL-6, CXCL-8* and *CCL-2* leading to expression of pro-inflammatory chemokines and cytokines that recruit granulocytes and T lymphocytes.	Understanding the molecular basis of differences in tick-resistant and susceptible cattle for anti-tick vaccine development	Brazil (Franzin et al., 2017)
F2 (Gir × Holstein) of contrasting phenotypes	Characterization of early (0, 12h & 48h) immune responses triggered by *R. microplus* ticks	Lipid metabolism genes & impaired acute phase responses	Improvement & development of novel tick control tools using immunomodulation tools	Brazil (Carvalho et al., 2014)
Brahman & Holstein-Friesian	Describe processes responsible for superior tick resistance in Brahman compared to low-resistance Holstein–Friesian cattle	Gene involved in inflammatory processes and immune responsiveness were upregulated only in the Holstein-Friesian hosts; genes encoding constituents of the extracellular matrix were upregulated in Brahmans	Predictive biomarker development for use in selective breeding programs and anti-tick vaccine development	Australia (Piper et al., 2010)
Brahman & Holstein-Friesian	Identify genes that are differentially expressed in the skin of tick resistant and tick susceptible cattle following infestation with *R. microplus*	138 significantly differentially expressed genes and 3 pathways were found including immunological/host defence genes, extracellular matrix proteins, and transcription factors as well as genes involved in lipid metabolism.	Exploration of potential mechanisms of tick resistance in cattle	Queensland (Kongsuwan et al., 2008)
Hereford Shorthorn	Define the nature of Bos taurus tick resistance (0 & 24h post-infestation)	Expression of keratin genes or mitochondrial genes, odorant-binding protein & major allergen *BDA20* were significantly altered post-infestation	Marker-assisted selection strategies for cattle breeding	Queensland (Wang et al., 2007)

The trade-off between good meat quality and reproductive traits with adaptability and survival traits is an impediment that hinders the extensive use of tick-resistant *Bos indicus* breeds for beef

production in areas such as northern Australia where the *R. microplus* tick species prevails (Piper et al., 2017). Although recognised for their desirable meat quality and reproductive efficiency, herds produced from European breeds (*B. taurus*) suffer heavy tick loads and inevitably tick-induced production losses. To introduce *B. taurus* superior genetics of reproduction and production while retaining the superior tick resistance of *B. indicus* breeds, the Santa Gertrudis composite breed was produced. Composed of approximately 5/8 *B. taurus* and 3/8 *B. indicus*, this stable composite breed boasts a wide range of resistance to *R. microplus* infestations (Seifert, 1971; Utech et al., 1978a). Unlike the Bonsmara composite breed (5/8 Afrikaner, 3/16 Shorthorn and 3/16 Hereford) that exhibits great productivity in variable climate but inferior tick resistance and resilience to tick-borne diseases (Marufu et al., 2011), the Santa Gertrudis offers an outstanding blend of good reproductive traits and productive characteristics with an increased ability to acquire superior resistance to ticks (Piper et al., 2017).

Genetic variabilities exist between breeds and within a breed, resulting in different tick-resistance phenotypes even within the breed (Frisch, 1999; Mapholi et al., 2014; Cardoso et al., 2015). No single breed is completely resistant to ticks (Frisch et al., 2000). As evidenced by the Belmont Adaptaur, *B. taurus* breeds can acquire total resistance to ticks upon long term exposure (Frisch et al., 2000), however, the resistance is often not sustained leading to a subsequent relapse and increased susceptibility (Tabor et al., 2017). Furthermore, purebred *B. taurus* breeds rarely even reach the superior resistance exhibited by *B. indicus* and *B. taurus africanus* breeds. There are many immune and non-immune factors contributing to the genetic variabilities within and between breeds. However, due to the increased interest in vaccination against ticks, it is important to understand the potential contribution of immune-related responses to resistance status.

The immunogenetic mechanisms that distinguish tick-resistance animals from tick-susceptible ones require further elucidation. Using microarray technology this study aimed to profile the genetic modulation of host immunity induced by *R. microplus* ticks. This was achieved by examining the differential gene expression and the relevant pathways enrichment between resistant and susceptible phenotypes in Santa Gertrudis cattle, pre- and post-tick infestation at different time points. Therefore, this study aimed to understand, at a systems biology level, the extent to which immunity differentiates the tick-resistant phenotypes from the tick-susceptible phenotype.

4.2 Material and methods

The experimental material and methods referred to in this chapter were described in detail by Piper et al. (2009) and Piper et al. (2017) but are briefly summarized here.

4.2.1 Experimental animals

Thirty-five tick-naïve Santa Gertrudis heifers aged 12 months were used. The animals were sourced from a tick-free region in Australia and vaccinated against tick fever pathogens (*Babesia bovis, Babesia bigemina and Anaplasma marginale*) four weeks before the commencement of the trial. Of the 35 animals, five were kept in a quarantine camp and maintained as the tick-free control group while the remaining 30 animals underwent artificial infestation. The environmental conditions and feeding regimes were similar between the control and treatment groups. Ethical approval to conduct the study was granted by the University of Queensland Animal Ethics Production and Companion Animals Committee.

4.2.2 Artificial infestation and animal ranking

The animals were phenotyped as tick-resistant and tick-susceptible following a tick infestation trail carried out over 105 days. The animals were simultaneously subjected to both artificial infestation and natural infestations in tick-infested pastures. For 13 weeks, each animal was artificially infested with approximately 10 000 (0.5g) *R. microplus* larvae weekly. Laboratory prepared larvae, free of Babesia and Anaplasma organisms, prepared from the aseptic colonies at the Queensland Department of Agriculture and Fisheries' Biosecurity Science Laboratories, were used for all artificial infestations. The larvae were aged between 7- and 14-days post-hatching upon application on the animals.

Tick count data was collected 21 days following the first artificial infestation and thereafter collected weekly for 13 weeks. Only engorged ticks sized between 4.5 and 8 mm were counted on one side of the animal. The data were used to rank the animals into resistance groups according to their ability to resist tick infestations. Six animals were classified as exhibiting the highest level of resistance to ticks following a 10-week artificial infestation trial coupled with an initial seven-week adaption period after the initial artificial infestation. An additional six animals were classified as lowly resistant and thus "susceptible" and the remaining 18 animals fell into the "middle" class. The detailed results of the tick counts and resistance ranking can be found in the paper by (Piper et al., 2017).

4.2.3 Blood sampling and RNA extraction

Blood samples were obtained via the jugular into EDTA tubes from all animals (both the treatment and control groups). These consisted of blood samples collected starting 21 days after the first artificial infestation and then weekly for a further 9 weeks. Only samples from Day 0 (pre-infestation), Day 21 and Day 91 post initial infestation were used for downstream analysis.

RNA was extracted from white blood cells following the Trizol reagents (Invitrogen) manufacturer's protocol. This involved lysing 5 ml of blood with 45 ml RBC lysing buffer, incubating the mixture at room temperature for 10 min then centrifuging it at 250 × g for 10 min followed by the removal of the supernatant. The remaining pellet was then resuspended in 4ml of Trizol reagents and total RNA

extraction was carried out according to the manufacturer's protocol. The resulting pellet was eluded in 30 µl of RNase-free water and subjected to DNase digestion and removal of genomic DNA contamination, following the Turbo DNase (Ambion) and RNeasy minicolumns (Qiagen) manufacturer's protocols, respectively.

4.2.4 Microarray assay and data pre-processing

Transcriptome profiling was conducted using the Affymetrix GeneChip bovine genome array platform (Affymetrix) that contained 24 128 probe sets. The probe sets represented 11 255 gene identities from *Bos taurus* build 4.0 and 10 777 annotated UniGene identities as well as a total number of 133 control probes. All microarray analyses (RNA hybridization to slides, target preparation and microarray processing) were conducted by the Australian Genome Research Facility, Melbourne, Australia, and scanning was performed with an Agilent microarray scanner (Agilent Technologies).

The statistical computing language R and Bioconductor packages were used for all standard quality control measures, preprocessing, and differential expression profiling. Standard quality control measures were carried out to assess the quality of the array. Quality control tests were carried out on the probe level data and the summarized data. These included intensity correlations between the arrays, pseudo-images of the arrays (based on PLM fit for detection of spatial effects), MA scatter plots of arrays versus a pseudo-median reference chip, boxplots and histograms of raw log intensities, boxplots of relative log intensities, boxplots of normalized unscaled standard errors (NUSE), plots of principal component analysis (PCA) for Person perfect match (PM) and Mismatch (MM) raw probes, summary and plots of RNA degradation per chip, and additional Affymetrix suggested quality control checks.

Six summarization methods were used to estimate transcription intensities in the $\log_2$ scale employing the R *affy* package: MAS 5.0, RMA, GCRMA, PLIER, VSN and DCHIP. MAS parameters included background correction for both PM and MM. The lowest 2% of the probe intensities were used as a background value for each of the 16 grids. Each probe was then adjusted based upon a weighted average of the background of each grid. Scale normalization was performed on all arrays using the same mean value (200). The arrays were then summarized using an adjusted $\log_2$ scale average using one-step Tukey biweight (Welsh et al., 2013). RMA parameters included convolution background correction, quantile normalization and median polish summarization. GCRMA parameters involved background correction using an affinity model based on probe sequences and MM intensities. This was followed by quantile normalization and median polish summarization. PLIER accounts for experimentally observed patterns for feature behavior and handling error at the appropriately low and high signal values. The parameters include quantile normalization and both PM and MM background correction. DCHIP parameters include PM-MM background correction, pair-wise rank invariant normalization and MBEI model summarization (Welsh et al., 2013). Summary statistics including boxplots, histograms and PCA plots of the distribution of normalized $\log_2$ probe

intensities were produced for each summarization method to determine the effectiveness of each method and detect outlier slides.

4.2.5 Differential gene expression and gene ontology enrichment analysis

Twelve experiments (Table 4.2) were set up to investigate the differential expression of genes in the blood of the highly resistant and lowly resistant heifers following tick infestations. Differential gene expression was tested on $\log_2$ expression intensities obtained using the summarization methods listed in 4.2.4 using the *Limma* package. Only probe sets that were differentially expressed across all six summarization methods, with convergence value of 100, six stars for the false discovery rate (FDR) and flagged as present in at least 50% of the samples were considered significant. Before the differential expression analyses, the Affymetrix control probes were filtered out from the data. A total of 6 701 probe sets were detected as absent or marginal on all slides and thus removed from downstream analyses. The remaining 17 295 probes were used for further analyses.

Table 4.2: Summary of the descriptions of the experimental contrasts & abbreviations referred to in the text

Experiment	*Abbreviation*	*Description*
1	S0 vs R0	*Which genes are differentially expressed between susceptible and resistant animals pre-infestation (day 0)?*
2	S21 vs R21	*Which genes are differentially expressed between susceptible and resistant animals at day 21?*
3	S91 vs R91	*Which genes are differentially expressed between susceptible and resistant animals at day 91?*
4	*S0 vs S21*	*Which genes are differentially expressed in susceptible animals between day 0 and day 21?*
5	*S0 vs S91*	*Which genes are differentially expressed in susceptible animals between day 0 and day 91?*
6	*S21 vs S91*	*Which genes are differentially expressed in susceptible animals between day 21 and day 91?*
7	*R0 vs R21*	*Which genes are differentially expressed in resistant animals between day 0 and day 21?*
8	*R0 vs R91*	*Which genes are differentially expressed in resistant animals between day 0 and day 91?*
9	*R21 vs 91*	*Which genes are differentially expressed in resistant animals between day 21 and day 91?*
10	S(0vs21) vs R(0vs21)	*Which genes are differentially expressed between susceptible and resistant animals between day 0 and day 21?*
11	S(0vs91) vs R(0vs91)	*Which genes are differentially expressed between susceptible and resistant animals between day 0 and day 91?*
12	S(21vs91) vs R(21vs91)	*Which genes are differentially expressed between susceptible and resistant animals between day 21 and day 91?*

The Affymetrix online platform (http://www.affymetrix.com/products/index.affx) was used to perform gene annotation of the differentially expressed probe sets. The Database for Annotation, Visualization, and Integrated Discovery (DAVID)(https://david.ncifcrf.gov/tools.jsp) web-based tool was used to perform gene-annotation enrichment analysis of the differentially expressed genes (DEGs) and pathway over-representation analysis. The probability scores presented in the DAVID over-representation analysis were corrected for false discovery rate (FDR) using the Benjamini-Hochberg method. Gene ontology (GO) biological processes/terms were derived for the DEGs. Functional profiling for each of the DEGs was performed and classified as molecular function (MF), biological process (BP), and cellular components (CC).

4.3 Results

4.3.1 Phenotyping animals according to tick count data

The results of the tick count data were described extensively in Piper et al. (2017). Briefly, the 30 infested animals displayed divergent tick counts following the 13 weeks of exposure to ticks. Although the animals produced similar tick counts on the first and second tick counting sessions, by the third tick counting session, corresponding to 35 days after the first artificial infestation, the resistant animals started diverging ($p < 0.001$) from the rest of the animals (Figure 3.1). Of the 30 animals that underwent artificial infestation, six exhibited a high level of resistance and six were lowly resistant and the remaining 18 animals displayed moderate levels of resistance. On average, the six tick-susceptible animals had 296 (±31) adult ticks per side by week 13 and the six tick-resistant animals had 27 (±22) adult ticks per side. Using the Utech and Wharton method (Utech et al., 1978b, 1978a) for calculating percentage resistance, the tick-susceptible group corresponded to 88% resistance while the tick-resistant group corresponded with 99% resistance to ticks. Presented in Figure 4.1 is the trend showing increasingly lowered tick counts in the tick-resistant animals with each successive week, reflecting their developing resistance to the biting ticks. The susceptible animals maintained similar tick loads throughout the trial.

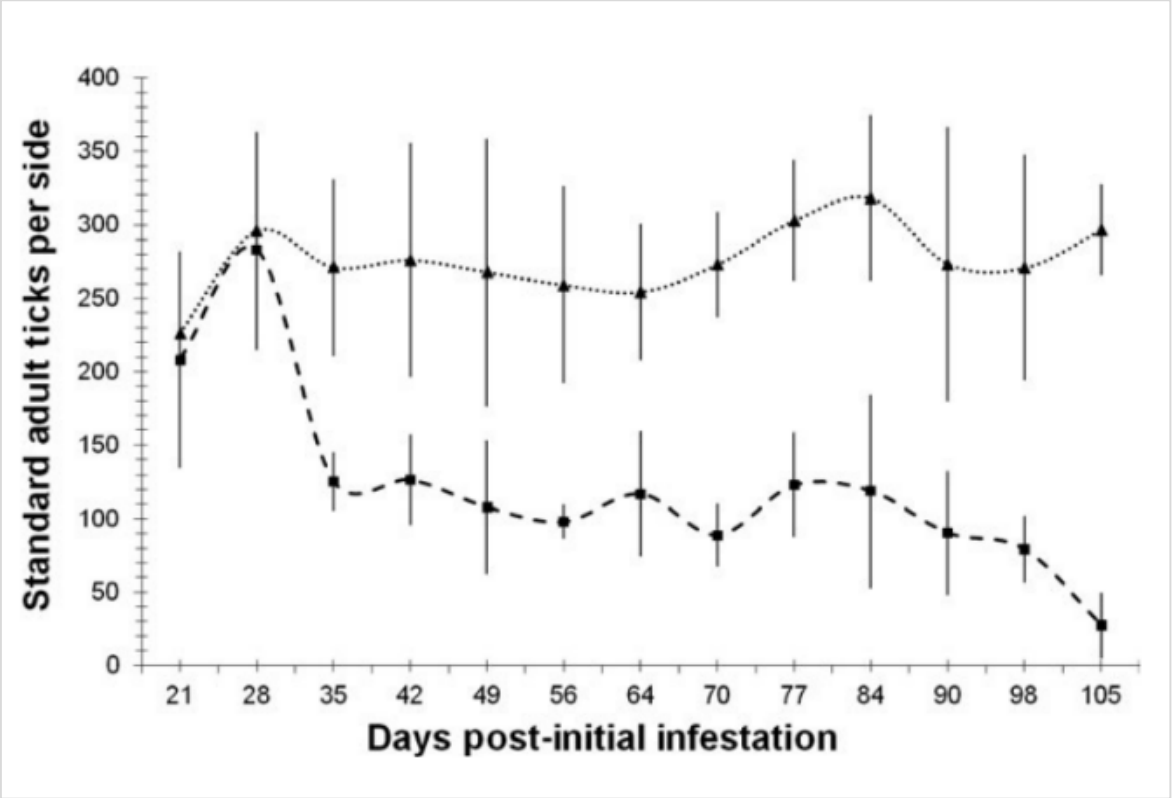


Figure 4.1: Weekly tick count data for the six tick-resistant (dashed line with squares) and six tick-susceptible (dotted line with triangles) animals used in this study. The data is presented as the average of the group (mean ± standard deviation from the group mean). From Piper et al. (2017).

4.3.2 Microarray data quality control analysis

The quality control parameters demonstrated that the arrays were within normal boundaries and of good quality, with no identifiable outliers in the samples. Figures 4.2 and 4.3 represent heat map graphs of intensity correlations between arrays. It appears in Figure 4.2 that slides 809Rt21 and 607St21 may be outliers. These two arrays have the least correlation with other arrays, and this may pose a data quality concern. This is further evidenced in the summary statistics in Figures 4.4 A and C where especially slide 607St21 stands outside the normal trend. However, this issue was successfully addressed by normalization with the six summarization methods, see Figures 4.4 B and D. It is also evident in Figure 4.3 that the sample treatments do not group together across treatment groups. No major clusters could be highlighted, however, the arrays containing post-infestation samples of both phenotypes (susceptible and resistant) and both time points (Day21 and Day91) seemingly cluster together and away from the pre-infestation samples. All the RNA samples showed good integrity indices according to the Agilent Bioanalyzer assays (Figure 4.4E) and hybridized successfully to the Affymetrix chips.

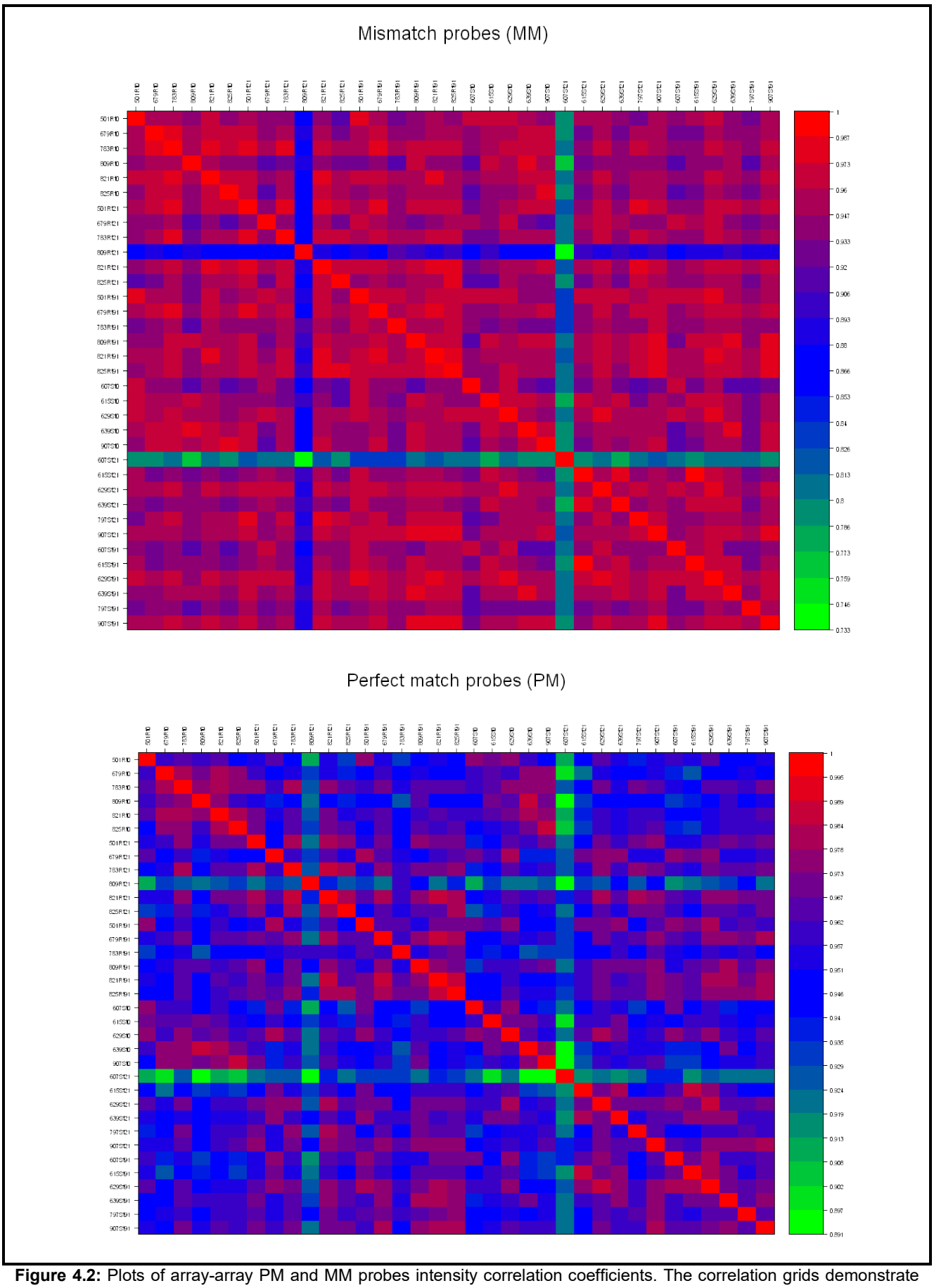


Figure 4.2: Plots of array-array PM and MM probes intensity correlation coefficients. The correlation grids demonstrate the degree of similarity between samples and are useful for the detection of outlier slides. The ranges are 0.733 to 1 in the first plot and 0.891 to 1 in the second plot as represented by the green to red colour, respectively. A correlation approaching 1 indicates increasing similarity.

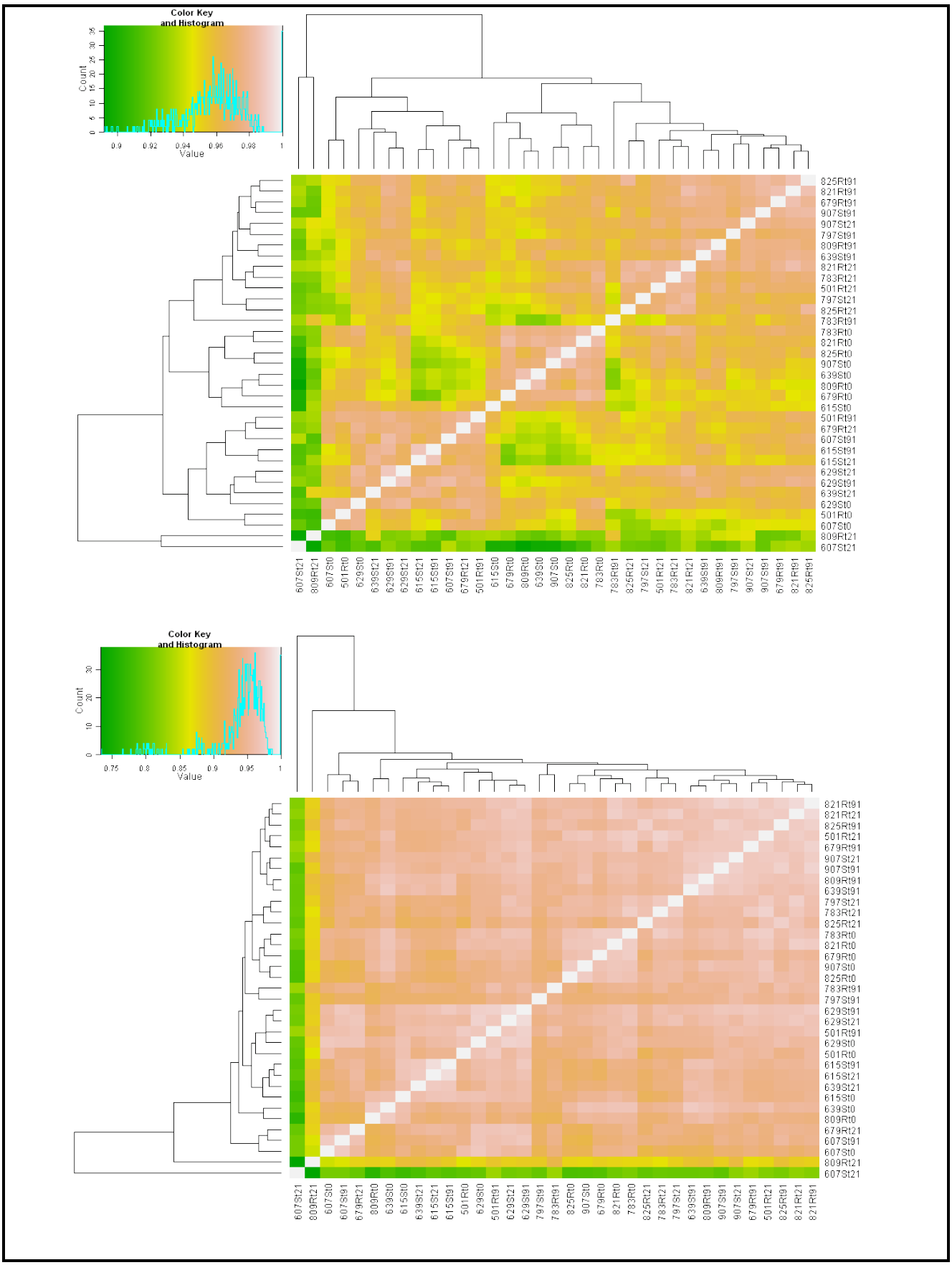


Figure 4.3: Hierarchical clustering of the correlations of PM and MM intensities using positive correlation and complete clustering parameters. Arrays are clustered based on similarity. In principal slides within a treatment group should be more similar than across treatments. These are useful to detect if the sample treatments group together.

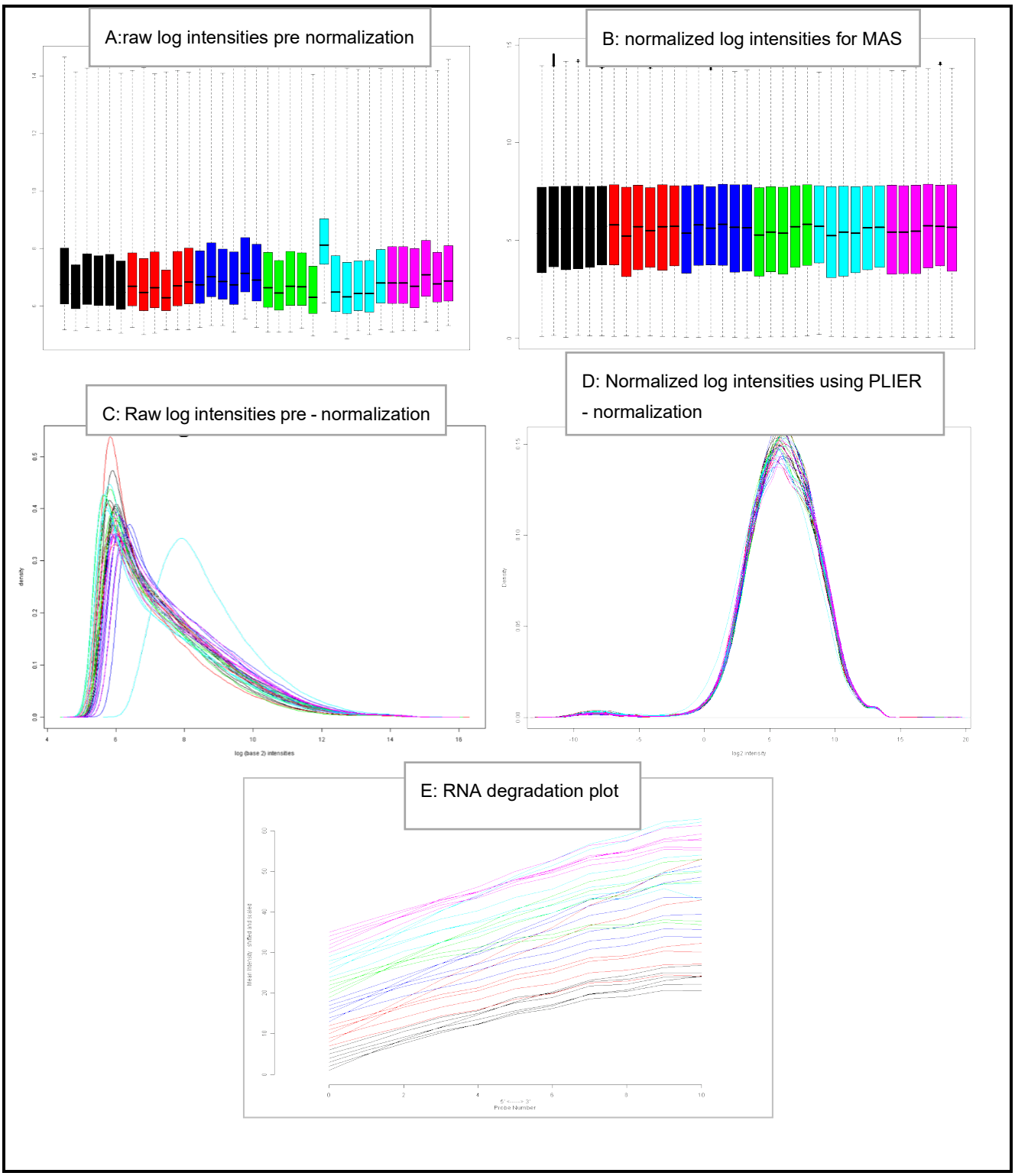


Figure 4.4: Summary statistics. (A) Boxplot of raw log intensities. Indication of quality problems: slides that show high levels of discrepancy in relation to the other slides. (B) Boxplot of normalized log intensities for the MAS summarization method. The outlier slide falls in line with the other slides post normalization. (C) Histogram of raw log intensities. Smoothed histogram of the probe intensities across arrays. Indication of quality problems: slides that show high levels of discrepancy in relation to the other slides and slides that show abnormal distributions (for example a bimodal distribution). (D) Histogram of the normalized log intensity displaying uniform distribution across all slides. (E) RNA degradation plot. Each line represents one of the chips. Mean intensities by probe set position are plotted on the Y-axis. Intensities have been shifted to improve visualization, but the slope remains unchanged. Indication of quality problems: very steep slopes (high level of RNA degradation) and slides that are very different from the other ones.

4.3.3 Annotation of significantly differentially expressed probes

Table 4.3 summarizes the average multiple testing adjusted p-values for the significantly differentially expressed probes identified from the intergroup comparisons between tick-susceptible and tick-resistant animals at the three different time points and between time points. The *p*-values shown, averaged across the six summarization methods, are for the moderated t-statistic which stabilizes the gene-specific variance using an empirical Bayes method to shrink the probe-wise sample variances towards a common value and to augment the degrees of freedom for the individual variances (Smyth, 2004). Significance after correction for FDR using the method *none* for a targeted *p*-value of 0.01 is also shown for each summarization set (ns - not significant, * - significant; this means that if the gene has six stars it is significant for all six summarization methods, and a very reliable indication that the gene is truly differentially expressed). Average $\log_2$ fold changes are also shown in the table. Probes were filtered for a $\log_2$ fold change of at least zero. The average fold changes are arranged as left-hand side minus right-hand side on the log scale: Plus = left up-regulated; minus = right up-regulated, where the tick-susceptible samples fall on the left side of the contrast and tick-resistant falls on the right side of the contrast. The Convergence column shows the convergence in the percentage of the gene across the six methods. A value of 100 indicates that the gene was detected as significantly differentially expressed for all summarizations. The tables were filtered for genes with a convergence equal or greater than 100%, called as present in at least 50% of the samples, and FDR equal to six stars. The criteria were relaxed for experiments 10 to 12 due to the low numbers of DE probes that were commonly found in all six methods. Therefore, probes that were commonly significantly differentially expressed in at least three of the six summarization methods were taken as significant overall.

The animals used for this experiment were of the same breed with contrasting phenotypes. Therefore, the influence of breed on the gene patterns observed was considered insignificant. The DEGs were interpreted in alignment with tick resistance and tick susceptibility to *R. microplus* infestations. The intragroup comparisons within tick-resistant animals produced more DEGs than within tick-susceptible animals. In general, the intragroup comparisons yield more DEGs than the intergroup comparisons. The Day0 versus Day21 and the Day0 versus Day91 intragroup comparisons yielded the most significantly differentially expressed probe sets and subsequently the most DEGs (Figure 4.5). There were notable differences in the expression profiles between the Day0 samples and samples from both the post-infestation time point samples. Figures 4.6B and 3.6C show that the number of unique DEGs identified in the Day0 vs Day21 (434; 384) and Day0 vs Day 91 (340; 670) contrasts greatly surpassed the Day21 vs Day91 (17; 72) contrast in tick-susceptible and tick-resistant animals. Gene B4GALT1 was commonly differentially expressed in the intragroup comparisons of tick-susceptible animals across all time-point comparisons (Figure 4.6B), while the comparison within tick-resistant animals produced 11 commonly differentially expressed genes (Figure 4.6C).

Table 4.3: List of probes that were significantly differentially expressed in susceptible and resistant cattle at each of the three time-points and between time points

AFFY	Gene Symbol	Gene Title	P-value (Average)	Fold Change (Average)	FDR	Convergence
Susceptible - Day0 vs Resistant - Day0 (pre-infestation)						
Bt.1932.1.S1_at	AHSA1	AHA1, activator of heat shock 90kDa protein ATPase homolog 1 (yeast)	3.02E-04	0.84	* * * * * *	100
Bt.2556.1.S1_at	HPGD	hydroxyprostaglandin dehydrogenase 15-(NAD)	1.31E-03	-1.98	* * * * * *	100
Bt.20318.1.S1_a_at	NQO2	NAD(P)H dehydrogenase, quinone 2	5.19E-03	-0.73	* * * * * *	100
Bt.5323.1.S1_at	SH3YL1	SH3 and SYLF domain containing 1	1.51E-03	0.77	* * * * * *	100
Bt.21118.1.S1_at	VIPAS39	VPS33B interacting protein, apical-basolateral polarity regulator, spe-39 homolog	4.86E-03	0.58	* * * * * *	100
Bt.23980.1.A1_at	---	---	2.86E-03	-2.09	* * * * * *	100
Bt.29182.1.A1_at	---	---	6.79E-03	0.57	* * * * * *	100
Susceptible - Day21 vs Resistant - Day21						
Bt.25023.1.A1_at	CLEC6A	C-type lectin domain family 6, member A	6.21E-04	-0.971	* * * * * *	100
Bt.25923.1.S1_at	FNBP4	formin binding protein 4	2.00E-03	-0.560	* * * * * *	100
Bt.16324.1.A1_at	LOC512867	GTPase, IMAP family member 1-like	3.81E-03	0.612	* * * * * *	100
Bt.26711.1.S1_at	LRRC20	leucine rich repeat containing 20	1.74E-03	1.599	* * * * * *	100
Bt.1536.2.S1_a_at	NFKBIE	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	6.30E-03	-0.915	* * * * * *	100
Bt.4706.1.S1_at	PPP1R1B	protein phosphatase 1, regulatory (inhibitor) subunit 1B	4.93E-03	-1.309	* * * * * *	100
Bt.7325.1.S1_at	PRRC2A	proline-rich coiled-coil 2A	4.52E-03	-0.449	* * * * * *	100
*Bt.4270.1.S1_at	WDFY1	Bos indicus WD repeat and FYVE domain containing 1	1.53E-03	0.417	* * * * * *	100
Susceptible – Day91 vs Resistant - Day91						
Bt.4599.1.S1_at	CROT	carnitine O-octanoyltransferase	1.68E-03	0.798	* * * * * *	100
Bt.11194.1.S1_at	DHRS4	dehydrogenase/reductase (SDR family) member 4	3.71E-03	0.901	* * * * * *	100
Bt.5525.1.S1_at	ECM1	extracellular matrix protein 1	4.92E-03	0.814	* * * * * *	100
Bt.16945.1.S1_at	ETHE1	ethylmalonic encephalopathy 1	4.84E-03	0.490	* * * * * *	100
Bt.14061.1.A1_at	FAM105A	family with sequence similarity 105, member A	2.89E-03	0.541	* * * * * *	100
Bt.6000.1.A1_at	LGALSL	lectin, galactoside-binding-like	5.13E-03	0.771	* * * * * *	100
Bt.28518.1.S1_s_at	PTI	spleen trypsin inhibitor /// pancreatic trypsin inhibitor	4.55E-03	1.126	* * * * * *	100

Bt.20339.1.S1_at	PAX5	paired box 5	3.76E-03	-0.582	* * * * * *	100
Bt.9542.1.S1_at	PTPN1	protein tyrosine phosphatase, non-receptor type 1	6.64E-03	-0.713	* * * * * *	100
Bt.13906.1.S1_at	SRXN1	sulfiredoxin 1	4.22E-03	0.772	* * * * * *	100
Bt.23348.1.S1_a_at	ZYX	zyxin	4.70E-03	0.733	* * * * * *	100
Bt.20745.1.A1_at	---	---	3.22E-03	-0.724	* * * * * *	100
Bt.23980.1.A1_at	---	---	9.89E-04	-2.226	* * * * * *	100
Susceptible Day0 – Day21 vs Resistant Day0 – Day21						
Bt.21292.2.S1_at	GEMIN4	gem (nuclear organelle) associated protein 4	5.12E-02	0.457	* * * * ns ns	66.67
Bt.26742.1.S1_at	LOC539383	alpha-amylase 2B	1.91E-01	-1.840	* * ns ns * ns	50
Bt.22561.1.A1_at	---	---	1.65E-01	0.345	ns * * * * *	83.33
Susceptible Day0 – Day91 vs Resistant Day0 – Day91						
Bt.27823.1.A1_at	LOC112586436	Bubalus bubalis uncharacterized LOC112586436 (LOC112586436), transcript variant X5, ncRNA	2.01E-02	0.456	ns * * ns * ns	50
Bt.26742.1.S1_at	LOC539383	alpha-amylase 2B	2.59E-01	-1.114	* * ns ns * ns	50
Bt.4943.3.S1_a_at	PVRL2	poliovirus receptor-related 2 (herpesvirus entry mediator B)	9.37E-02	-0.708	ns * * * ns ns	50
Susceptible Day21 – Day91 vs Resistant Day21 – Day91						
Bt.9182.1.S1_at	ARF3	ADP-ribosylation factor 3	8.88E-03	-0.644	ns * ns * ns *	50
Bt.2889.1.S1_at	CFAP20	cilia and flagella associated protein 20	1.59E-02	0.406	* * * ns ns ns	50
Bt.15804.1.S1_at	CLCN3	chloride channel, voltage-sensitive 3	7.09E-03	0.556	* * * * ns *	83.33
Bt.25923.1.S1_at	FNBP4	formin binding protein 4	3.62E-03	-0.740	* * * * * *	100
Bt.19334.3.S1_at	JUND	jun D proto-oncogene	1.32E-02	-0.738	* ns ns * * *	66.67
Bt.7325.1.S1_at	PRRC2A	proline-rich coiled-coil 2A	1.05E-02	-0.560	ns * * * ns *	66.67
Bt.4943.3.S1_a_at	PVRL2	poliovirus receptor-related 2 (herpesvirus entry mediator B)	4.01E-02	-0.751	* * * * ns ns	66.67
Bt.5135.1.S1_at	RAD23A	RAD23 homolog A (S. cerevisiae)	8.75E-03	-0.857	* * * ns * ns	66.67

*The gene symbol and title for this probe were recovered by putting the probe sequence through NCBI BLAST.

* The average fold changes are arranged as left-hand side minus right-hand side on the log scale. Plus = left up-regulated; minus = right up-regulated, where the tick-susceptible samples fall on the left side of the contrast and tick-resistant falls on the right side of the contrast.

*Gene symbols and gene titles containing “---” are probe sets that are not annotated by Affymetrix, and their sequences could not be linked to any gene ID using NCBI BLAST.

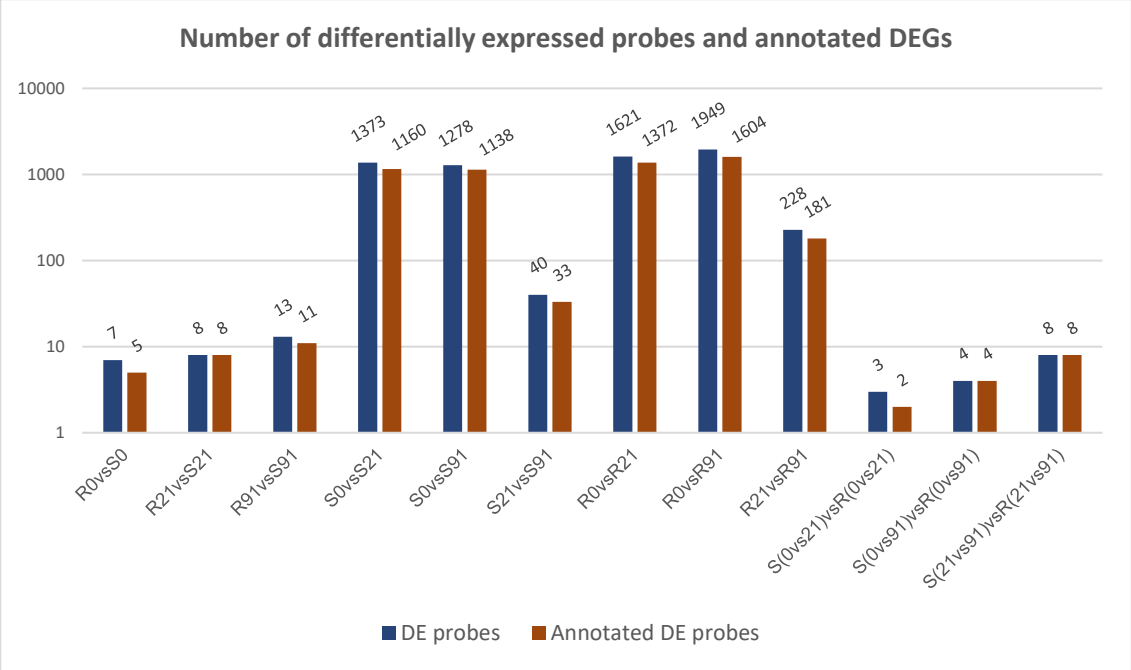


Figure 4.5: Number of significantly differentially expressed probes ("DE probes") common among the different summarization methods and the annotated genes thereof ("Annotated DE probes"). R0 = Samples from resistant animal at Day 0; R21 = Samples from resistant animal at Day 21; R91 = Samples from resistant animal at Day 91; S0 = Sample from susceptible animal at Day 0; S21 = Sample from susceptible animal at Day 21; S91 = Sample from susceptible animal at Day 91.

While a higher number DEGs were identified in the intragroup contrasts between time points (Figure 4.6B & C) the number of DEGs that were common in both the tick-susceptible and tick-resistant animals between time points was diminished (Figure 4.6D); S(0vs21) vs R(0vs21) = 2; S(0vs91) vs R(0vs91) = 4; S(21vs91) vs R(21vs91) = 8. The intergroup comparisons between tick-resistant and tick-susceptible animals at different time points produced fewer DEGs and none of which were found to be common across groups in the binary comparisons of the different time points (Figure 4.5A & 4.5D). While fewer genes (5 unique DEGs) differed between the two phenotypes before infestation (Day0), the number of DEGs had more than doubled (11 unique DEGs) by Day91.

The analysis of changes in gene expression intensities over time showed similar trends in the way the DEGs behaved in both the tick-susceptible and tick-resistant animals (Table 4.4). In the first 21 days post infestation, most of the genes were either upregulated or downregulated, followed by no change over the remaining subsequent infestations until Day91 (Day0 ↓ Day21 ↔ Day91 and Day0 ↑ Day21 ↔ Day 91, where ↑ = upregulation, ↓ = downregulation, ↔ = no change) in both phenotypes. Very few genes displayed the Day0 ↑ Day21 ↑ Day91 and Day0 ↓ Day21 ↓ Day 91 trend over the course of the infestations. This implies that most of the alterations in the gene expression profiles happened in the earlier days of tick infestation rather than later infestations.

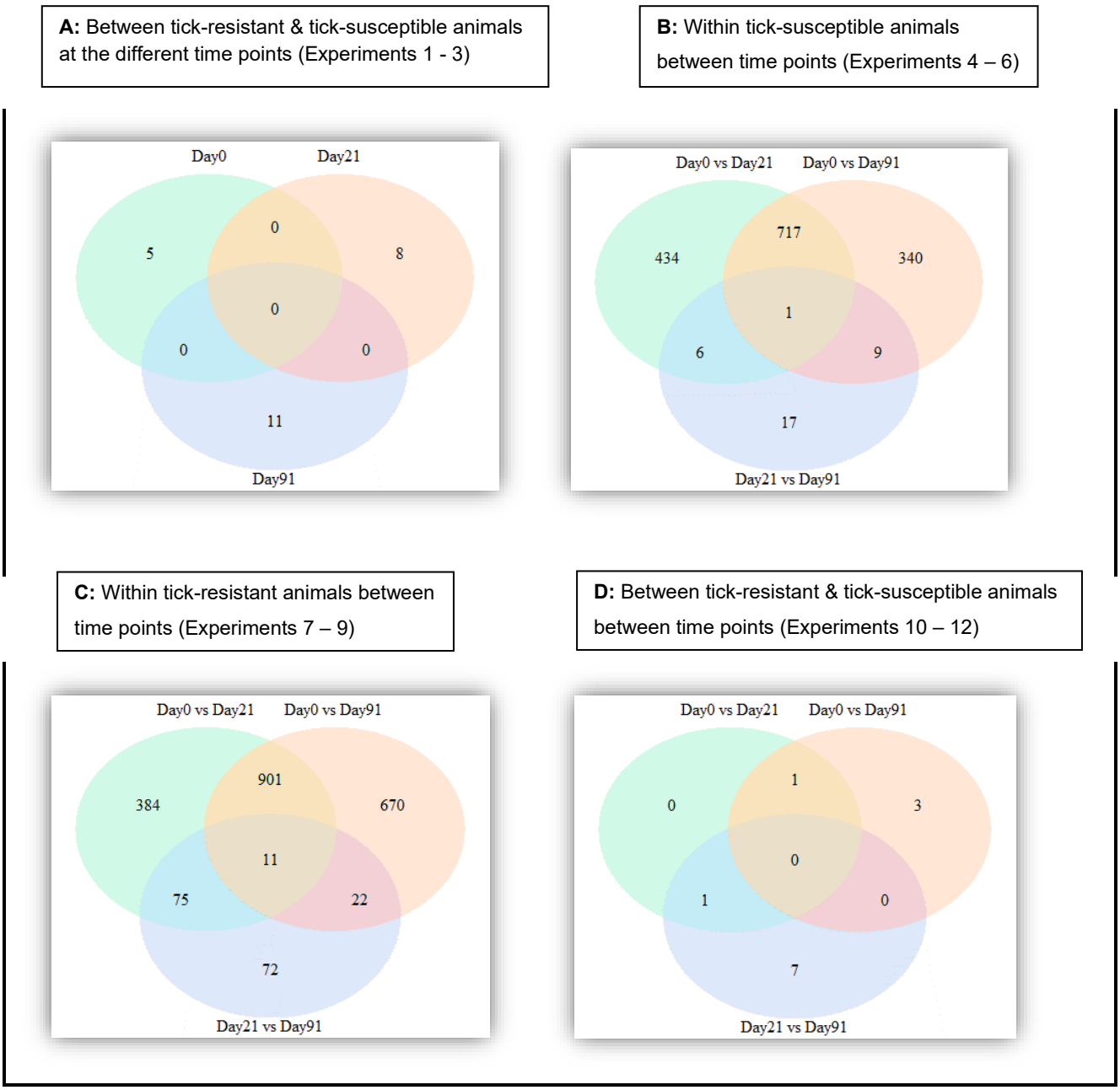


Figure 4.6: Venn diagrams illustrating the distributions of DEGs derived from the different intergroup (A & D) and intragroup (B & C) comparisons. (A) Number of DEGs in tick-resistant versus tick-susceptible animals at the different time points; (B) Number of DEGs in tick-susceptible samples between time points; (C) Number of DEGs in tick-resistant animals between time points; (D) Number of DEGs found when contrasting samples from tick-resistant animals to those of tick-susceptible animals between time points. Only the annotated DEGs were used in constructing the diagrams.

Summarized in Table 4.5 is the number of DE probes that were upregulated and downregulated in the different experiments. Concerning the intergroup comparisons, more DE probes were upregulated in the tick-susceptible animals. This excluded the Day21 contrast between the two phenotypes where more DEGs were upregulated in the tick-resistant animals. As shown in the table, more probes were highly expressed on Day21 and Day91 post infestation as opposed to before the

initial infestation. This was in agreement with the time course changes illustrated in Table 4.4 where most of the changes in gene expression were observed on Day 21 post-infestation.

Table 4.4: Changes in gene expression intensity over time between Day0 to Day21 to Day91 (↑ = upregulation, ↓ = downregulation, ↔ = no change)

Trend	Tick-resistant	Tick-susceptible
Down > Down ↓↓	7	6
Down > Flat ↓↔	345	328
Down > Up ↓↑	72	72
Flat > Down ↔ ↓	134	102
Flat > Up ↔ ↑	145	154
Up > Down ↑↓	39	42
Up > Flat ↑↔	546	531
Up > Up ↑↑	2	7

Table 4.5: Summary of the number of DE probes split into positive and negative fold changes. In the intergroup contrasts, the negative fold change means that DE probes were upregulated in the tick-resistant animals while the positive fold change means that the DE probes we upregulated in the tick-susceptible animals. Alternately, in the intragroup contrasts, a negative fold change means that the right side of the contrast was upregulated and vice versa.

Experiment	Negative Fold Change	Positive fold change
S0 vs R0	3	4
S21 vs R21	5	3
S91 vs R91	4	9
S0 vs S21	771	389
S0 vs S91	954	324
S21 vs S91	31	9
R0 vs R21	1043	578
R0 vs R91	1395	554
R21 vs 91	187	41
S(0vs21) vs R(0vs21)	1	2
S(0vs91) vs R(0vs91)	3	1
S(21vs91) vs R(21vs91)	6	2

4.3.4 GO annotation and Pathway analysis.

To reduce the probability of GO terms appearing as strongly enriched by chance, only experiments with a minimum number of 15 DEGs were used for GO annotation and pathway analysis. This approach was further strengthened using correction for FDR using the Benjamini-Hochberg method ($p < 0.05$ but FDR > 0.05) that ensured that only genes that were found as significantly differentially expressed in all six summarization methods were used for downstream analyses. Due to the few numbers of DEGs retrieved in the intergroup comparisons, it was not possible to correlate the genes to any of the three functional categories using DAVID.

4.3.4.1 Intragroup comparisons of DEGs within tick-susceptible animals (Exp 4 – 6) and within tick-resistant animals (Exp 7 – 9) between time points

The DEGs identified in the intragroup comparisons of tick-susceptible animals between time points correlated with numerous pathways using the DAVID functional analysis platforms (see Table 4.6).

Table 4.6: List of top GO Direct terms enriched by the DEGs for the three functional categories; Biological Process, Cellular Components and Molecular Function

Experiment	Functional category	*Total number of GO Terms enriched by the DEGs	Topmost enriched GO Term in each functional category (number of DEGs out of the total number of DEGs enriching the term)	p-value	Benjamini-Hochberg (FDR)
S0 vs S21	Biological Process	110 (4)	Cell division (27/912)	4.944 E-6	0.013
	Cellular Component	63 (12)	Nucleoplasm (150/995)	1.694 E-12	9.282 E-10
	Molecular Function	45 (2)	Poly(A) RNA binding (93/862)	6.412 E-7	5.431 E-4
S0 vs S91	Biological Process	68	Fatty acid beta-oxidation (10/836)	8.140 E-5	0.194
	Cellular Component	46 (6)	Nucleoplasm (134/920)	3.391 E-10	1.831 E-7
	Molecular Function	45 (1)	poly(A) RNA binding (90/775)	3.661 E-8	2.922 E-5
S21 vs S91	Biological Process	14 (2)	Response to cholesterol (3/29)	8.256 E-5	0.022
	Cellular Component	7	chromosome, centromeric region (3/30)	0.002	0.092
	Molecular Function	1	Receptor signalling protein serine/threonine kinase activity (2/25)	0.094	1.0
R0 vs R21	Biological Process	144 (4)	Cell division (34/1079)	3.786 E-8	1.084 E-4
	Cellular Component	73 (13)	Nucleus (322/1169)	5.575 E-15	3.390 E-12
	Molecular Function	71 (3)	Poly(A) RNA binding (113/987)	9.988 E-10	8.999 E-7
R0 vs R91	Biological Process	140 (5)	Cellular response to DNA damage stimulus (28/1251)	2.44 E-6	0.008
	Cellular Component	70 (9)	Nucleoplasm (208/1366)	4.677 E-18	3.119 E-15
	Molecular Function	65 (2)	Poly(A) RNA binding (145/1153)	1.031 E-15	1.041 E-12
R21 vs R91	Biological Process	24	T cell receptor V(D)J recombination (3/144)	0.001	0.726
	Cellular Component	8	Nucleoplasm (26/144)	3.858 E-4	0.062
	Molecular Function	7	Poly(A) RNA binding (18/128)	0.003	0.611

*The total number of GO terms, with $p < 0.05$, that were enriched by the DEGs from the various contrasts.

*The number in the bracket refers to the total number of GO terms that were enriched and had both a $p =< 0.05$ and FDR =< 0.05 (the relevant rows have been highlighted in dark grey).

*GO: Gene Ontology; DEGs: Differentially Expressed Genes; FDR: False Discovery Rat

The most significantly enriched GO categories (GO Direct) directly annotated by the source database in DAVID, were summarized in Figures 4.7A and 4.8A. None of the GO Direct terms were significantly enriched by the DEGs identified in the Day21 vs Day91 contrast. In general, a greater number of DEGs correlated with GO terms belonging to the CC category and the least number of DEGs were found to correlate with GO terms from the BP category in all intragroup contrasts between tick-susceptible animals. However, given that the study aimed to elucidate the underlying biology of the tick resistance trait, the BP category of significantly enriched terms was further expanded to a more specific level. A further expansion of the biological processes to the second level ontology tree depth is illustrated in Figures 4.7B, 4.8B and 4.9.

The DEGs from the S0 vs S21 contrast correlated with four BP, 12 CC and two MF functional pathways, while the DEGs from the R0 vs R21 contracts correlated with four BP, 13 CC and three MF pathways (Figure 4.7A). The top BP associated terms that were correlated to the S0 vs S21 contrast correlated with cell division. At the second ontology tree specificity, it was evident that a greater number of genes were involved in metabolic and cellular function, however, some immune function pathways were enriched in both the tick-resistant and tick-susceptible animals (Figure 4.7B). A total of 43 GO terms correlating to the biological process functional category were significantly enriched in the intragroup comparison between Day0 and Day21. A similar trend was observed in the GO analysis of the DEGs identified in the Day0 vs Day 91 intragroup comparison. Eight GO terms were exclusively enriched in the tick-resistant animals in the Day0 vs Day 21 contrast, including regulation of homeostatic process and establishment of localization. In the same contrast, three GO terms, including immune response, positive regulation of response to stimulus and macromolecule localization, were exclusively enriched in the tick-susceptible animals. However, multiple other immunoregulatory terms were enriched in both tick-resistant and tick-susceptible animals. These included the activation of immune responses, immune effector process, positive regulation of immune system process, immune responses, regulation of immune system process, positive and negative regulation of the biological process. Also noteworthy was the enrichment of the biological processes involved in the host's regulation of response to biotic stimulus and stress, the regulation of multi-organism process and the interspecies interaction between organisms in both tick-resistant and tick-susceptible animals.

The DEGs from the S0 vs S91 contrast correlated to none of the BP functional pathways but enriched six CC and one MF pathway (Figure 4.8A). Conversely, the DEGs from the R0 vs R91 contrast enriched all three functional categories with five BP, nine CC and two MF pathways. Similar to the enrichment analysis of the Day0 vs Day21 contrast, a greater number of biological processes correlating to metabolic and cellular functions were enriched as in both tick-resistant and tick-susceptible animals as opposed to immune function pathways at the second ontology tree depth (Figure 3.8B). More than half of the BP terms enriched by the DEGs identified in the tick-resistant animals were not enriched in the tick-susceptible animals. The pathways that were exclusively

enriched in the tick-resistant animals included interspecies interaction between organisms, modification of morphology or physiology of the other organism, negative regulation of the biological process, response to abiotic stimulus and stress, activation of immune responses and positive regulation of immune system process.

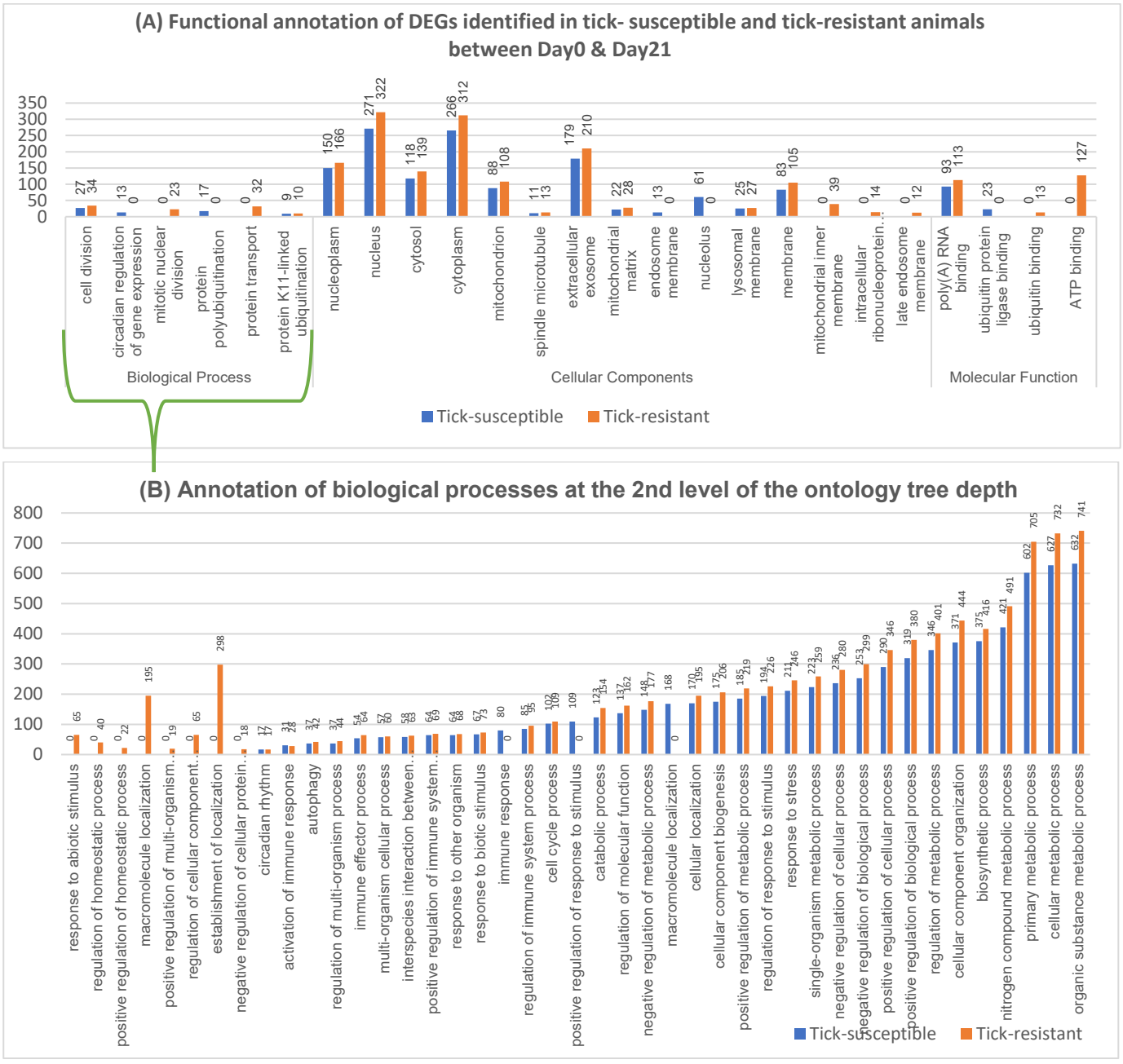


Figure 4.7: A) Gene Ontology terms that were significantly enriched (p =< 0.05, FDR =< 0.05) by the significantly differentially expressed genes identified in the intragroup contrasts within tick-resistant animals (orange bars) and within tick-resistant animals (blue bars) between Day 0 and Day 21, grouped according to most relevant functional categories (BP, CC & MF). B) More detailed representation of the Biological Processes that were significantly enriched by the DEGs at the second level of the ontology tree depth. The y-axis and the numbers above the bars in the graph indicate the number of DEGs correlated with each of the enriched terms. The data was generated using the DAVID functional annotation web-based tool.

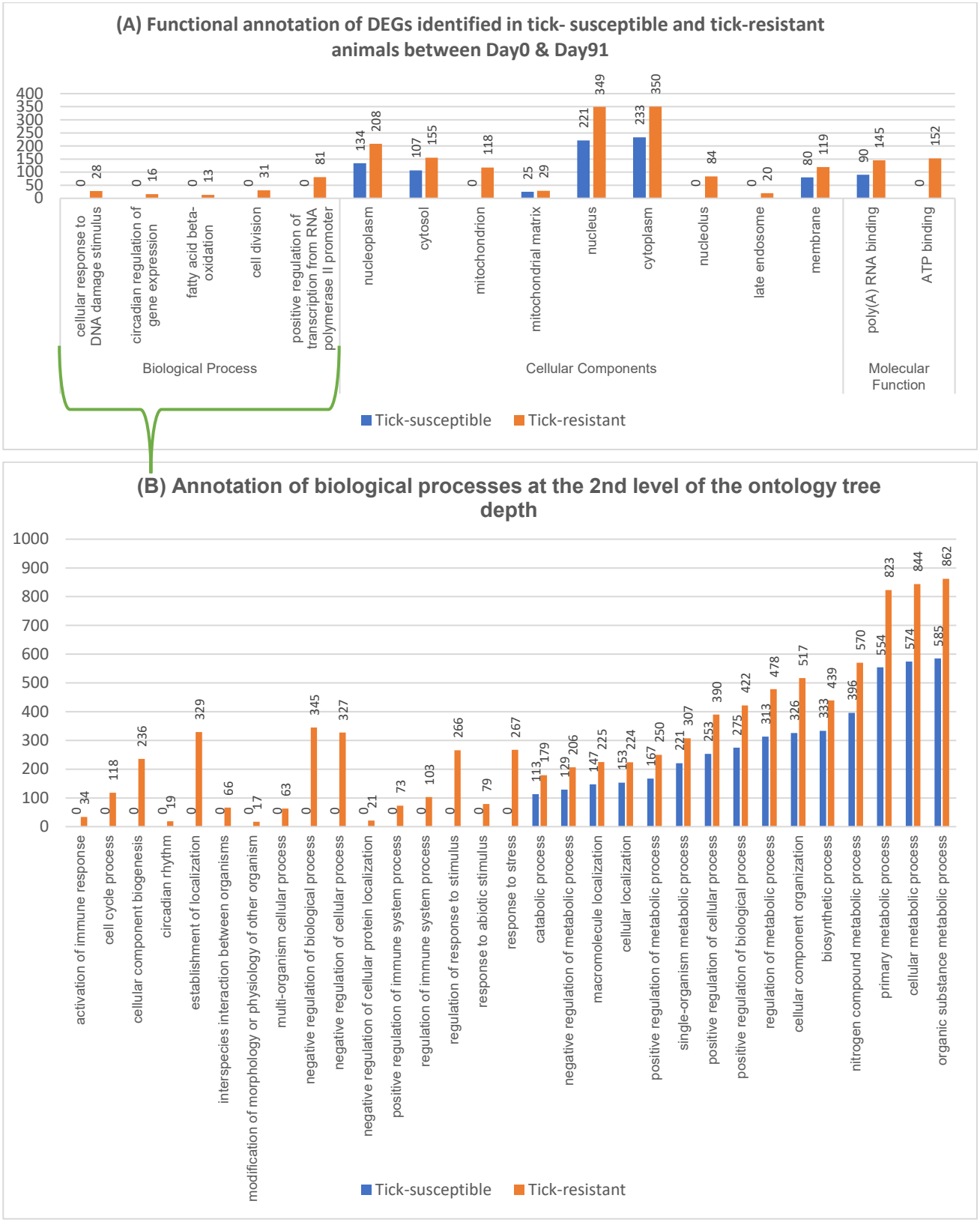


Figure 4.8: A) Gene Ontology terms that were significantly enriched (p =< 0.05, FDR =< 0.05) by the significantly differentially expressed genes identified in the intragroup contrasts within tick-resistant animals (orange bars) and within tick-resistant animals (blue bars) between Day 0 and Day 11, grouped according to most relevant functional categories (BP, CC & MF). B) More detailed representation of the Biological Processes that were significantly enriched by the DEGs at the second level of the ontology tree depth. The y-

axis and the numbers above the bars in the graph indicate the number of DEGs correlated with each of the enriched terms. The data was generated using the DAVID functional annotation web-based tool.

Although none of the GO Direct terms were enriched, at the second ontology tree depth of the biological process three pathways were significantly enriched by the DEGs identified in the Day21 vs Day91 contrast within tick-resistant animals. These include pathways correlated to primary metabolic process, cellular metabolic process and organic substance metabolic process. Once again these pathways were correlated with metabolic and cellular processes more than immune system processes.

4.4 Discussion

4.4.1 Phenotypic data based on tick counts

Tick resistance in cattle is a trait currently quantified based on the percentage of larval ticks which fail to survive to maturity following artificial infestation with a known number of larvae (Jonsson, 2006). However, as observed in the current study, with successive infestations of the same host, the level of tick resistance is likely to increase relative to the first infestation. This is attributable to the variable responses of the animal's innate or acquired immunity to the biting tick (Bonsma and Pretorius, 1943).

Under the assumption that the animals have the same genetic background and were reared under the same conditions, the Santa Gertrudis breed presents an ideal candidate for studies aimed at investigating within breed variations in tick resistance while eliminating the confounding effects of breed (Piper et al., 2017). Highlighted in the introduction is the fact that *Bos taurus* breeds can acquire some resistance to ticks upon long term exposure although the acquired element of resistance is not substantial and is often not sustained. In the case of the Santa Gertrudis heifers in this study, which were composed of 5/8 *Bos taurus*, a selection of the animals in the experimental herd displayed increased resistance following multiple exposures to the *R. microplus* ticks. This was evidenced by the decreased tick burdens on these animals from the third tick counting session and onwards.

The source of the blood meal, whether tick-resistant or tick-susceptible, may affect the different developmental stages of the *R. microplus* thus determining attachment and feeding success on the host animal (Garcia et al., 2020). In this study, the tick-resistant and tick-susceptible animals only displayed diverging phenotypes (i.e., tick counts) after the third consecutive artificial infestation. The Santa Gertrudis breed of cattle displays divergent degrees of the tick-resistance phenotype, with resistance estimates ranging from 81% to 99,7% (Utech et al., 1978b). Similar estimates were observed in the current study, with 88% and 99% for tick-susceptible and tick-resistant animals, respectively. To some extent, these results contradicted the resistance estimates reported by Wang

et al. (2007) who reported 98.5-99% and 95% resistance in the high resistance and low resistance Hereford Shorthorn cattle, respectively.

The genetic background behind these changing levels of tick resistance may help clarify the role that either the innate or the acquired immune system responses play in this regard. This may provide insight into the varied responsiveness of individuals of varying phenotypes to vaccines to aid in future attempts for vaccine development.

4.4.2 Differential gene expression profiles

Regulation of the host's immune system has been implicated in the divergent nature of the tick-resistance phenotype in cattle (Moré et al., 2019). This includes the regulation of hemostatic and inflammatory responses as well as the modulation of both the innate and adaptive immune systems (Kongsuwan et al., 2010). The highly variable nature of the tick-resistance phenotype within the Santa Gertrudis breed points towards the complex assembly of the genes and mechanisms actively regulated to produce the varying levels of resistance (Regitano and Prayaga, 2011). The animals used in the current study were tick-naïve with no previous exposure to any tick species. Therefore, through the intragroup comparisons between time points the study aimed to investigate responses acquired by the hosts following multiple tick infestations from a starting point of tick naivety. Conversely, the intergroup comparisons aimed to characterize the immunogenetic components differentiating the tick-resistant phenotype from the tick-susceptible phenotype.

The intragroup comparisons within the tick-resistant and tick-susceptible animals between time points (R0 vs R21, R0 vs R91, R21 vs R91; S0 vs S21, S0 vs S91, S21 vs S91) produced higher numbers of DEGs than the intergroup comparisons at any of the time points. The number of DEGs identified in each of the intragroup comparisons were similar in both the tick-resistant and tick-susceptible animals, with cytokines, chemokines, and toll-like receptors accounting for a small portion of the total DEGs. However, the intergroup contrasts between phenotypes and between time points [S(0vs21) vs R(0vs21), S(0vs91) vs R(0vs91), S(21vs91) vs R(21vs91)] produced two, three, and eight significantly differentially expressed genes, respectively. *GEMIN4* was upregulated in the tick-susceptible animals while *LOC539383* was upregulated in the tick-resistant animals in the S(0vs21) vs R(0vs21) contrast. Both genes have no immune regulatory functions but could be associated with cellular functions correlated to the RNA transport and gene expression pathways. *LOC539383* was again found to be upregulated, together with *PVRL2*, in tick-resistant animals in the S(0vs91) vs R(0vs91) contrast, while *LOC112586436* was upregulated in the tick-susceptible animals. *PVRL2* was again upregulated in the tick-resistant animals in the S(21vs91) vs R(21vs91) contrast. Also known as *NECTIN2*, *PVRL2* modulates T-cell signaling resulting in either the co-stimulation of T-cell function or a co-inhibition thereof, depending on the receptor to which it binds. The interactions with the receptors are competitive. Interaction with the *CD226* receptor stimulates T-cell proliferation and cytokine production including that of *IL2*, *IL5*, *IL10*, *IL13*, and *IFNG*.

Conversely, binding to the *PVRIG* receptor inhibits T-cell proliferation (Warner et al., 1998; Zhu et al., 2016). This corroborates the finding of Domingues et al. (2014), Franzin et al. (2017) and (Piper et al., 2008) who observed elevated levels of different T cell populations in tick resistant animals following infestation with *R. microplus* ticks. Six out of the eight DEGs identified in the S(21vs91) vs R(21vs91) were upregulated in the tick-resistant animals. Products encoded by genes *ARF3* and FNBP4 participate in cellular modulation rather than immune response regulation. Conversely, *JUND*, *PRRC2A*, *PVRL2* and *RAD23A* may play a role in immune response modulation. JUND encodes a protein that forms a functional component of the *AP-1* transcription factor complex that regulates gene expression in response to stimuli that include stress, infections and cytokines (Agarwal et al., 1999; Hess et al., 2004). On the contrary Carvalho et al. (2014) reported the upregulation of genes that encode components that trigger *NFKB* and *AP-1* in tick-susceptible animals. Although gene *PRRC2A* was upregulated in the tick-resistant animals within this contrast as well as in the S21 vs R21i contrast, its contribution to immune system responses is unclear. *PRRC2A* is located within the bovine major histocompatibility complex. The gene was shown to have a significant positive correlation (correlation = 0.61) with lymphocyte counts in cattle infected with the bovine leukemia virus and was thought to contribute to a reduced number of infected cells in the peripheral blood (Petersen et al., 2021). Lastly, proteins encoded by *RAD23A* are critical for nucleotide excision repair which is a pathway employed in the removal of bulky DNA lesions created by environmental mutagen such as tick infestation (Watkins et al., 1993). Pro-inflammatory neutrophils are the most abundant immune response cells that can infiltrate the site of tissue damage with haste. Their role in the innate immune system, involving the recognition and destruction of pathogens and tissue repair at the tick bite site, is vital as the first line of defense against the biting tick. Supporting this observation were the findings by Marima et al. (2020) who noted an increase in segmented neutrophils in the blood of tick-resistant Brahman cattle following 12 hours tick infestation. However, ticks have developed mechanisms used to delay tissue repair at the host-tick interface, thereby prolonging access to their bloodmeal (Francischetti et al., 2009; Kongsuwan et al., 2010; Kotál et al., 2015).

Upon attachment and insertion of the hypostome, the tick mouthparts come into contact with mast cells that signal the activation and mobilization of circulating host immune regulatory cells (dendritic cells, neutrophils, macrophages, keratinocytes, and fibroblasts) to the tick bite site (Kazimírová et al., 2017). This response together with the expression of proinflammatory chemokines, cytokines, anti-microbial peptides and wound healing components offer the first line of the innate immune system's inflammatory defenses against the biting tick (Franzin et al., 2017; Marufu et al., 2014; Severo, 2013). B and T lymphocytes are then recruited and activated to mount the cell-mediated immune responses of the adaptive immune system (Franzin et al., 2017; Robbertse et al., 2018). Consequently, tick-resistant animals use these initial encounters with the tick antigens to build memory and solidify the adaptive immune responses to the specific tick species in future encounters.

Innate immune responses are mounted faster and are stronger than adaptive immune responses although not specific to the biting tick.

The delayed production of inflammatory molecules and downregulation of transcripts of the toll-like receptor pathways has been implicated in the expression of the superior tick-resistance phenotype (Franzin et al., 2017; Piper et al., 2008). Samples collected from tick-resistant animals pre-infestation (S0 vs R0) showed higher activity of genes *HPGD* and *NQO2*. *HPGD* encodes an enzyme that is involved in prostaglandin inactivation. Prostaglandins are lipids secreted in the tick saliva that control inflammation, blood flow and formation of blood clots at the site of tissue damage to ensure a constant supply of fluid host blood (Ensor and Tai, 1994; Kongsuwan et al., 2008). *NQO2* encodes an enzyme that serves as a quinone reductase in connection with the conjugation of hydroquinones involved in detoxification pathways and biosynthetic processes such as the vitamin K-dependent gamma-carboxylation of glutamate residues in prothrombin synthesis (Calamini et al., 2008). The expression of products of both these genes suggests a higher efficiency of coagulation and shorter clotting time which in turn may restrict the tick's blood meal in tick-resistant animals. This was in agreement with the perception that the immune profiles of some hosts may affect the expression of salivary components in the tick that may impair coagulation, vasoconstriction and wound repair in the host (Franzin et al., 2017; Nuttall, 2017). The expression and effects of these salivary components may be more intense in tick susceptible animals where there is seemingly reduced activity of transcripts that can counteract the salivary components. Conversely, a higher expression of VIPAS39 was observed in the tick-susceptible animals pre-infestation. This gene encodes proteins involved in lysosomal trafficking and transcription regulation of E-cadherin, functions that contribute to cell and tissue structure repair and homeostasis thereof (Graziano, 2013). Also observed is the higher expression of the protein coding *AHSA1* gene that has been correlated to the T cell receptor signaling pathway. The establishment of initial tick attachment through the successful creation of lesions as well as gasket and holdfast creation is crucial for successful tick infestation (Kongsuwan et al., 2008). However, the robust inflammatory response of the innate immune system observed in tick-resistant animals may destabilize the tick's cement cone thus lowering the number of ticks able to complete their parasitic life cycle (Franzin et al., 2017).

Of the eight genes that were significantly differentially expressed in the S21 vs R21 contrast, five were upregulated in the tick-resistant animals. Among the upregulated genes was *CLEC6A*, a gene that plays an important role in the recognition of tick antigens in a mannose-dependent manner culminating in cysteinyl leukotriene synthesis (a family of inflammatory lipid mediators) (Zhu et al., 2013). When stimulated or upregulated the protein products of *CLEC6A* initiate signally through the CARD9-Bcl10-Malt1 pathways, consequently inducing cytokines to achieve immune homeostasis. This corroborated the finding by Carvalho et al. (2014) who highlighted the importance of lipid metabolism in tick rejection as well as the positive regulatory activity of cysteinyl leukotriene in sustaining long term signaling in the host's immune system, characteristic of innate immune

responses. Veríssimo et al. (2008) also reported an increased mast cell count in tick resistant Nelore cattle. Mast cells are critical to establishing homeostasis in response to the tick antigens. It is thought that the activation of host defense signally genes coupled with the diminished immune responses and re-establishment of homeostasis following exposure to ticks while suppressing inflammatory responses may contribute to tick resistance (Kongsuwan et al., 2008). Similarly, the upregulation of the *NFkB1* gene points to the increased production of inflammatory cytokines, chemokines and adhesion molecules in tick-resistant animals. *NFkB1* is a pleiotropic transcription factor initiated by inflammation and immunity, among other stimuli, and plays a critical role in regulating immediate-early immune responses to stimuli such as tick antigens. Inhibition or underexpression of the *NFkB1* gene has been associated with inappropriate immune cell developments or delayed cell growth including the regulation of pro-inflammatory activation of monocytes (Beinke et al., 2004). These findings correlate with significant differential expression of response initiation factors reported by Kongsuwan et al. (2008). On the other hand, the genes that were upregulated in the tick-susceptible animals 21 days post-infestation included *LOC512867*. This gene encodes proteins belonging to the immuno-associated nucleotide subfamily of nucleotide-binding proteins and is thought to play a role in the differentiation of T helper cells of the Th1 lineage and is critical in the development of mature B and T lymphocytes in mice. The increased production of B and T lymphocytes is characteristic of adaptive immune system cellular immune responses. This was coupled with the upregulation of the *WDFX1* gene that positively regulates *TLR3* and *TLR4*-mediated signally pathways by bridging the interaction between *TLR3* and *TLR4* and *TICAM1*. Consequently, transcription factors *IRF3* and *NF-kappa-B* are activated coupled with the production of INF-beta and inflammatory cytokines. It was reasonable to expect the downregulation of *TL3* and *TLR4* in the tick susceptible animals, given they play a fundamental role in pathogen recognition and activation of innate immunity. However, similar to the current study, Piper et al. (2008) and Kongsuwan et al. (2008) observed an upregulation of some members of the *TLR* signally family and pro-inflammatory pathways in tick-susceptible Holstein-Friesian cattle. This suggest that some members of the *TLR* family may not contribute to mounting high levels of resistance.

Eleven significantly differentially expressed genes were identified in the S91 vs R91 contrast, with only two genes (*PAX5* and *PTPN1*) being upregulated in the tick-resistant animals. Stanley et al. (2017) also reported the upregulation of the *PTPN1* gene in tick-resistant animals 91 days post-infestation. Contradicting expression patterns are defined for the *PAX5* and *PTPN1* genes. The upregulation of the *PAX5* gene in tick-resistant animals suggests that there may be an increased activation of B-lineage-specific genes resulting in the adhesion, migration and propagation of immune factors to the site of tick attachment (Gu et al., 2017). On the contrary, the activation of *PTPN1* has been associated with the positive regulation of *IFN-γ*- induced *STAT1*-dependent gene expression with enhanced destruction of B-cells, while the deactivation thereof results in the protection of B-cells from cytokine-mediated cell death. Carvalho et al. (2014) reported inhibition of the *IFN-γ* signaling pathways in tick-susceptible animals in Gir × Holstein F1 cattle 24 hours post-

infestation with *R. microplus*. While this demonstrates the participation of B cells in mounting immune responses against tick infestation, the simultaneous upregulation of these conflicting genes limits the ability to assign causality in the tick-resistance phenotype of the Santa Gertrudis animals in this study. Of the genes that were upregulated in the tick-susceptible animals, *CROT* was included. The protein encoded by the *CROT* gene plays an important role in lipid metabolism and fatty-acid beta-oxidation (Ferdinandusse et al., 1999). This contradicts the findings of previous studies that highlighted the importance of lipid metabolism in the expression of super host resistance to ticks in cattle. Kongsuwan et al. (2008) reported the upregulation of the transcripts encoding enzymes that synthesize and bind fatty acids resulting in cellular trafficking and lipid storage in tick-resistant Brahman. Also upregulated were genes *ECM1* and *ZYX*. *ECM1* encodes soluble proteins involved in stimulating the proliferation of endothelial cells and promoting angiogenesis (Han et al., 2001) while *ZYX* encodes zinc-binding phosphoproteins that concentrate at focal adhesions that enable adherence of cells to the extracellular matrix. *ECM1* has been shown to interact with a variety of extracellular and structural proteins contributing to the maintenance of skin integrity and homeostasis (Deckers et al., 2001; Sercu et al., 2008). The expression of this gene inhibits matrix metalloproteinase-9 (*MMP9*) proteolytic activity. *MMP9* was shown to regulate pathological remodeling processes involving inflammation and fibrosis while directly degrading the extracellular matrix, activating the *IL-1β* and cleaving cytokines and chemokines that regulate tissue remodeling (Yabluchanskiy et al., 2013). While Kongsuwan et al. (2008) reported results that contradicted the finding of the current study where genes involved in the production of lipid mediators that regulate pro-inflammatory cytokines and chemokine production were upregulated in tick-resistant animals Brahman cattle. The authors also noted the upregulation of transcripts relating to the extracellular matrix in both tick-resistant and tick-susceptible animals. Contributing to the innate immune responses are the components of the extracellular matrix that assist with tissue remodeling to form and maintain a protective barrier to prevent tick attachment and feeding.

4.4.3 Pathway enrichment and functional annotation

The main aim of this study was to unpack the systems biology of host resistance to ticks in phenotypically divergent Santa Gertrudis animals. Therefore, the distribution according to the biological process of the significantly differentially expressed genes was studied at different ontology tree depths. The annotated DEGs identified in the intergroup comparisons were too few to be significantly correlated with biological functions. Overall, the intragroup comparisons correlated to multiple biological processes. However, very few pathways correlating to immune system processes were enriched by the DEGs. This was more evident in the S21 vs S91 and R21 vs R91 contrasts, where no immune regulatory pathways were significantly enriched. On the contrary, many of the significantly differentially expressed genes correlated with the regulation of metabolic and cellular components of the biological processes instead. This corresponded with the finding by Carvalho et

al. (2014) who reported different patterns of cell response to *R. microplus* attachment. All of the most highly enriched terms correlated to the metabolic process terms.

In response to the biting tick, multiple pro-inflammatory immune regulatory and effector pathways are confronted and activated leading to the chemotaxis of antigen-presenting cells and antibodies to the tick bite site (Garcia et al., 2020). It was thus expected that a notable activation of the complement cascade would be observed in both tick-resistant and tick-susceptible animals. In the current study, only the tick-resistant animals seemed to exhibit a positive regulation of the response to stimulus in the Day0 vs Day21 contrast. Furthermore, two GO terms correlating to the regulation of the homeostatic process were exclusively significantly enriched in the tick-resistant animals. This may be indicative of the increased activation of processes involved in the regulation and re-establishment of homeostasis in tick-resistant animals following the recognition of external stressors. This corroborated the findings from previous literature (Brossard and Wikel, 2016; Carvalho et al., 2014; Wambura et al., 1998; Wikel, 1996). The activation or suppression of components of the host's inflammatory, hemostatic, and immune defenses determine the success of the biting tick's life cycle. The bioactive molecules in the saliva of the tick contain immunosuppressive molecules that can modulate the host's local hemostatic reactions (Kotál et al., 2015). Tick-resistant hosts have been shown to influence the downregulation of the expression of the anti-hemostatic in the ticks salivary glands while this reaction may be delayed in tick-susceptible animals consequently availing hemorrhagic pools at the tick bite site (Carvalho et al., 2010).

Following 91 days of consecutive weekly tick infestations, the tick-resistant animals presented a more sustained activation of immune regulatory pathways. The GO analyses demonstrated the enrichment of pathways relating to response to stress and stimulus, the activation of immune responses and the regulation of biological and immune system processes exclusively in the tick-resistant animals. These may be characteristic of the development of more robust adaptive immune system responses in the tick resistant Santa Gertrudis cattle following multiple encounters with the *R. microplus* tick species. The competent response of the host biological processes to stimulation of the epidermal differentiation may contribute to the activation of the interspecies interaction between organisms pathway thus mounting stronger innate immune responses in the tick-resistant animals as the initial defense mechanism (Kongsuwan et al., 2010). This consequently results in the maintenance of an intact epidermal barrier. Contrary to previous studies (Kongsuwan et al., 2010), the enrichment of the response to stress and stimulus pathways 91 days post multiple infestations points towards the continued attempt of the host's biological processes to maintain the epidermal barrier post successful tick attachment. Therefore, the sustained activation of the abovementioned pathways may lead to the development of strong adaptive immune responses. They may explain the divergence of the tick-resistant animals from the rest of the group by the third weekly artificial infestation. Consequently, mechanisms of the adaptive immune system are mounted and sustained, as is evidenced by the GO analysis results of the Day0 vs Day91 contrast.

4.5 Conclusions

The study evidence the presence of within-breed differences in tick counts observed in the different animals and this could be correlated with varying levels of tick resistance exhibited by the cattle. While tick naïve cattle display similar tick counts at first infestation, some cattle may acquire immunity following consecutive exposure to the same tick. Upon long term exposure to the *R. microplus* the tick-resistant Santa Gertrudis animals displayed an increased ability to develop and mount more robust adaptive responses against the tick infestations than the tick-susceptible animals. This indicates that selective breeding for natural host resistance is possible. Genes that were significantly differentially expressed in the tick-resistant animals pre-infestation involved factors involved in response initiations and pro-inflammatory immune responses. It was highlighted that both the innate and the adaptive immune system responses that may function collaboratively with non-immune responses in determining the various levels of host resistance to tick infestation.

While interesting immune response results were recovered in the current study where blood samples from animals with varied tick resistance phenotypes were considered, a comparative study of the skin, blood and serum from tick-infested animals may offer a more comprehensive elucidation of the underlying mechanisms. It may also guide future research advances regarding the choice of sample to examine to best characterize tick resistance within and between cattle breeds in search of biomarkers for host resistance to ticks for improved selective breeding for the trait.

4.6 References

Agarwal, S.K., Guru, S.C., Heppner, C., Erdos, M.R., Collins, R.M., Park, S.Y., Saggar, S., Chandrasekharappa, S.C., Collins, F.S., Spiegel, A.M., Marx, S.J., Lee Burns, A., 1999. Menin interacts with the AP1 transcription factor JunD and represses JunD-activated transcription. Cell 96, 143–152. https://doi.org/10.1016/S0092-8674(00)80967-8

Beinke, S., Robinson, M.J., Hugunin, M., Ley, S.C., 2004. Lipopolysaccharide Activation of the TPL-2/MEK/Extracellular Signal-Regulated Kinase Mitogen-Activated Protein Kinase Cascade Is Regulated by IκB Kinase-Induced Proteolysis of NF-κB1 p105. Mol. Cell. Biol. 24, 9658–9667. https://doi.org/10.1128/mcb.24.21.9658-9667.2004

Bonsma, J., Pretorius, A., 1943. Influence of colour coat cover on adaptability of cattle. Farming in South Africa 18, 101–120.

Brossard, M., Wikel, S.K., 2016. Tick immunobiology. https://doi.org/10.1017/S0031182004004834

CALAMINI, B., SANTARSIERO, B.D., BOUTIN, J.A., MESECAR, A.D., 2008. Kinetic, thermodynamic and X-ray structural insights into the interaction of melatonin and analogues with quinone reductase 2. Biochem J. 413, 81–91. https://doi.org/10.1042/BJ20071373.Kinetic

Cardoso, F.F., Gomes, C.C.G., Sollero, B.P., Oliveira, M.M., Roso, V.M., Piccoli, M.L., Higa, R.H., Yokoo, M.J., Caetano, A.R., Aguilar, I., 2015. Genomic prediction for tick resistance in Braford and Hereford cattle. J. Anim. Sci. 93, 2693–2705. https://doi.org/10.2527/jas.2014-8832

Carvalho, W.A., Domingues, R., de Azevedo Prata, M.C., da Silva, M.V.G.B., de Oliveira, G.C., Guimarães, S.E.F., Machado, M.A., 2014. Microarray analysis of tick-infested skin in resistant and susceptible cattle confirms the role of inflammatory pathways in immune activation and larval rejection. Vet. Parasitol. 205, 307–317. https://doi.org/10.1016/j.vetpar.2014.07.018

Carvalho, W.A., Franzin, A.M., Abatepaulo, A.R.R., de Oliveira, C.J.F., Mor??, D.D., da Silva, J.S., Ferreira, B.R., de Miranda Santos, I.K.F., 2010. Modulation of cutaneous inflammation induced by ticks in contrasting phenotypes of infestation in bovines. Vet. Parasitol. 167, 260–273. https://doi.org/10.1016/j.vetpar.2009.09.028

Deckers, M.M.L., Smits, P., Karperien, M., Ni, J., Tylzanowski, P., Feng, P., Parmelee, D., Zhang, J., Bouffard, E., Gentz, R., Löwik, C.W.G.M., Merregaert, J., 2001. Recombinant human extracellular matrix protein 1 inhibits alkaline phosphatase activity and mineralization of mouse embryonic metatarsals in vitro. Bone 28, 14–20. https://doi.org/10.1016/S8756-3282(00)00428-2

Domingues, R., Wohlres-Viana, S., Reis, D.R.L., Teixeira, H.C., Ferreira, A.P., Guimarães, S.E.F., Prata, M.C.A., Furlong, J., Verneque, R.S., Machado, M.A., 2014. Expression of immune response genes in peripheral blood of cattle infested with Rhipicephalus microplus. Genet. Mol. Res. 13, 4013–4021. https://doi.org/10.4238/2014.May.23.12

Ensor, C.M., Tai, H.H., 1994. Bacterial expression and site-directed mutagenesis of two critical residues (tyrosine-151 and lysine-155) of human placental NAD+ -dependent 15-hydroxyprostaglandin dehydrogenase. Biochim. Biophys. Acta (BBA)/Protein Struct. Mol. 1208, 151–156. https://doi.org/10.1016/0167-4838(94)90172-4

Ferdinandusse, S., Mulders, J., Ijlst, L., Denis, S., Dacremont, G., Waterham, H.R., Wanders, R.J.A., 1999. Molecular cloning and expression of human carnitine octanoyltransferase: Evidence for its role in the peroxisomal β-oxidation of branched-chain fatty acids. Biochem. Biophys. Res. Commun. 263, 213–218. https://doi.org/10.1006/bbrc.1999.1340

Francischetti, I.M.B., Sa-Nunes, A., Mans, B.J., Santos, I.M., Ribeiro, J.M.C., 2009. The role of saliva in tick feeding. Front. Biosci. 14, 2051–88. https://doi.org/3363 [pii]

Franzin, A.M., Maruyama, S.R., Garcia, G.R., Oliveira, R.P., Ribeiro, J.M.C., Bishop, R., Maia, A.A.M., Moré, D.D., Ferreira, B.R., De Miranda Santos, I.K.F., 2017. Immune and biochemical responses in skin differ between bovine hosts genetically susceptible and resistant to the cattle tick Rhipicephalus microplus. Parasites and Vectors 10, 1–24. https://doi.org/10.1186/s13071-016-1945-z

Frisch, J.E., 1999. Towards a permanent solution for controlling cattle ticks. Int. J. Parasitol. 29, 57–71.

Frisch, J.E., O'Neill, C.J., Kelly, M.J., 2000. Using genetics to control cattle parasites - The Rockhampton

experience. Int. J. Parasitol. 30, 253–264. https://doi.org/10.1016/S0020-7519(00)00010-2

Garcia, G.R., Marcos Chaves Ribeiro, J., Regina Maruyama, S., Gustavo Gardinassi, L., Nelson, K., Ferreira, B.R., Galdino Andrade, thales, Ferreira de Miranda Santos, I.K., 2020. A transcriptome and proteome of the tick Rhipicephalus microplus shaped by the genetic composition of its hosts and developmental stage 10, 12857. https://doi.org/10.1038/s41598-020-69793-3

Graziano, F., 2013. The E-cadherin gene, structure and function, in: Spotlight on Familial and Hereditary Gastric Cancer. Springer Netherlands, pp. 27–33. https://doi.org/10.1007/978-94-007-6570-2_4

Gu, J., Li, T., Zhao, L., Liang, X., Fu, X., Wang, J., Shang, Z., Huang, W., Zhou, J., 2017. Identification of significant pathways induced by PAX5 haploinsufficiency based on protein-protein interaction networks and cluster analysis in Raji cell line. Biomed Res. Int. 2017. https://doi.org/10.1155/2017/5326370

Han, Z., Ni, J., Smits, P., Underhill, C.B., Xie, B., Chen, Y., Liu, N., Tylzanowski, P., Parmelee, D., Feng, P., Ding, I., Gao, F., Gentz, R., Huylebroeck, D., Merregaert, J., Zhang, L., 2001. Extracellular matrix protein 1 (ECM1) has angiogenic properties and is expressed by breast tumor cells. FASEB J. 15, 988–994. https://doi.org/10.1096/fsb2fj990934com

Hess, J., Angel, P., Schorpp-Kistner, M., 2004. AP-1 subunits: Quarrel and harmony among siblings. J. Cell Sci. 117, 5965–5973. https://doi.org/10.1242/jcs.01589

Jenner, R.G., Young, R.A., 2005. Insights into host responses against pathogens from transcriptional profiling. Nat. Rev. Microbiol. 3, 281–294. https://doi.org/10.1038/nrmicro1126

Jonsson, N.N., 2006. The productivity effects of cattle tick (Boophilus microplus) infestation on cattle, with particular reference to Bos indicus cattle and their crosses. Vet. Parasitol. https://doi.org/10.1016/j.vetpar.2006.01.010

Kazimírová, M., Thangamani, S., Bartíková, P., Hermance, M., Holíková, V., Nuttall, Š.I., 2017. Tick-Borne Viruses and Biological Processes at the Tick-Host-Virus Interface 7. https://doi.org/10.3389/fcimb.2017.00339

Kongsuwan, K., Josh, P., Colgrave, M.L., Bagnall, N.H., Gough, J., Burns, B., Pearson, R., 2010. Activation of several key components of the epidermal differentiation pathway in cattle following infestation with the cattle tick, Rhipicephalus (Boophilus) microplus. Int. J. Parasitol. 40, 499–507. https://doi.org/10.1016/j.ijpara.2009.10.013

Kongsuwan, K., Piper, E.K., Bagnall, N.H., Ryan, K., Moolhuijzen, P., Bellgard, M., Lew, A., Jackson, L., Jonsson, N.N., 2008. Identification of genes involved with tick infestation in Bos taurus and Bos indicus. Anim. Genomics Anim. Heal. 132, 77–88.

Kotál, J., Langhansová, H., Lieskovská, J., Andersen, J.F., Francischetti, I.M.B., Chavakis, T., Kopecký, J., Pedra, J.H.F., Kotsyfakis, M., Chmelař, J., 2015. Modulation of host immunity by tick saliva. J. Proteomics. https://doi.org/10.1016/j.jprot.2015.07.005

Machado, M.A., Azevedo, A.L., Teodoro, R.L., Pires, M.A., Peixoto, M.G., de Freitas, C., Prata, M.C., Furlong, J., da Silva, M. V, Guimaraes, S.E., Regitano, L.C., Coutinho, L.L., Gasparin, G., Verneque, R.S., 2010. Genome wide scan for quantitative trait loci affecting tick resistance in cattle (Bos taurus x Bos indicus). BMC Genomics 11, 280. https://doi.org/10.1186/1471-2164-11-280 [doi]

Mackinnon, M.., Meyer, K., Hetzel, D.J.., 1991. Genetic variation and covariation for growth, parasite resistance and heat tolerance in tropical cattle. Livest. Prod. Sci. 27, 105–122.

Mapholi, N.O., Marufu, M.C., Maiwashe, A., Banga, C.B., Muchenje, V., Macneil, M.D., Chimonyo, M., Dzama, K., 2014. Ticks and Tick-borne Diseases Towards a genomics approach to tick (Acari : Ixodidae) control in cattle : A review. Ticks Tick. Borne. Dis. 5, 475–483. https://doi.org/10.1016/j.ttbdis.2014.04.006

Marima, J.K., Nel, C.L., Marufu, M.C., Jonsson, N.N., Dube, B., Dzama, K., 2020. A genetic and immunological comparison of tick-resistance in beef cattle following artificial infestation with Rhipicephalus ticks. Exp. Appl. Acarol. 80, 569–590. https://doi.org/10.1007/s10493-020-00480-8

Marufu, M.C., Chimonyo, M., Mapiye, C., Dzama, K., 2011. Tick loads in cattle raised on sweet and sour rangelands in the low-input farming areas of South Africa. Trop. Anim. Health Prod. 43, 307–313. https://doi.org/10.1007/s11250-010-9690-3

Marufu, M.C., Dzama, K., Chimonyo, M., 2014. Cellular responses to Rhipicephalus microplus infestations in pre-sensitised cattle with differing phenotypes of infestation. Exp. Appl. Acarol. 62, 241–252. https://doi.org/10.1007/s10493-013-9723-5

Moré, D.D., Cardoso, F.F., Mudadu, M.A., Malagó-Jr, W., Gulias-Gomes, C.C., Sollero, B.P., Ibelli, A.M.G., Coutinho, L.L., Regitano, L.C.A., 2019. Network analysis uncovers putative genes affecting resistance to tick infestation in Braford cattle skin. BMC Genomics 20, 1–20. https://doi.org/10.1186/s12864-019-6360-3

Morris, C. a., 2007. A review of genetic resistance to disease in Bos taurus cattle. Vet. J. 174, 481–491. https://doi.org/10.1016/j.tvjl.2006.09.006

Nuttall, P.A., 2017. The wonders of tick saliva, in: One Health 9th Tick and Tick-Borne Pathogen Conference & 1st Asia Pacific Rickettsia Conference. Cairnes, Australia, p. 13.

Petersen, M.I., Carignano, H.A., Suarez Archilla, G., Caffaro, M.E., Alvarez, I., Miretti, M.M., Trono, K., 2021. Expression-based analysis of genes related to single nucleotide polymorphism hits associated with bovine leukemia virus proviral load in Argentinean dairy cattle. J. Dairy Sci. 104, 1993–2007. https://doi.org/10.3168/jds.2020-18924

Piper, E.K., Jackson, L. a., Bagnall, N.H., Kongsuwan, K.K., Lew, A.E., Jonsson, N.N., 2008. Gene expression in the skin of Bos taurus and Bos indicus cattle infested with the cattle tick, Rhipicephalus (Boophilus) microplus. Vet. Immunol. Immunopathol. 126, 110–119. https://doi.org/10.1016/j.vetimm.2008.06.011

Piper, E.K., Jackson, L. a., Bielefeldt-Ohmann, H., Gondro, C., Lew-Tabor, A.E., Jonsson, N.N., 2010. Tick-

susceptible Bos taurus cattle display an increased cellular response at the site of larval Rhipicephalus (Boophilus) microplus attachment, compared with tick-resistant Bos indicus cattle. Int. J. Parasitol. 40, 431–441. https://doi.org/10.1016/j.ijpara.2009.09.009

Piper, E.K., Jonsson, N.N., Gondro, C., Lew-Tabor, A.E., Moolhuijzen, P., Vance, M.E., Jackson, L. a, 2009. Immunological profiles of Bos taurus and Bos indicus cattle infested with the cattle tick, Rhipicephalus (Boophilus) microplus. Clin. Vaccine Immunol. 16, 1074–86. https://doi.org/10.1128/CVI.00157-09

Piper, E.K., Jonsson, N.N., Gondro, C., Vance, M.E., Lew-Tabor, A., Jackson, L.A., 2017. Peripheral cellular and humoral responses to infestation with the cattle tick Rhipicephalus microplus in Santa Gertrudis cattle. Parasite Immunol. 39, 1–12. https://doi.org/10.1111/pim.12402

Porto Neto, L.R., Jonsson, N.N., D'Occhio, M.J., Barendse, W., 2011. Molecular genetic approaches for identifying the basis of variation in resistance to tick infestation in cattle. Vet. Parasitol. 180, 165–172. https://doi.org/10.1016/j.vetpar.2011.05.048

Regitano, L., Prayaga, K., 2011. Ticks and tick-borne diseases in cattle, in: Bishop, S.C., Axford, R.F.E., Nicholas, F.W., Owen, J.B. (Eds.), Breeding for Disease Resistance in Farm Animals. CABI, St Lucia, pp. 295–314.

Robbertse, L., Richards, S.A., Clift, S.J., Barnard, A.C., Leisewitz, A., Crafford, J.E., Maritz-Olivier, C., 2018. Comparison of the differential regulation of T and B-lymphocyte subsets in the skin and lymph nodes amongst three cattle breeds as potential mediators of immune-resistance to Rhipicephalus microplus. Ticks Tick. Borne. Dis. 9, 976–987. https://doi.org/10.1016/j.ttbdis.2018.03.034

Seifert, G.W., 1971. Variations between and within breeds of cattle in resistance to field infestations of the cattle tick (Boophilus microplus). Aust J Agric Res 22, 159–168. https://doi.org/10.1071/AR9710159

Sercu, S., Zhang, M., Oyama, N., Hansen, U., Ghalbzouri, A.E.L., Jun, G., Geentjens, K., Zhang, L., Merregaert, J.H., 2008. Interaction of extracellular matrix protein 1 with extracellular matrix components: ECM1 is a basement membrane protein of the skin. J. Invest. Dermatol. 128, 1397–1408. https://doi.org/10.1038/sj.jid.5701231

Severo, M.S., 2013. UC Riverside UC Riverside Electronic Theses and Dissertations Author.

Smyth, G.K., 2004. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. 3. https://doi.org/10.2202/1544-6115.1027

Stanley, W.J., Trivedi, P.M., Sutherland, A.P., Thomas, H.E., Gurzov, E.N., 2017. Differential regulation of pro-inflammatory cytokine signalling by protein tyrosine phosphatases in pancreatic β-cells. J. Mol. Endocrinol. 59, 325–337. https://doi.org/10.1530/JME-17-0089

Tabor, A., Ali, A., Rehman, G., Rocha Garcia, G., Zangirolamo, A.F., Malardo, T., Jonsson, N.N., 2017. Cattle Tick Rhipicephalus microplus-Host Interface: A Review of Resistant and Susceptible Host Responses. Front. Cell. Infect. Microbiol. https://doi.org/10.3389/fcimb.2017.00506

Utech, K.B., Seifert, G.W., Wharton, R.H., 1978a. Breeding Australian Illawarra Shorthorn cattle for resistance to Boophilus microplus. I. Factors affecting resistance. Aust. J. Agric. Res. 29, 411–422. https://doi.org/10.1071/AR9780411

Utech, K.B., Wharton, R.H., Kerr, J.D., 1978b. Resistance to Boophilus microplus (Canestrini) in different breeds of cattle. Aust. J. Agric. Res. 29, 885–895. https://doi.org/10.1071/AR9780885

Veríssimo, C., Bechara, G., Mukai, L., 2008. Mast cell counts correlate with Rhipicephalus (Boophilus) microplus tick load in different cattle breeds. Brazilian J. … 1, 81–87.

Wambura, P.N., Gwakisa, P.S., Silayo, R.S., Rugaimukamu, E. a., 1998. Breed-associated resistance to tick infestation in Bos indicus and their crosses with Bos taurus. Vet. Parasitol. 77, 63–70.

Wang, Y.H., Reverter, A., Kemp, D., McWilliam, S., Ingham, A., Davis, C., Moore, R., Lehnert, S., 2007. Gene expression profiling of Hereford Shorthorn cattle following challenge with Boophilus microplus tick larvae. Aust. J. Exp. Agric. 47, 1397–1407. https://doi.org/10.1071/EA07012

Warner, M.S., Geraghty, R.J., Martinez, W.M., Montgomery, R.I., Whitbeck, J.C., Xu, R., Eisenberg, R.J., Cohen, G.H., Spear, P.G., 1998. A cell surface protein with herpesvirus entry activity (Hveb) confers susceptibility to infection by mutants of herpes simplex virus type 1, herpes simplex virus type 2, and pseudorabies virus. Virology 246, 179–189. https://doi.org/10.1006/viro.1998.9218

Watkins, J.F., Sung, P., Prakash, L., Prakash, S., 1993. The Saccharomyces cerevisiae DNA repair gene RAD23 encodes a nuclear protein containing a ubiquitin-like domain required for biological function. Mol. Cell. Biol. 13, 7757–7765. https://doi.org/10.1128/mcb.13.12.7757-7765.1993

Welsh, E.A., Eschrich, S.A., Berglund, A.E., Fenstermacher, D.A., 2013. Iterative rank-order normalization of gene expression microarray data. BMC Bioinformatics 14. https://doi.org/10.1186/1471-2105-14-153

Wikel, S.K., 1996. HOST IMMUNITY TO TICKS. Annu. Rev. Entomol. 41, 1–22.

Yabluchanskiy, A., Ma, Y., Iyer, R.P., Hall, M.E., Lindsey, M.L., 2013. Matrix metalloproteinase-9: Many shades of function in cardiovascular disease. Physiology. https://doi.org/10.1152/physiol.00029.2013

Zhu, L.-L., Zhao, X.-Q., Jiang, C., You, Y., Chen, X.-P., Jiang, Y.-Y., Jia, X.-M., Lin, X., 2013. Article C-Type Lectin Receptors Dectin-3 and Dectin-2 Form a Heterodimeric Pattern-Recognition Receptor for Host Defense against Fungal Infection. https://doi.org/10.1016/j.immuni.2013.05.017

Zhu, Y., Paniccia, A., Schulick, A.C., Chen, W., Koenig, M.R., Byers, J.T., Yao, S., Bevers, S., Edil, B.H., 2016. Identification of CD112R as a novel checkpoint for human T cells. J. Exp. Med. 213, 167–176. https://doi.org/10.1084/jem.20150785

Chapter 5:
Transcriptome analysis of ancient and modern host-tick interactions in South African beef cattle using RNA sequencing

Abstract

Cattle ticks (Acari: Ixodidae) are one of the biggest impediments currently threatening the economic viability of the beef production industry. Chemical acaricides used to control tick burdens are unstainable, costly, and toxic to the environment. The use of tick-resistant indigenous cattle breeds as opposed to susceptible European breeds has been identified as a potential option to combat heavy tick burdens. To develop cost-effective, chemical-free, and sustainable alternative tick control strategies, the fundamental aspects of the molecular mechanisms of host immune responses to tick infestations must be elucidated. This study aimed to profile candidate genes and pathways associated with tick resistance in the Angus, Brahman and Nguni breeds. Bull calves aged between 12 and 15 months were artificially infested with 1000 unfed tick larvae of the species *R. microplus* and *R. decoloratus* and skin biopsy samples were collected on day-10 and day-20 post-infestation. Transcriptome analysis was conducted on the samples following RNA sequencing at a depth of 15 million reads per sample. The differential gene expression and pathway enrichment analysis highlighted the role of genes encoding components of the extracellular matrix with wound healing and tissue remodelling properties together with the participation of pro-inflammatory cytokines. Gene *CCL26* and *MZB1* were significant in the inter-tick species contrast while *MMP9* was highlighted for its importance in the inter-breed contrasts. Distinct differences in gene expression patterns were revealed between the Angus breed and both the Brahman and Nguni breeds. Differences were observed between the Brahman and the Nguni breeds as well. Further research is required to validate the identified genes.

5.1 Introduction

Tick and tick-borne diseases (TTBDs) are a major cause of cattle mortalities, crippled livestock production efficiency, devaluation of hides and the contamination of animal products and the environment with chemical residues from tick control. Ticks have been shown to rapidly evolve to produce strains that can adapt to different host environments by altering their feeding signatures for successful feeding. The observed increased rates of evolution and adaptation in ticks are thought to be facilitated by the loss and gain of genes in the tick's genetic makeup resulting in the reprogramming of gene expression networks (Fournier, 2017). This allows ticks to transition between various cattle breeds, ultimately achieving successful feeding endeavours on breeds with which they have not co-evolved. Due to this fast evolution time, the tick species evolve to produce acaricide-

resistant strains at a rate faster than new acaricides and vaccines can be developed thereby making it difficult to control the tick population below a certain threshold in most regions.

The *Rhipicephalus microplus* (*R. microplus*) is one of the most successful invasive tick species which has shown rapid adaptation to different climatic conditions especially in tropical and sub-tropical regions (Nyangiwe et al., 2013; Horak et al., 2015). Alongside causing economical anxieties in the cattle production sector, the *R. microplus* has also been displacing the indigenous *R. decoloratus* species in some regions (Nyangiwe et al., 2013). These two tick species can be found feeding alongside each other on the same host. During mixed infestations the *R. microplus* will opportunistically mate with the *R. decoloratus* females to create infertile hybrids, consequently interfering with the expansion of the *R. decoloratus* populations (Nyangiwe et al., 2017). The investigation of the gene expression profiles at the tick bite site on the host may be informative in clarifying the tissue-specific transcriptional programs at the host-tick interface.

Tick infestations produce distinguishable breed-specific responses that stem from diverse gene expression profiles. Both innate and adaptive immune responses are involved in the host's attempt to fight off ticks. Tick-induced immunological changes are accompanied by altered gene expression profiles (Wang et al., 2014). Whereas hypersensitivity type II responses are thought to govern adaptive immunity in the presence of tick infestations, inflammatory cytokines and chemokines and components of the major histocompatibility (MHC) class I and II play an important role in the activation and mediation of the innate immune responses. Although not inherited, adaptive immunity is an important contributor to infection resolution and provides the host animal with long term immunological memory to protect against subsequent encounters with the same antigen (Wang et al., 2014). The precise pathogenic contribution of currently known tick resistance genes is poorly understood and their value as targets for gene introgression for the tick resistance component into susceptible breeds has yet to meet expectations. The study of the systems biology, immunology and vaccinology of host immune correlates of protection to tick infestations provides an integrated approach to identifying tick species-specific signatures of immunogenicity and predicting the overall dynamics of ancient and modern host-tick associations (Kuleš et al., 2016). This approach will provide a comprehensive understanding of the different host-tick interactomes and the regulations of the immune system to characterize novel candidate genes and pathways while informing the development of new vaccines.

Several studies have documented gene expression patterns in several breeds by tick species interactions. However, the comparison of the gene expression profiles and immune responses elicited by two closely related tick species is yet to be done. Piper et al., (2008, 2009), Kongsuwan et al., (2008, 2010) and (Wang et al., 2007) identified numerous genes associated with tick resistance in cattle by profiling skin biopsies at the host-tick interface of the cattle skin. (Wang et al., 2007) examined transcription activity in Hereford shorthorn infested with *R. microplus*, while Piper et

al. (2008, 2009) investigated the response of Brahman and Angus to *R. microplus* infestation and Kongsuwan et al. (2008; 2010) dissected the inflammatory responses of Angus cattle to *R. microplus* tick species. Table 5.1 summarises the studies that have been conducted using RNA-seq to investigate the tick resistance phenotype in cattle.

Table 5.1: Summary of gene expression studies using RNA sequencing through Next-generation sequencing to characterize host resistance to ticks in cattle

Breed/Tick species	Aim	Outcomes	Applications	Country
Haemaphysalis longicornis	Investigate the physiological response of the tick to cypermethrin	Detoxification metabolism genes were upregulated in the cypermethrin-tolerant group coupled with the heightened expression of genes encoding CYP450, GST, and ABC transporters	Tick control strategies	South Korea (Moon et al., 2021)
R. microplus	To identify candidates for novel tick control approaches by characterizing the tick salivary gland gene expression in tick-susceptible and tick resistant hosts	Putative targets for infestation control included genes involved in stress response during blood-feeding; Coordinated regulation of a small heat shock protein and negative elongation factor B-like genes along with upregulation of a 26S proteasome subunit and calnexin were identified in ticks fed on tick-susceptible cattle	Tick control strategies	Brazil (Giachetto et al., 2020)
Braford	Identify genetic pathways and transcription factors affecting host resistance to ticks	Members of the Ap1 and NF-kB families were the most relevant transcription factors predicted in both resistant and susceptible animals; Leucocyte chemotaxis was over-represented in both phenotypes and skin degradation and remodelling was over-represented in resistant hosts; activation of the WNT-signaling pathway was identified as a potential candidate mechanism of resistance	Search for candidate genes for tick resistance in bovine	Brazil (Moré et al., 2019)
R. decoloratus	Elucidate the complex mechanism underlying amitraz resistance	A potential relationship exists between the α-adrenergic-like octopamine receptor and ionotropic glutamate receptors in establishing amitraz resistance; it is proposed that in amitraz-susceptible ticks Ca2+ entry into cells is inhibited followed by membrane hyperpolarization which prevents the release of neurotransmitters; Ionotropic glutamate receptors (NMDA and AMPA) prevent this action in amitraz-resistant ticks	Drug development for tick control	South Africa (Baron et al., 2018)
R. microplus fed on Nelore and Holstein calves	Examine the expression profiles of genes encoding secreted tick proteins that mediate parasitism in larvae and nymphs feeding of the two breeds	Transcripts encoding immunomodulatory molecules secreted in the tick saliva were more abundant in ticks fed on tick-susceptible hosts; Resistant hosts expose ticks to an earlier inflammatory response that results in significantly lower expression of genes encoding salivary proteins that suppress host immunity, coagulation, and inflammation	Elucidation of the molecular basis of the differences between tick-susceptible and tick-resistant bovine hosts and the associated modulation of the tick secretome	Brazil (Franzin et al., 2017)

While the severity and ultimate outcome of tick infestations are known, the molecular basis and the genetic mechanisms underlying tick resistance and susceptibility in cattle remains incompletely defined. To understand the genetic mechanisms of tick resistance in cattle it is critical to elucidate the global expression profiles of the different host-tick associations using RNA-sequencing (RNA-seq). Given the limitations and low dynamic range associated with other transcriptome analysis technologies, including microarray analysis and RT-qPCR, RNA-seq has proven more efficient at providing a global picture of the differential expression patterns. The introduction of RNA-seq using next-generation sequencing technology (NGS) has provided a platform to dissect the complex details of tick resistance while addressing the biological heterogeneity of different cattle breeds challenged with various cattle tick species (Colgan et al., 2017). The transcriptional outputs produced by RNA-seq may be instrumental in providing insight into the complex genetic networks mediating host-tick interactions in cattle.

The current study aimed to profile the tick-induced differential gene expression patterns and enriched pathways associated with tick resistance in three cattle breeds; the tick-susceptible Angus (British, Bos taurus) breed; the highly tick-resistant Brahman (Asian Bos indicus) breed; and the moderate to high tick-resistant Nguni (indigenous Bos taurus africanus) breed, following artificial infestation with *R. microplus* and *R. decoloratus* ticks. RNA sequencing was used as an exploratory tool to isolate and define the transcriptional programs at the host-tick interface that may be used to discriminate resistance cattle biotypes from susceptible ones. Given the indiscriminate morphological difference between the closely related *R. microplus* and the *R. decoloratus*, it is hypothesised that their feeding signatures, and consequently the host immune responses they trigger, will not differ.

5.2 Material and methods

5.2.1 The research site, experimental animals, and tick species.

The research site, experimental animals and tick species used in this study were described in detail sections 3.2.1 and 3.2.2 (Chapter 3). Briefly, a group of 30 pure-bred bull calves, made up of 10 Angus (*Bos taurus taurus*), 10 Brahman (*Bos taurus indicus*) and 10 Nguni (*Bos Taurus africanus*) were investigated. The animals were between the ages of 12 and 15 months with prior exposure to both *R. microplus* and *R. decoloratus* tick infestations. Freshly hatched aseptic unfed tick larvae (UFL) of the *R. microplus* and *R. decoloratus* tick species were used for all artificial infestations.

The study was conducted at the Mariendal experimental farm of Stellenbosch University, located 33.8452599419S 18.834721521E in the Western Cape Province of South Africa. The animals were housed individually for the duration of the trial and allowed adlib access to a Custom mix of dairy fibremax pellets with a portion of hay (Nova feeds, Kingswell Boerdery). The pens were fitted with animal-operated taps to supply each animal with an adlib supply of fresh clean water. The concrete

floor in the pens were layered with wood shavings that were changed daily to prevent injuries of the animal due to wet floors.

Ethical clearance was granted by the Stellenbosch University Research Ethics Committee: Animal Care and Use (**REC: ACU Reference #**: ACU-2018-1719) and the South African Department of Agriculture, Forestry and Fisheries (Section 20 permit). Furthermore, the procedures of this study complied with internationally accepted standards for animal welfare and ethics (Austin et al., 2005).

5.2.2 Artificial infestation and tick counting

It has been established that the rate at which the host acquires immunity to tick exposure greatly affects the degree of resistance the host exhibits (Burrow et al., 2019). It takes approximately nine days, the average age of an *R. microplus* or *R. decoloratus* nymph, for a host animal to mount adaptive immune responses (Franzin et al., 2017). Animals with acquired immunity to one tick species, resulting from repeated exposure to a particular tick species, often exhibit a degree of susceptibility to other tick species equal to that of tick-naïve cattle (Burrow et al., 2019).

Therefore, the animals were all subjected to full body infestation cycles with both tick species to allow all animals to mount adaptive immunity against both tick species. The tick counts from the initial full-body infestations were then used to inform the selection of animals used for the two patch infestation cycles. Five hundred milligrams (0.5g), approximately 10 000 UFL, aliquots were placed into small vials and used for the full body infestations infestation. Five animals per breed were infested with *R. microplus* UFL and the other five were infested with *R. decoloratus*. The cattle groups were then exchanged, where those which were challenged with *R. microplus* were infested with *R. decoloratus* and vice versa. Each animal underwent a full-body pre-infestation with 10 000 UFL on two separate occasions. Portions of the UFL were released for feeding on the soft areas of the animal and the rest were emptied on the back (Figure 3.1). The first two infestations were conducted 24 days apart where semi- and fully engorged (length ≥4mm – 8mm) were counted on the whole body of each animal between days 16 to 23 post-infestation (eight counts).

Based on ranking from the first two full-body infestations, a total of 24 animals (4 animals per breed and tick species) were selected consisting of two top-ranking individuals and two bottom ranking individuals. The animals were then subjected to patch infestations where a calico bag was attached to a shaved area on the animal's back and tick released end enclosed in that area to attach and engorge (Figure 5.1). Each animal underwent patch infestation first with approximately 1 000 UFL (0.05g) using the *R. microplus* larvae and then with the *R. decoloratus* larvae, with 14 days of acaricide-free rest in between the two patch infestations. Tick count data were collected on day 20 post-infestation with each of the two tick species, where only fully engorged (≥5mm) female ticks were counted. All animals were treated with a short-acting acaricide (Drastic Deadline, Bayer®) following the last patch infestation.

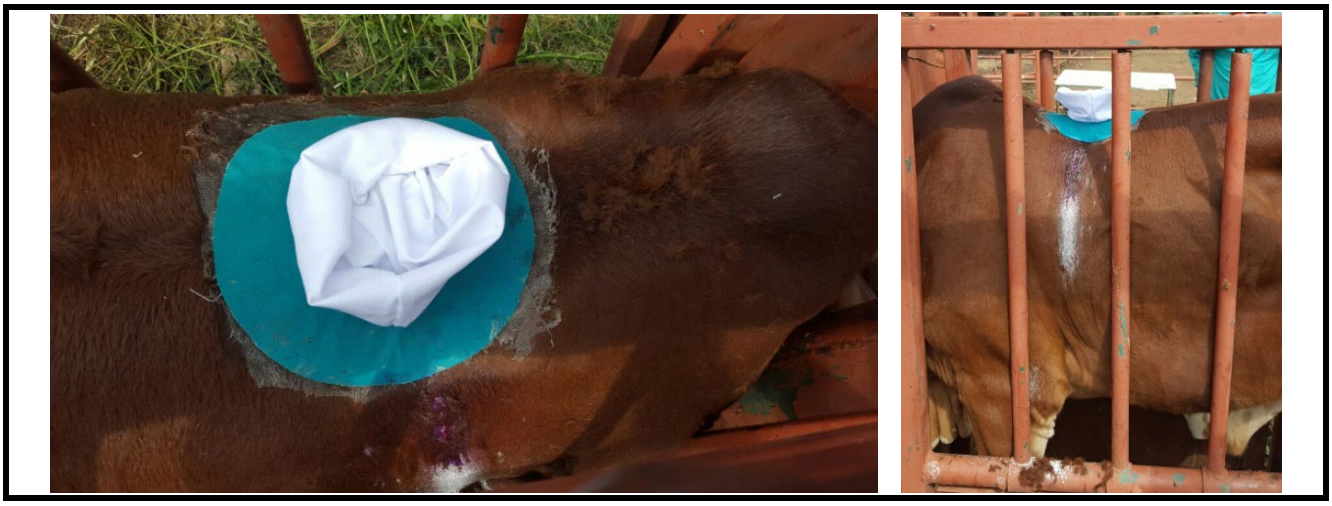


Figure 5.1: Patch infestation, and the calico bag attached to the shaven area on the animal's back for artificial infestation.

5.2.3 Skin biopsy sampling

Using disposable 5mm biopsy punches 9 tissue samples were collected from each animal following patch infestation with each of the tick species: three from non-parasitized skin on the crops area before the patch infestation, three with 10 days post-infestation and another three at 20 days from the feeding of visibly engorging adult female ticks. The diameter on the punch was ideal to capture the area of inflammation from the host-tick interface (Franzin et al., 2017). A local anaesthetic injection, at a dose of 0.1ml/site of 2% lignocaine hydrochloride (Lignocaine®, Bayer, South Africa) was administered subcutaneously to desensitize the area around the biopsy collection site for sampling. Directly from the collection, the biopsies were placed in separate 15ml tissue-protect, RNase- DNase-free tubes (CELLSTAR® tubes, Greiner Bio-One) containing 2ml RNALater solution (ThermoFisher) and stored at -80 °C until processing.

5.2.4 RNA extraction

The RNeasy® Mini kit Quick-Start protocol for RNA purification from tissue samples was followed for isolation of total RNA from the skin biopsies samples. The skin biopsies were removed from the stabilization solution and 30mg of each sample was used for RNA extraction. The skin biopsy samples were each placed in a 2 ml specialized screwcap along with 2 ceramic beads. Using the Geno/Grinder 2010 machine (SPEX SamplePrep, Vacutec), set to run at 1750 rpm for a maximum of 10 min, the samples were macerated, disrupted and the lysate homogenized with 600 µl of Buffer RLT. The homogenized samples were transferred to a new 2 ml Eppendorf tube using a needle and syringe. This was followed by the removal of insoluble material from the homogenate by centrifugation at maximum speed (± 12 000 x g) for 3 min and pipetting of the supernatant. One volume of 70% ethanol was added to the supernatant, mixed by pipetting and 700 µl of the resulting mixed sample was transferred to an RNeasy Mini spin column. The tubes were centrifuged for 15 s at 8 000 x g and the flow-through was discarded.

The RNeasy On-column DNase digestion protocol was followed to perform the removal of genomic DNA contamination. Briefly, 350 µl of Buffer RW1 was added to the RNeasy column and centrifuged at 8 000 x g for 15 s. Then 80 µl of DNase I incubation mix, composed of 10 µl DNase I stock solution and 70 µl Buffer RDD, was added to the column membrane and incubated at room temperature for 15 min. Lastly, 350 µl of Buffer RW1 was added to the column and centrifuged 15 s at 8 000 x g. The RNeasy Mini Kit Quick-Start protocol was further followed to perform total RNA clean-up. This included treating the spin column with 700 µl of Buffer RW1, 700 µl and 500 µl of Buffer RPE with centrifugation at 8 000 x g for 15 s, 15s and 2 min after the addition of every buffer, respectively. The total RNA was eluted with 30 µl of RNase-free water to achieve a higher concentration of total RNA and stored at -80 °C.

5.2.5 Library preparation and RNA sequencing

Library preparation and RNA sequencing were all performed at the Agricultural Research Council's Biotechnology Platform. The library preparation and sequencing protocols are described in detail by (Malatji, 2017) and briefly described here. The TruSeq RNA Library Preparation kit manufacturer's protocol (Illumina, San Diego, CA, USA) was followed to prepare mRNA libraries from 10 µl of total RNA per sample. The ligation of TruSeq adapters to the cDNA fragments was performed followed by PCR to produce the final sequencing libraries. The TruSeq Stranded mRNA preparation kit was used to construct sequencing libraries for the 72 samples. Reverse transcription was performed to synthesize double-stranded cDNA fragments using randomly primed RNA fragments. The quality and quantities of the starting RNA were checked using the Labchip GX Touch Nucleic Acid Analyzer. The generated cDNA was colligated, nebulized, and fragmented, followed by adapter ligation to both ends of the fragmented nucleic acid. The fragments were hybridized to a flow cell that performed bridge amplification, generating thousands of single-stranded DNA copies in preparation for sequencing. Sequencing was performed using the Illumina HiSeq 2500 machine.

5.2.6 Data trimming, filtering and mapping of reads

Data quality control, trimming, filtering and read mapping were done using shell scripts (ssh) on the Mac IOS terminal. The pre-and-post trimming quality of the sequence data was visualized using FastQC software v. 0.11.9 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Adapters were trimmed from the reads and poor-quality reads and bases filtered from the samples using Trimmomatic v. 0.36. (http://www.usadellab.org/cms/uploads/supplementary/Trimmomatic/TrimmomaticManual_V0.32.pdf) (Bolger et al., 2014) and BBDuk function from BBmap v. 37.90 (https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbduk-guide/), following default parameter settings.

Using the Salmon software v1.6.0 (https://salmon.readthedocs.io/en/latest/salmon.html and https://www.hadriengourle.com/tutorials/rna/), the reads generated from the Angus breed grouped were mapped against the *B. taurus* reference genome (GCF_002263795.1_ARS-UCD1.2) and

reads from the Brahman samples were mapped against the Bos indicus reference genome (GCF_000247795.1_Bos_indicus_1.0). Given the lack of a *B. taurus* africanus reference genome, reads from the Nguni breed group were mapped against both the Bos taurus and *Bos indicus* reference genomes to investigate which genome fit the reads best. The samples mapped best against the B. indicus genome and were then used for downstream analysis. Transcript abundance was also quantified using the Salmon tools.

5.2.7 Transcriptome analysis

5.2.7.1 Differential gene expression analysis

Differential expression analysis was performed using DESeq2 Bioconductor package v.3.13. in RStudio (https://www.hadriengourle.com/tutorials/rna/ and https://sbc.shef.ac.uk/ workshops/2020-02-13-rnaseq-r/rna-seq-preprocessing.nb.html#fixing_the_problem_with_ transcript_ names_-_the_hard_way). The nine contrasts that are listed in Table 5.2 were analysed. Read count data was transformed on a $\log_{10}$ scale to compensate for the different library sizes. The significance of the differentially expressed gene was corrected for multiple testing using the Benjamini-Hochberg method. Only genes with a significant p-value ($p < 0.05$) and adjusted p-value (FDR < 0.05) were classified as differentially expressed genes (DEGs). Genes with a $\log_2$ fold change less than one ($\log_2FC < 1$) were classified as downregulated while those with $\log_2$ fold change greater than one ($\log_2FC > 1$). The DEGs were annotated using the NCBI Entrez identifiers.

5.2.7.2 Pathway enrichment analysis

Pathway enrichment analyses were conducted following the methods described in section 4.2.5 of Chapter 4. Briefly, the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/tools.jsp) web-based tool was used to perform gene-annotation enrichment analysis DEGs and pathway over-representation analysis. The probability scores presented in the DAVID over-representation analysis were corrected for false discovery rate (FDR) using the Benjamini-Hochberg method. Functional profiling for the DEGs was performed and classified according to biological, molecular, and cellular processes. However, only the biological processes are discussed in this study.

Table 5.2: Summary of the different contrasts that were used to investigate differential expression across the various host tick associations

Analysis	Abbreviation	Contrast
1	AM vs AD	Angus vs Angus between tick species (*R. microplus* vs *R. decoloratus*)
2	BM vs BD	Brahman vs Brahman between tick species (*R. microplus* vs *R. decoloratus*)
3	ND vs ND	Nguni vs Nguni between tick species (*R. microplus* vs *R. decoloratus*)
4	AM vs BM	Angus vs Brahman post infestation with *R. microplus*
5	AD vs BD	Angus vs Brahman post infestation with *R. decoloratus*
6	AM vs NM	Angus vs Nguni post infestation with *R. microplus*
7	AD vs ND	Angus vs Nguni post infestation with *R. decoloratus*
8	BM vs NM	Brahman vs Nguni post infestation with *R. microplus*
9	BD vs ND	Brahman vs Nguni post infestation with *R. decoloratus*

*Listed in the parentheses are the directions of the contrast

5.3 Results

5.3.1 Tick count data

No significant differences in tick counts were observed between tick species (p = 0.441, df = 1). Tick counts differed significantly between breeds for both tick species (*R. decoloratus*: p =0.005 and *R. microplus*: p = 0.007, df = 2). The Angus cattle, a tick susceptible breed, exhibited significantly higher mean tick counts (*R. decoloratus*: 139.75 ± 33.28, *R. microplus*: 215.5 ± 54.259 ticks per animal) than both the tick resistant Brahman (*R. decoloratus*: 35.25 ± 5.65, *R. microplus*: 53.75 ± 10.778) and Nguni breeds (*R. decoloratus*: 2.25 ± 1.244, *R. microplus*: 1.5 ±0.75) that did not differ significantly from each other.

5.3.2 Differentially expressed genes analyses

The genetic differences underlying the difference observed in tick counts across breeds was addressed using the RNA sequencing of skin biopsy samples post artificial infestation with two tick species. Similar to the microarray analysis presented in Chapter 4, the RNA-seq analysis examined the differential gene expression patterns expressed in the various host-tick associations post-infestation. The following host-tick associations were considered in differential expression analysis: Angus x *R. decoloratus*, Angus x *R. microplus,* Brahman x *R. decoloratus*, Brahman x *R. microplus,* Nguni x *R. decoloratus* & Nguni x *R. microplus*. The contrasts investigated are detailed in Table 5.2.

5.3.2.1 Differentially expressed genes between tick species

Distinct differences in the three inter-tick species comparisons were observed. At a false discovery rate (FDR) < 0.05, a total of 51, 9, and 6 DEGs were found in the AM vs AD, BM vs BD and NM vs ND contrasts, respectively. Of these 45, 5, and 3 genes, respectively, were annotated (Table 5.3). Considering the AM vs AD, the *CCL26* gene was the only DEG that was downregulated in the Angus x *R. decoloratus* association as compared to the Angus x *R. microplus* animals. The gene had a log_2FC of -1.721 and a baseMean of 2033.966, notably higher than any of the DEGs within this contrast. The rest of the genes within this contrast were upregulated. From the total annotated DEGs in the BM vs BD contrast, two were upregulated and three were downregulated, with genes *ASB2* and *PBX4* possessing the highest baseMean estimates of 323.696 and 265.223, respectively. The NM vs ND contrast produced three annotated DEGs, all of which were upregulated in the Nguni x R. microplus. Notably, *MZB1* had the highest baseMean estimate of 1688.62 and was upregulated in the Nguni x *R. decoloratus* host-tick association. None of the DEGs were commonly observed across the different contrast.

Table 5.3: Annotated significant DEGs found in the Inter tick-species comparisons within the breed. A negative log_2FC means that the gene was down-regulated in the latter host-tick association of the contrast (i.e. the Angus x *R. decoloratus,* Brahman x *R. decoloratus,* Nguni x *R. decoloratus*).

Gene ID	Gene Description	baseMean	log_2 FC	FDR
Angus*_R. microplus_ vs Angus*_R. decoloratus_				
ACTBL2	Actin beta like 2	13.434	7.765	0.026
APCDD1	APC Down-Regulated 1	409.88	1.935	0.036
BICDL2	BICD Family Like Cargo Adaptor 2	63	4.486	0.003
BNC1	Basonuclin 1	23.503	4.374	0.021
BNIPL	BCL2 Interacting Protein Like	109.275	3.654	0.004
C23H6orf132	Chromosome 23 C6orf (open reading frame) 132 homolog	200.164	2.187	0.002
CCL26	**C-C Motif Chemokine Ligand 26**	**2033.966**	**-1.721**	**0.019**
DUSP2	Dual Specificity Phosphatase 2	685.388	2.199	0.041
EPHA1	Epiplakin 1	96.727	2.981	0.019
EPHB6	EPH Receptor B6	59.039	3.112	0.038
EPPK1	Epiplakin 1	85.514	2.468	0.012
ESRP1	Epithelial splicing regulatory protein 1	134.433	3.771	0.013
FAM83G	Family With Sequence Similarity 83 Member G	205.996	2.247	0.033
FOXE1	Forkhead Box E1	30.152	8.158	0.002
FUT2	Fucosyltransferase 2	14.563	7.777	0.041
FZD10	Frizzled Class Receptor 10	214.965	2.458	0.017
GJB6	Gap Junction Protein Beta 6	129.336	5.89	0.012
GNMT	Glycine N-Methyltransferase	55.023	4.465	0.037
GOLGA7B	Golgin subfamily A member 7B	129.957	4.181	0.001
GPR143	G Protein-Coupled Receptor 143	30.985	5.374	0.006
GRHL1	Grainyhead Like Transcription Factor 1	150.467	2.722	0.039

HPDL	4-Hydroxyphenylpyruvate Dioxygenase Like	35.76	4.628	0.026
HR	HR Lysine Demethylase and Nuclear Receptor Corepressor	454.346	3.233	0.001
ID1	Inhibitor Of DNA Binding 1, HLH Protein	789.024	1.477	0.039
IGSF9	Immunoglobulin Superfamily Member 9	27.897	3.936	0.049
IRF6	Interferon Regulatory Factor 6	56.191	4.325	0.010
KRT80	Keratin 80	122.81	3.548	0.021
MC1R	Melanocortin 1 Receptor	62.928	4,.878	0.002
MPZL2	Myelin protein zero-like protein 2	152.568	2.257	0,041
MSX2	Muscle Segment Homeobox (MSH homeobox 2)	121.583	3.906	0.036
MYH14	Myosin Heavy Chain 14	210.616	3.308	0.000
NTS	Neurotensin	51.652	4.651	0.014
PADI3	Peptidyl Arginine Deiminase 3	243.297	3.286	0.008
PLCH2	Phospholipase C Eta 2	51.825	4.139	0.031
PMEL	Premelanosome Protein	644.289	4.629	0.010
RAB15	RAB15, Member RAS Oncogene Family	181.935	2.178	0.039
RBBP8NL	RBBP8 N-Terminal Like	35.648	5.752	0.016
SLC24A4	Solute Carrier Family 24 (Sodium/Potassium/ Calcium Exchanger), Member 4	24.933	9.018	0.000
SLC24A5	Solute Carrier Family 24 (Sodium/Potassium/ Calcium Exchanger), Member 5	82.64	10.687	0.002
TFAP2C	Transcription factor AP-2 gamma	29.437	4.683	0.021
TP63	Tumor Protein p63	89.921	4.617	0.003
TRPM1	Transient Receptor Potential Cation Channel Subfamily M Member 1	20.926	6.912	0.038
TSPAN10	Tetraspanin 10	10.759	7.49	0.047
TYRP1	Tyrosinase Related Protein 1	368.644	7.608	0.016
WNT3	Wnt Family Member 3	37.838	6.081	0.003
	Brahman*_R. microplus_ vs Brahman*_R. decoloratus_			
ALPK2	Alpha Kinase 2	23.435	2.097	0.029
AMPD3	Adenosine Monophosphate Deaminase 3	323.696	-1.255	0.029
ASB2	Ankyrin Repeat and SOCS Box Containing 2	265.223	-1.341	0.017
PBX4	PBX Homeobox 4	20.928	-2.742	0.027
SST	Somatostatin	12.453	4.775	0.017
	Nguni*_R. microplus_ vs Nguni*_R. decoloratus_			
IBSP	Integrin Binding Sialoprotein	31.579	7.173	0.020
MZB1	**Marginal Zone B And B1 Cell Specific Protein**	**1688.62**	**5.334**	**0.044**
TNFRSF11B	TNF Receptor Superfamily Member 11b	153.002	5.083	0.001

*FDR = False Discovery Rate, the adjected p-value using the Benjamini-Hochberg method
*Significant based of p-value < 0.05 and FDR < 0.05

5.3.2.2 Differentially expressed genes between breeds

Using the inter-breed contrasts within the different tick species, this study investigated the breed-specific differential gene expression patterns. The highest number of significant DEGs was found in the contrast between the Brahman and Nguni breeds infested with the *R. microplus* ticks (1409

genes; FDR < 0.05) and the least amount found in the Brahman and Nguni contrast within the *R. decoloratus* species (Table 5.4).

Table 5.4: Inter-breed comparisons of DEGs within tick species

Contrast	Total DEGs (FDR < 0.10)	Total DEGs (FDR <0.05)	Upregulated (FDR < 0.10)	Downregulated (FDR < 0.10)
Angus x *R. microplus* vs Brahman x *R. microplus*	589	432	208	381
Angus x *R. decoloratus* vs Brahman x *R. decoloratus*	241	160	140	101
Angus x *R. microplus* vs Nguni x *R. microplus*	587	414	400	187
Angus x *R. decoloratus* vs Nguni x *R. decoloratus*	570	332	337	233
Brahman x *R. microplus* vs Nguni x *R. microplus*	1846	1409	1276	570
Brahman x *R. decoloratus* vs Nguni x *R. decoloratus*	16	12	16	0

Figure 5.2 shows the intersections between the DEGs found in the different inter-breed contrasts within the *R. microplus* (Venn diagram of the left) and the *R. decoloratus* (Venn diagram on the right) tick species. A total of 21 DEGs, 12 of which are annotated, were found to be commonly significantly expressed across all inter-breed contrasts within the *R. microplus* species (Table 5.5). Three of these genes (*KRT80, GRHL1* and *TFAP2C*) also appeared as DEGs in the AM vs AD inter-tick species contrast and were all upregulated in the Angus x *R. decoloratus* host-tick association. Conversely, none of the DEGs were shared across all three contrasts within the *R. decoloratus* species.

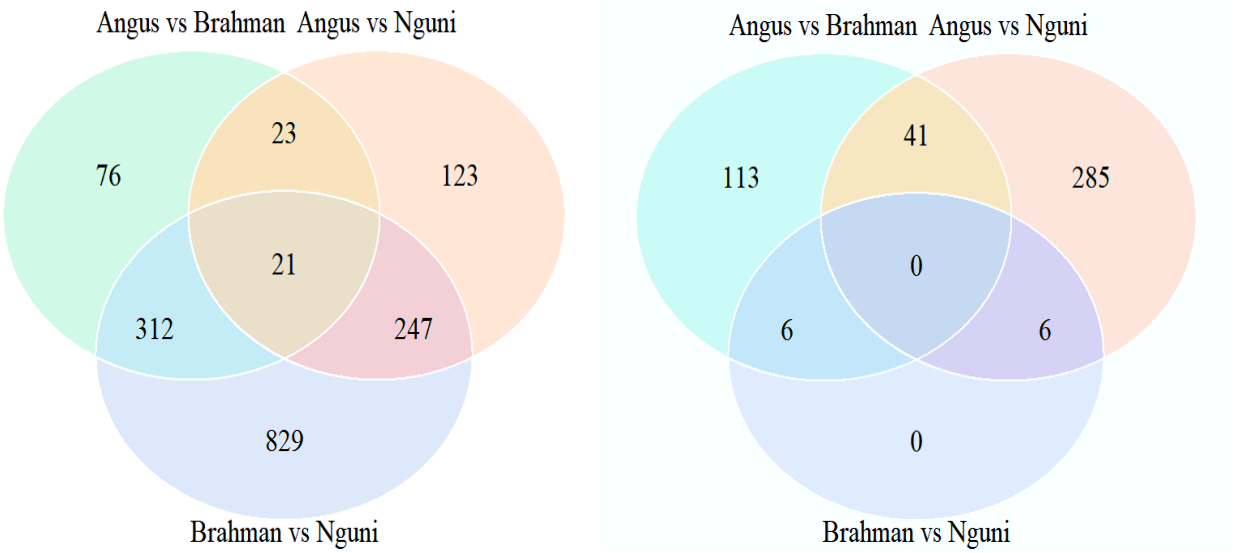


Figure 5.2 On the left: Venn diagram representing the intersection between the different breeds within the *R. microplus* tick species (analyses 4, 6 and 8 from Table 5.2). On the right: Venn diagram of the intersection of the different breed contrasts within the *R. decoloratus* species (analyses 5, 7 and 9 from Table 5.2).

Table 5.5: List of the 12 annotated genes of 21 found commonly significantly differentially expressed across the inter-breed contrasts in association with *R. microplus.*

Gene ID	Gene Description	Gene Function	Angus vs Brahman		Angus vs Nguni		Brahman vs Nguni	
			Log_2 FC	FDR	Log_2 FC	FDR	Log_2 FC	FDR
ATG9B	Autophagy Related 9B	Autophagy and cytoplasm to vacuole transport (Cvt) vesicle formation; Organization of the autophagosomal structure/ phagophore assembly site (PAS), the nucleating site for the formation of the sequestering vesicle.	-3.523	0.002	4.089	0.016	7.625	5.746e-10
CLIC3	Chloride Intracellular Channel 3	Regulate fundamental cellular processes including stabilization of cell membrane potential, transepithelial transport, maintenance of intracellular pH, and regulation of cell volume; Stimulates chloride ion channel activity.	-3.001	0.004	4.385	0.010	7.441	1.123e-10
DMKN	Dermokine	Soluble regulator of keratinocyte differentiation that is upregulated in inflammatory diseases and was first observed as expressed in the differentiated layers of skin. The most interesting aspect of this gene is the differential use of promoters and terminators to generate isoforms with unique cellular distributions and domain components.	-3.135	0.001	6.473	0.034	9.645	8.074e-07
ESRP2	Epithelial Splicing Regulatory Protein 2	Regulates the formation of epithelial cell-specific isoforms. Specifically regulates the expression of *FGFR2-IIIb*, an epithelial cell-specific isoform of *FGFR2*. Also regulates the splicing of *CD44, CTNND1, ENAH*, 3 transcripts that change in splicing during the epithelial-to-mesenchymal transition (EMT).	-4.049	0.003	3.821	0.047	7.871	5.717e-09
GRHL1*	Grainyhead Like Transcription Factor 1	Functions as a transcription factor during development.	-3.199	9.443e-05	3.322	0.047	6.527	3.255e-12
GSDMA	Gasdermin A	Constitutes the precursor of the pore-forming protein, that homopolymerizes within the membrane triggering inflammatory-	-4.432	5.125e-07	4.079	0.0168	8.553	1.746e-05

		mediated cell death (pyroptosis) and causing membrane permeabilization.						
KRT80*	Keratin 80	Responsible for the structural integrity of epithelial cells and are subdivided into epithelial keratins and hair keratins; Involved in cell differentiation, localizing near desmosomal plaques in earlier stages of differentiation but then dispersing throughout the cytoplasm in terminally differentiating cells.	-3.790	3.716e-05	4.610	0.006	8.392	1.946e-14
MMP12	**Matrix Metallopeptidase 12**	Involved in tissue injury and remodelling, displaying significant elastolytic activity. Capable of degrading several extracellular molecules and many bioactive molecules.	**2.057**	**0.033**	**4.651**	**6.400e-10**	**2.625**	**0.0005**
PENK	Proenkephalin	Encodes a preproprotein that is proteolytically processed to generate multiple protein products, including the pentapeptide opioids Met-enkephalin and Leu-enkephalin, which are stored in synaptic vesicles, then released into the synapse where they bind to mu- and delta-opioid receptors to modulate the perception of pain.	-4.246	1.291e-05	4.505	0.017	8.940	0.0006
PRSS8	Serine Protease 8	Encodes preproprotein that are proteolytically processed to generate light and heavy chains that associate via a disulfide bond to form the heterodimeric enzyme. Encoded protease partially mediates proteolytic activation of the epithelial sodium channel, a regulator of sodium balance, and may also play a role in epithelial barrier formation.	-4.358	0.004	4.240	0.034	8.596	3.418e-09
TFAP2C*	Transcription factor AP-2 gamma	Interacts with inducible viral and cellular enhancer elements to regulate the transcription of selected genes of important biological functions.	-4.528	0.001	4.249	0.013	8.790	4.639e-12
TMEM54	Transmembrane Protein 54		-3.608	0.001	3.820	0.031	7.409	2.807e-09

*Genes that were commonly differentially expressed in the inter-tick species contrasts within the Angus breed

***The gene functional descriptions were provided by the Genecards website (Weizmann Institute of Science., 2021) and cross-referenced with the NCBI gene annotation website** (NCBI-Refseq, 2020)

Of the 12 annotated genes that were shared across the different contrast, all but one were underexpressed in the AM vs BM contrast. The *MMP12* gene was upregulated in all the host tick associations and notably in the AM vs BM contrast, favouring the Brahman x *R. microplus* association. The other genes were all upregulated in both other contrasts, favouring the Nguni x *R. microplus* in both the AM vs NM and BM vs NM contrasts. *MMP12* was upregulated at a higher level (log_2FC = 4.651, FDR = 6.400e-10) in the AM vs NM contrast in comparison to the other two contrasts. In addition, it was upregulated at a relatively lower level in the BM vs NM contrast as compared to the other 11 genes.

5.3.3 Gene functional ontologies (Enrichment analysis)

The pathway enrichment analyses were conducted using the DEGs found in the different inter-breed contrasts. The results displayed in Figure 5.3 are those of the ontology enrichment analysis at the first level of the ontology tree produced by the DAVID online platform. The DEGs retrieved from the inter-tick species analysis were not sufficient to enrich any of the pathways of interest using DAVID. Similarly, the DEGs generated from the different inter-breed contrasts containing the *R. decoloratus* species did not significantly enrich any functional gene ontology (GO) terms, except in the contrasts between the Angus and the Nguni breeds. This contrast produced DEGs that were associated with a larger number of GO terms in the *R. decoloratus* containing contrast than in the *R. microplus* containing contrasts. An overlap was observed in the GO terms that were significantly enriched by the DEGs expressed in the various inter-breed contrasts. The overlap was especially apparent in the GO terms associated with the cellular processes category. Biological processes that were significantly enriched across all inter-breed contrasts included single organism processes, biological adhesion, and developmental process. The single organism process GO term was enriched by the largest number of DEGs in comparison to the other significantly enriched GO terms. The immune system process and response to stimulus terms were enriched by DEGs produced in the AM vs NM and BM vs NM contrasts. In the AD vs ND contrast, the immune system process term was enriched by 52 genes consisting of a mixture of chemokines, cytokines, and toll-like receptors. In the BD vs ND contrast, 117 genes enriched the same GO term. The list of DEGs consisted of a myriad of chemokines, cytokines, and toll-like receptors. It was then possible to conclude that DEGs presented in the contrast involving the Nguni breed were largely components of the immune system responses.

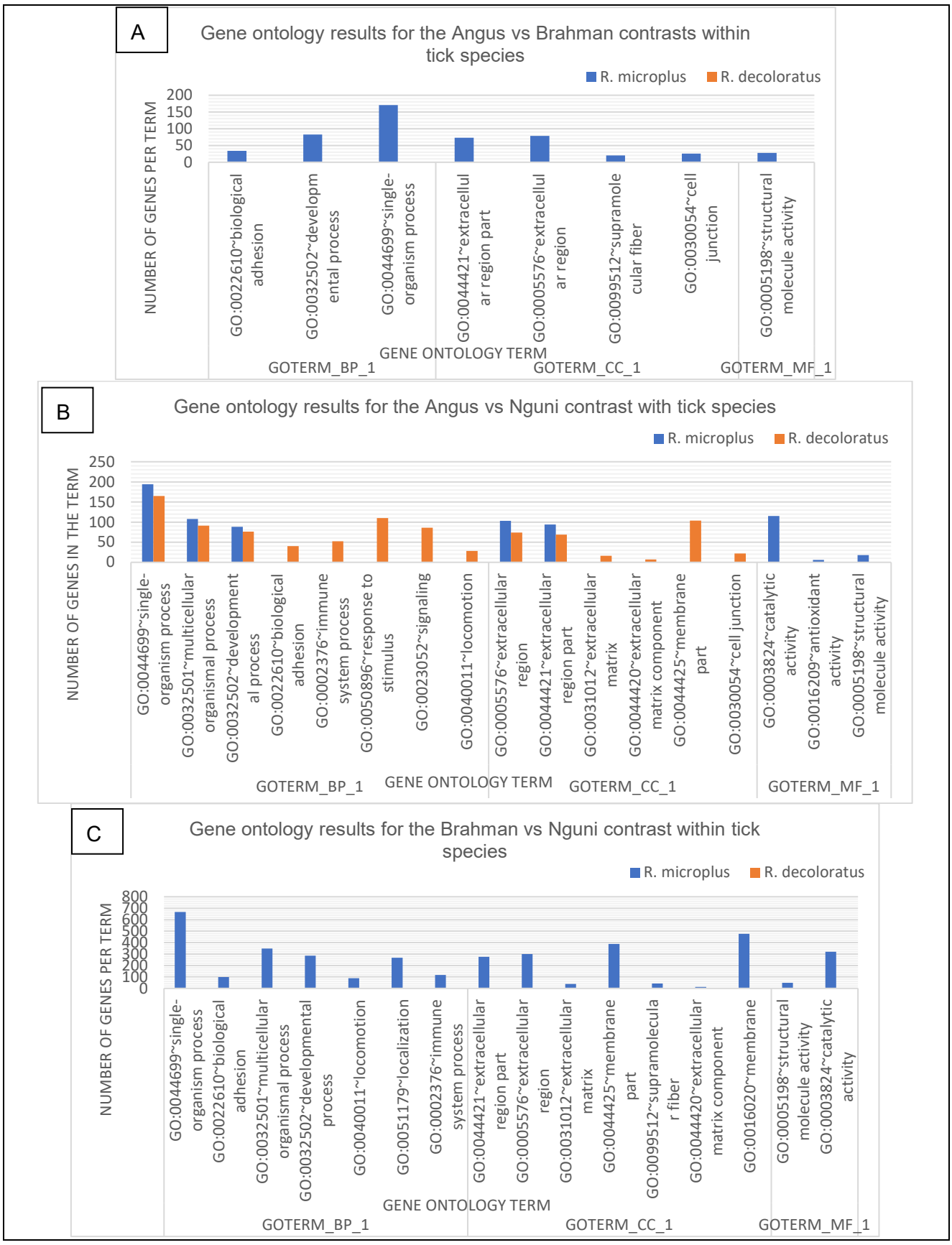


Figure 5.3: Functional ontology results, at the first level on the ontology tree, for the inter-breed comparisons within the *R. microplus* and *R. decoloratus* tick species. The contrast between the functional terms enriched by the DEGs that were found in the A) Angus vs Brahman contrast, B) Angus vs Nguni contrast and C) Brahman vs Nguni contrast. Only the gene ontology terms with a false discovery rate of less than 0.05 were plotted in the figures. BP refers to Biological Processes, CC refers to Cellular Components and MF refers to Molecular Functions.

5.4 Discussion

5.4.1 Tick count data

The *R. microplus* and the *R. decoloratus* have shared morphological characteristics and have been found to display great similarities in their feeding signatures (Walker et al., 2000; Barker and Walker, 2014). The two tick species have been found to coexist in certain areas and were even found to co-feed on the same host (Kopp et al., 2009). Therefore, it was expected that tick burdens inflicted by these species would not differ, thereby supporting the findings of this study.

According to Kaiser et al., (1982) and Rechav et al. (1990), breeds that display resistance to one tick species often exhibits the same level of resistance to other tick species. The *B. indicus breed* and *B.t africanus* breeds have been shown to on average be more resistant to ticks than *B. taurus* breeds. The results of the current study were partially consistent with the findings of Marima et al. (2020) who observed significant differences in tick counts between the Brahman and the Angus breeds and no difference in tick count between tick species. In similar previous studies, the Brahman displayed 99% resistance to the *R. microplus* following consecutive infestations with the *R. microplus* ticks (Frisch and O'Neill, 1998; Wambura et al., 1998; Shyma et al., 2013). The difference in tick counts is thought to be a consequence of different gene expression patterns presented in the different breeds that lead to the enrichment of various pathways, thereby making some breeds more resistant to ticks than others.

5.4.2 Differential expression analyses

5.4.2.1 Between tick species differences

The co-evolutionary history between the host and tick species has been shown to result in the host animal developing immuno-genetic responses against the specific tick species (Kazimírová et al., 2017). The natural acquisition of these genes, however, does not always offer cross-protection against multiple tick species. Given that the animals in this study were not naïve to any of the two tick species studied, it was expected that components of both the innate and adaptive immune response would be observed, as is characteristic of re-infested animals (Constantinoiu et al., 2010; Piper et al., 2010; Jonsson et al., 2014; Moré et al., 2019). Although there were no significant differences noticed in the tick counts between the *R. microplus* and *R. decoloratus* tick species, analyses of the DEGs expressed in the inter-tick species contrasts were conducted. With the observed range expansion of the *R. microplus* and the anticipated complete displacement of the indigenous *R. decoloratus* by the alien invasive *R. microplus* species (Nyangiwe et al., 2017, 2013), it was deemed necessary to characterize the differences in the mechanisms employed by the host animals during encounters with each of the tick species individually.

Most of the DEGs found in the inter-tick species contrast encode proteins with functions in regulating transcriptional activities, cytokinesis, and cell differentiation, while some have been found to mediate signal transduction to influence cell adhesion and migration. The proinflammatory cytokine *CCL26* functions as a chemoattractant for basophils and eosinophils resulting in the transendothelial migration of these cell subsets to the site of atopic infections and mounting cell-mediated inflammatory responses (Piper et al., 2008; NCBI-Refseq, 2020). This gene induces cell chemotaxis by acting as a ligand for *CX3C* chemokine receptor *CX3CR1* (Wang et al., 2007; NCBI-Refseq, 2020). Cytokines are secreted proteins that play a vital role in immunoregulation and inflammatory responses (Wang et al., 2007; Piper et al., 2008). Cytokines and chemokines are especially crucial in orchestrating wound-healing events at the tick bite site (Kazimírová et al., 2017). In their study on Holstein-Friesian and Brahman cattle, Piper et al. (2008) reported significant differences in the expression of the *CCL26* gene between breeds, sampling site and attachment site. A significant interaction between breed and attachment site was also observed. Upregulation of this gene at the attachment site in the Holstein-Friesian cattle post artificial infestation with the *R. microplus* species was observed. Wang et al. (2007) conversely, reported the downregulation of the gene in Hereford Shorthorn cattle post infestation, describing it as “the end of local inflammatory reactions”. However, this gene was not detected as a DEG in some of the more recent studies using Gir × Holstein (Carvalho et al., 2014), Braford (Moré et al., 2019) and Nelore and Holstein breeds (Franzin et al., 2017). Therefore, this transcript requires further validation. The results presented in this study suggest that there may be different bioactive molecules secreted in the saliva of the *R. decoloratus* that inhibit the activity of the *CCL26* gene and restrict inflammatory responses driven by eosinophil and basophil activity, as was observed the Angus.

Also of interest was the differential expression of the *MZB1* gene. This hormone-regulated proinflammatory cytokine has been implicated in causing chronic inflammation in diabetes patients (Bonfoco et al., 2001). The Biological processes associated with the *MZB1* gene include regulation of apoptosis, peripheral B-cell diversification through the regulation of the (Ca^{2+}) stores, antibody secretion and integrin activation as well as promoting immunoglobulin M (IgM) assembly and secretion through associations formed with IgM heavy and light chains (NCBI-Refseq., 2021). Therefore, the upregulation of this gene in the Nguni x *R. decoloratus* association indicates the presence of an inflammatory reaction in the host in response to the biting tick. The gene has not been reported in previous studies investigating host resistance to ticks. Therefore, it would also need further investigations and validations in other populations.

5.4.2.2 Between breed differences

The genetic differences in tick resistance across breeds have been immensely studied, yet little is understood. It is currently understood that the *Bos indicus*, *Zebu* and *Sanga* breeds, which are better adapted to the harsh environmental conditions of the tropical and subtropical regions, often

experience lighter tick burdens as opposed to their *Bos taurus* counterparts. However, the biological mechanisms governing these differences are yet to be elucidated.

Tick feed by injecting saliva containing a cocktail of bioactive molecules in the skin of the host to induce immunosuppressive mechanisms (Kazimírová et al., 2017). To combat the effects of these molecules the host animals will often mount cell-mediated inflammatory responses characterized by the recruitment and mobilization of circulating immune cells and tissue repair at the tick bite site (Franzin et al., 2017; Kazimírová et al., 2017; Nuttall, 2017). The gradual development of cutaneous reactions has been implicated in the observed vulnerability of tick-susceptible hosts to tick infestations. Although tick susceptible hosts have been shown to present more transcripts that encode enzymes responsible for tissue detoxification, it was also found that the same enzyme often produces semiochemicals that render susceptible hosts more attractive to ticks (Franzin et al., 2017). On the contrary, the intense formation of epidermal lesions, spongiosis, microvesicles, vesicle and bullae has been implicated in the mounting of robust tick-resistance in tick-resistant cattle (Franzin et al., 2017).

The 12 DEGs identified in this part of the study had biological functions that could be correlated to inflammatory mechanisms and the regulation of cellular processes mounted against the biting ticks. The DEGs are involved in functions ranging across the formation and differentiation of epithelial cell-specific isoforms (*DMKN, ESPRP2, GRHL1, KRT80*), monitoring the structural integrity of epithelial cells (*KRT80*), cell chemotaxis to the site of infection (*CLIC3*), pyroptosis (*GSDMA*), injury and tissue remodelling (*MMP12*), extracellular matrix degradation and remodelling (*MMP12*, *PRSS8*), and modulation of the perception of pain (*PENK*). The skin is the first line of defence against ticks and cutaneous immune cells play an important role in initiating, controlling and sustaining responses that may discourage tick feeding (Wikel, 1999; Kazimírová et al., 2017). Therefore, it was not surprising to see that genes that were commonly expressed across the different contrasts encoded elements involved in cutaneous immune response regulation.

Interestingly, *MMP12* was one of the genes that were notably differentially expressed in the Brahman and Nguni breeds and more so in the Nguni breed when contrasted with the Brahman. The MMP12 gene encodes proteins that are capable of degrading the extracellular matrix (ECM) molecules and many bioactive molecules as well as tissue remodelling in normal physiological processes (Weizmann Institute of Science., 2021). The Matrix Metallopeptidase (*MMP*) genes have been implicated in the regulation of immune responses against tick infestations. In their study, Moré et al. (2019) described the functions of the *MMP1* and MMP13 on tissue remodelling. The overexpression of the *MMP9* gene was found in tick-resistant Braford animals suggesting the presence of enriched connective tissue degradation and tissue remodelling following ticks attachment (Moré et al., 2019). The upregulation of this gene in the tick resistant Brahman and Nguni breeds suggests the presence of robust mechanisms that are not in core elements of the immune responses mount but rather

components of the ECM. Supporting the expression of the *MMP12* genes was the simultaneous upregulation of the *ESRP2* and *DMKN. ESPRP2* specifically regulates the expression of fibroblast growth factor receptor 2 (FGFR2). The *FGFR2* gene is essential in the proliferative phase of wound healing to facilitate fibroblast growth (Kazimírová et al., 2017). *DMKN* regulates keratinocyte differentiation and is often found to be upregulated in the differentiated layers of skin in inflammatory diseases (Weizmann Institute of Science., 2021). These findings correlated with those of a previous study where the *Lumican* gene (*LUM*), a gene that encodes constituents of the extracellular matrix, was significantly upregulated in the Nguni and Brahman breeds (Marima et al., 2020). Therefore, it was evident that one of the main factors distinguishing tick resistant hosts from susceptible hosts may be the differential regulation of the immune-biology mechanisms employed to impair tick attachment and feeding.

5.4.3 Functional enrichment analysis

Consistent with the results of the different gene expression analyses, the functional enrichment analysis allowed the identification of cellular component processes associated with the extracellular matrix region and components. *MMP12* was one of the genes enriching these GO terms. The MMPs formed part of the genes that enriched processes of development of the cardiovascular system, cellular movement, haematological system development and function, cell-To-cell signalling and interaction, and tissue morphology. Upon further investigation, Moré et al. (2019) reported the involvement of *MMP1* in one of the most relevant immune-response regulation pathways enriched in the tick susceptible Braford cattle. Franzin et al. (2017) also reported the production of *MMP13* and *MMP14* by members of the *MyD88* dependent toll-like receptor signally pathway. This pathway was one of the GO processes that were significantly enriched by the DEGs found between the Nelore and Holstein cattle infested with *R. microplus* ticks. The results of this study also corresponded with the results presented in Chapter 4, where the enrichment of the response to stimuli GO term suggested the continued attempts by the host's biological process to sustain the integrity of the epidermal barrier by mounting strong adaptive immune responses (Kongsuwan et al., 2010).

5.5 Conclusions

This study provided a new list of candidate genes and pathways abundantly expressed at the host-tick interface which may explain how the host immune responses function collaboratively to regulate host-tick interactions. Differences between the Nguni and the Angus breeds were presented at various stages of this study. Unexpectedly, differences were also observed between the Nguni and Brahman breeds, with the largest number of DEGs resulting from the contrast involving the two breeds. While it is assumed that components of the immune system responses characterize the between-breed differences in host resistance to ticks, this study confirmed the presence of constituents of the extracellular matrix and cutaneous inflammatory reactions. Further studies are

required to validate the participation of *CCL26, MZB1* and *MMP12* in conferring host resistance to ticks in the bovine species. It was also evidenced that although the *R. microplus* and *R. decoloratus* possess morphological similarity and display similar feeding signatures, there may be bioactive molecules in their saliva that set the *R. microplus* apart from the *R. decoloratus*, thereby enabling it to successfully displace the *R. decoloratus* in most regions. Furthermore, there was an indication that the existence of a co-evolutionary history between tick species and breed may offer cross-protection to the host against multiple tick species.

5.6 References

Austin, E.J.., Deary, I.J.., Edwards-Jones, G., Arey, D., 2005. Attitudes to Farm Animal Welfare: Factor Structure and Personality Correlates in Farmers and Agriculture Students. J. Individ. Differ. 26, 107–120.

Barker, S.C., Walker, A.R., 2014. Ticks of Australia. The species that infest domestic animals and humans, Zootaxa. https://doi.org/10.11646/zootaxa.3816.1.1

Baron, S., Barrero, R.A., Black, M., Bellgard, M.I., van Dalen, E., Maritz-Olivier, C., 2018. Differentially expressed genes in response to amitraz treatment suggests a proposed model of resistance to amitraz in R. decoloratus ticks. Int. J. Parasitol. Drugs Drug Resist. 8, 361–371. https://doi.org/10.1016/j.ijpddr.2018.06.005

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Bonfoco, E., Li, E., Kolbinger, F., Cooper, N.R., 2001. Characterization of a Novel Proapoptotic Caspase-2- and Caspase-9-binding Protein. J. Biol. Chem. 276, 29242–29250. https://doi.org/10.1074/jbc.M100684200

Burrow, H.M., Mans, B.J., Cardoso, F.F., Birkett, M.A., Kotze, A.C., Hayes, B.J., Mapholi, N.O., Dzama, K., Marufu, M.C., Githaka, N.W., Djikeng, A., 2019. Towards a new phenotype for tick resistance in beef and dairy cattle: a review. Anim. Prod. Sci. 59, 1401–1427. https://doi.org/10.1071/AN18487

Carvalho, W.A., Domingues, R., de Azevedo Prata, M.C., da Silva, M.V.G.B., de Oliveira, G.C., Guimarães, S.E.F., Machado, M.A., 2014. Microarray analysis of tick-infested skin in resistant and susceptible cattle confirms the role of inflammatory pathways in immune activation and larval rejection. Vet. Parasitol. 205, 307–317. https://doi.org/10.1016/j.vetpar.2014.07.018

Colgan, A.M., Cameron, A.D., Kröger, C., 2017. If it transcribes, we can sequence it: mining the complexities of host–pathogen–environment interactions using RNA-seq. Curr. Opin. Microbiol. 36, 37–46. https://doi.org/10.1016/j.mib.2017.01.010

Constantinoiu, C.C., Jackson, L. a., Jorgensen, W.K., Lew-Tabor, a. E., Piper, E.K., Mayer, D.G., Venus, B., Jonsson, N.N., 2010. Local immune response against larvae of Rhipicephalus (Boophilus) microplus in Bos taurus indicus and Bos taurus taurus cattle. Int. J. Parasitol. 40,

865–875. https://doi.org/10.1016/j.ijpara.2010.01.004

Fournier, P.-E., 2017. Genomics of Rickettsia species, in: One Health 9th Tick and Tick-Borne Pathogen Conference & 1st Asia Pacific Rickettsia Conference. Cairnes, Australia, p. 6.

Franzin, A.M., Maruyama, S.R., Garcia, G.R., Oliveira, R.P., Ribeiro, J.M.C., Bishop, R., Maia, A.A.M., Moré, D.D., Ferreira, B.R., De Miranda Santos, I.K.F., 2017. Immune and biochemical responses in skin differ between bovine hosts genetically susceptible and resistant to the cattle tick Rhipicephalus microplus. Parasites and Vectors 10, 1–24. https://doi.org/10.1186/s13071-016-1945-z

Frisch, J.E., O'Neill, C.J., 1998. Comparative evaluation of beef cattle breeds of African, European and Indian origins. 2. Resistance to cattle ticks and gastrointestinal nematodes. Anim. Sci. 67, 39–48. https://doi.org/10.1017/S1357729800009772

Giachetto, P.F., Cunha, R.C., Nhani, A.J., Garcia, M.V., Ferro, J.A., Andreotti, R., 2020. Gene Expression in the Salivary Gland of Rhipicephalus (Boophilus) microplus Fed on Tick-Susceptible and Tick-Resistant Hosts. Front. Cell. Infect. Microbiol. | www.frontiersin.org 9, 477. https://doi.org/10.3389/fcimb.2019.00477

Horak, I.G., Jordaan, A.J., Nel, P.J., van Heerden, J., Heyne, H., van Dalen, E.M., 2015. Distribution of endemic and introduced tick species in Free State Province, South Africa. J. S. Afr. Vet. Assoc. 86, 1–9. https://doi.org/10.4102/jsava.v86i1.1255

Jonsson, N.N., Piper, E.K., Constantinoiu, C.C., 2014. Host resistance in cattle to infestation with the cattle tick Rhipicephalus microplus. Parasite Immunol. 36, 553–559. https://doi.org/10.1111/pim.12140

Kaiser, M.N., Sutherst, R.W., Bourne, A.S., 1982. Relationship between ticks and zebu cattle in southern Uganda. Trop. Anim. Health Prod. 14, 63–74. https://doi.org/10.1007/BF02282583

Kazimírová, M., Thangamani, S., Bartíková, P., Hermance, M., Holíková, V., Štibrániová, I., Nuttall, P.A., 2017. Tick-borne viruses and biological processes at the tick-host-virus interface. Front. Cell. Infect. Microbiol. 7, 339. https://doi.org/10.3389/fcimb.2017.00339

Kongsuwan, K., Josh, P., Colgrave, M.L., Bagnall, N.H., Gough, J., Burns, B., Pearson, R., 2010. Activation of several key components of the epidermal differentiation pathway in cattle following infestation with the cattle tick, Rhipicephalus (Boophilus) microplus. Int. J. Parasitol. 40, 499–507. https://doi.org/10.1016/j.ijpara.2009.10.013

Kongsuwan, K., Piper, E.K., Bagnall, N.H., Ryan, K., Moolhuijzen, P., Bellgard, M., Lew, A., Jackson, L., Jonsson, N.N., 2008. Identification of genes involved with tick infestation in Bos taurus and Bos indicus. Anim. Genomics Anim. Heal. 132, 77–88.

Kopp, N., Diaz, D., Amacker, M., Odongo, D.O., Beier, K., Nitsch, C., Bishop, R.P., Daubenberger, C.A., 2009. Identification of a synthetic peptide inducing cross-reactive antibodies binding to Rhipicephalus (Boophilus) decoloratus, Rhipicephalus (Boophilus) microplus, Hyalomma anatolicum anatolicum and Rhipicephalus appendiculatus BM86 homologues. Vaccine 28, 261–269. https://doi.org/10.1016/j.vaccine.2009.09.085

Kuleš, J., Horvatić, A., Guillemin, N., Galan, A., Mrljak, V., Bhide, M., 2016. New approaches and omics tools for mining of vaccine candidates against vector-borne diseases. Mol. Biosyst. 12, 2680–2694. https://doi.org/10.1039/c6mb00268d

Malatji, D.P., 2017. A genomic insight into the diversity and pathogenesis of village chicken in a Ascaridia Galli infested environment. University of Pretoria.

Marima, J.K., Nel, C.L., Marufu, M.C., Jonsson, N.N., Dube, B., Dzama, K., 2020. A genetic and immunological comparison of tick-resistance in beef cattle following artificial infestation with Rhipicephalus ticks. Exp. Appl. Acarol. 80, 569–590. https://doi.org/10.1007/s10493-020-00480-8

Moon, K.H., Kim, W.J., Lee, J.S., Kim, D., Choi, K.S., Lee, S.H., Kim, Y.H., 2021. Transcriptomic comparison of cypermethrin-susceptible and -tolerant Asian longhorned ticks (Haemaphysalis longicornis Neumann). Entomol. Res. 2017. https://doi.org/10.1111/1748-5967.12511

Moré, D.D., Cardoso, F.F., Mudadu, M.A., Malagó-Jr, W., Gulias-Gomes, C.C., Sollero, B.P., Ibelli, A.M.G., Coutinho, L.L., Regitano, L.C.A., 2019. Network analysis uncovers putative genes affecting resistance to tick infestation in Braford cattle skin. BMC Genomics 20, 1–20. https://doi.org/10.1186/s12864-019-6360-3

NCBI-Refseq, 2020. Entrez Gene Summary for CCL26 Gene [WWW Document]. URL https://www.ncbi.nlm.nih.gov/refseq/

Nuttall, P.A., 2017. The wonders of tick saliva, in: One Health 9th Tick and Tick-Borne Pathogen Conference & 1st Asia Pacific Rickettsia Conference. Cairnes, Australia, p. 13.

Nyangiwe, N., Harrison, a., Horak, I.G., 2013. Displacement of Rhipicephalus decoloratus by Rhipicephalus microplus (Acari: Ixodidae) in the Eastern Cape Province, South Africa. Exp. Appl. Acarol. 61, 371–382. https://doi.org/10.1007/s10493-013-9705-7

Nyangiwe, N., Horak, I.G., Van der Mescht, L., Matthee, S., 2017. Range expansion of the economically important Asiatic blue tick, *Rhipicephalus microplus*, in South Africa. J. S. Afr. Vet. Assoc. 88, e1–e7. https://doi.org/10.4102/jsava.v88i0.1482

Piper, E.K., Jackson, L. a., Bagnall, N.H., Kongsuwan, K.K., Lew, A.E., Jonsson, N.N., 2008. Gene expression in the skin of Bos taurus and Bos indicus cattle infested with the cattle tick, Rhipicephalus (Boophilus) microplus. Vet. Immunol. Immunopathol. 126, 110–119. https://doi.org/10.1016/j.vetimm.2008.06.011

Piper, E.K., Jackson, L. a., Bielefeldt-Ohmann, H., Gondro, C., Lew-Tabor, A.E., Jonsson, N.N., 2010. Tick-susceptible Bos taurus cattle display an increased cellular response at the site of larval Rhipicephalus (Boophilus) microplus attachment, compared with tick-resistant Bos indicus cattle. Int. J. Parasitol. 40, 431–441. https://doi.org/10.1016/j.ijpara.2009.09.009

Piper, E.K., Jonsson, N.N., Gondro, C., Lew-Tabor, A.E., Moolhuijzen, P., Vance, M.E., Jackson, L. a, 2009. Immunological profiles of Bos taurus and Bos indicus cattle infested with the cattle tick, Rhipicephalus (Boophilus) microplus. Clin. Vaccine Immunol. 16, 1074–86. https://doi.org/10.1128/CVI.00157-09

Rechav, Y., Dauth, J., Els, D.A., 1990. Resistance of Brahman and Simmentaler cattle to southern African ticks. Onderstepoort J. Vet. Res. 57, 7–12.

Shyma, K.P., Gupta, J.P., Singh, V., 2013. Breeding strategies for tick resistance in tropical cattle: a sustainable approach for tick control. J. Parasit. Dis. 39, 1–6. https://doi.org/10.1007/s12639-013-0294-5

Walker, J.B., Keirans, J.E., Horak, I.G., 2000. The Genus Rhipicephalus (Acari, Ixodidae): A Guide to the Brown Ticks of the tick of the world. Cambridge University Press, United States of America.

Wambura, P.N., Gwakisa, P.S., Silayo, R.S., Rugaimukamu, E. a., 1998. Breed-associated resistance to tick infestation in Bos indicus and their crosses with Bos taurus. Vet. Parasitol. 77, 63–70.

Wang, J., Lin, R., Zhang, W., Li, L., Gottstein, B., Blagosklonov, O., Lu, G., Zhang, C., Lu, X., Vuitton, D.A., Wen, H., 2014. Transcriptional profiles of cytokine/chemokine factors of immune cell-homing to the parasitic lesions: A comprehensive one-year course study in the liver of E. multilocularis-infected mice. PLoS One 9. https://doi.org/10.1371/journal.pone.0091638

Wang, Y.H., Reverter, A., Kemp, D., McWilliam, S., Ingham, A., Davis, C., Moore, R., Lehnert, S., 2007. Gene expression profiling of Hereford Shorthorn cattle following challenge with Boophilus microplus tick larvae. Aust. J. Exp. Agric. 47, 1397–1407. https://doi.org/10.1071/EA07012

Weizmann Institute of Science., 2021. Genecard [WWW Document]. URL www.genecard.org

Wikel, S.K., 1999. Tick modulation of host immunity: an important factor in pathogen transmission. Int. J. Parasitol. 29, 851–859. https://doi.org/10.1016/S0020-7519(99)00042-9

Chapter 6:
General Conclusions and Recommendations

6.1 Scientific contribution

With the *R. microplus* appearing as the main ectoparasite commonly affecting cattle of Brazil, Australia and South Africa, these countries have had to join efforts in searching for genetic enhancements that may render their herds more resistant growing threat of heavy tick burdens. The three countries have unified genomic data to produce genomic prediction information on the level of tick resistance displayed by the different cattle breeds. The current study aimed to contribute to this collaboration between Brazil, Australia, and South Africa. The aim was to generate transcriptional information that will help define new biomarkers and validate existing ones that are participating in biological processes that render some breeds more resistant to ticks than others. The identification of potential biomarkers linked to host resistance in ticks can be used to inform the development of new vaccine candidates as well as for the development of prediction tests that can be used the aid marker-assisted selected breeding.

6.2 Conclusions

Gene expression studies including RNA sequencing using Next-generation sequencing, microarray analyses and RT-qPCR analysis, in conjunction with genome-wide association studies and proteomic studies are approaches that have been instrumental in the study of bovine host resistance to ticks. It is proven that incorporating data from different molecular approaches increases the credibility of the conclusion drawn for the investigation. The current study combined two transcriptomic studies and an SNP detection study to present a robust perspective of the molecular processes differentiating the Angus, Brahman, Nguni, and Santa Gertrudis cattle in terms of their ability to resist tick infestations. Using data from a study originally performed in Australia on Santa Gertrudis cattle combined with a study conducted in South Africa using Angus, Brahman and Nguni cattle, guided by insights from literature generated by Brazilian studies, the immuno-genetic component of host resistance to the *R. microplus* and *R. decoloratus* tick species were examined, and the following conclusions were drawn:

- The SNP discovery study facilitated the identification of a gene (*SATB2*) that has been extensively studied in oncogenesis in humans but was no information connecting it with the expression of the host resistance to ticks. The gene displayed a significant slightly positive correlation with tick count and significant differences in allele frequencies in the Angus breed compared to both the Brahman and Nguni breeds. Upon further investigations, it may be found that the gene may elicit interesting immune events in the bovine species that will render it a potential biomarker.

- The microarray study on the Santa Gertrudis revealed factors involved in response initiations and pro-inflammatory immune responses following consecutive artificial infestations. The Santa Gertrudis is evidence that upon long term exposure to a particular tick species some individuals within the breed may display an increased ability to develop and mount more robust adaptive responses against the tick infestations than the tick-susceptible animals.

- The RNA sequencing study produced results that resonated with earlier studies of host resistance to tick. The *CCL26, MMP12* and *MZB*1 genes displayed characteristics as potential as potential biomarkers for host resistance to ticks. Marked differences were observed in the differential gene expression patterns of a myriad of immunoregulatory, inflammatory and extracellular matrix genes. This served as another confirmation that the Bos indicus and Bos taurus africanus breed possess superior tick resistance governed by associated genetic mechanisms.

Overall, it could be concluded that the Nguni and Brahman breeds express distinct immunogenetic reactions governed by both the adaptive and innate immune systems accompanied by a cascade of cutaneous events primarily involved in tissue repair and remodelling. Additionally, it could be believed that the existence of a co-evolutionary history between a particular tick species and breed may offer cross-protection to the host against a specific tick species and all others.

6.3 Recommendations for future studies

- It is recommended that a more comparative genome-wide association study be conducted to isolate and validate candidate genes and SNPs found in Angus, Brahman, and Nguni breeds within a single study.
- It is also recommended that a follow-up study be conducted to validate the active functions of genes *SATB2, MMP12, CCL26* and *MZB1* within the context of host resistance to ticks.
- More studies need to be conducted to investigate the effect of co-evolutionary history between the host and tick species on the exhibited degree of resistance and the extent to which it may offer cross-protection to multiple tick species.
- It is also important to develop new tick resistance phenotypes or more accurate tick counting tools or methods to increase the accuracy of selective breeding programs for host resistance to ticks. This is possible through the incorporation of all populations, novel phenotypes and the gold-standard tick count phenotype.