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Mechanisms of resistance to Rhipicephalus ticks in Nguni cattle reared in the semiarid areas of South Africa

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School of Agricultural, Earth and Environmental Sciences

Doctor of Philosophy Animal Science

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Promoter: Professor Michael Chimonyo

Co-Promoter: Professor Kennedy Dzama

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Declaration

I, Munyaradzi Christopher Marufu, declare that this research has not been previously accepted for any degree and is not being currently considered for any other degree at any other University. I declare that this Dissertation contains my own work except where specifically acknowledged.

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Professor Michael Chimonyo   Professor Kennedy Dzama

(Promoter)   (Co-Promoter)

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List of Abbreviations

BCS   body condition score

BW   body weight

cELISA   complement enzyme-linked immunosorbant assay

CS   coat score

DNA   deoxyribonucleic acid

DTH   delayed type hypersensitivity

EDTA   ethylene diamine tetra acetic acid

GLM   generalised linear model

HL   hair length

Mab   monoclonal antibody

MSP   major surface protein

PCR   polymerase chain reaction

PCV   packed cell volume

PI   post inoculation

SAS   Statistical Analysis Systems

ST   skin thickness

TBD   tick-borne disease

TC   tick count

UV   ultraviolet

ULE   unfed larval extract

Abstract

Resistance to *Rhipicephalus* ticks in Nguni cattle reared in the semiarid areas of

South Africa

By

M. C. Marufu

Ticks and tick borne-diseases (TBD) are major challenges to cattle production among smallholder farmers in the semiarid areas of South Africa. Nguni cattle have been reported to be resistant to ticks and TBD, however, the mechanisms responsible for the trait are not fully understood. The broad objective of this study was to determine the mechanisms of resistance to ticks in Nguni cattle reared in the semiarid areas of South Africa. Tick infestation levels, body condition scores (BCS), packed cell volumes (PCV) and the molecular prevalence of *A. marginale* were determined in Nguni (n = 70) and local crossbred (n = 79) cattle reared in the semiarid areas of South Africa. Relationships among skin thickness, hair length, coat score and tick counts were assessed in seven to nine month old Nguni (n = 12) and Bonsmara (n = 12) heifers. As a follow up, cutaneous hypersensitivity responses to unfed larval extracts (ULE) of the ticks *Rhipicephalus decoloratus* and *Rhipicephalus microplus* were examined in heifers to determine host immunity to the ticks. Tick counts and inflammatory cell infiltrates in skin biopsies from feeding sites of adult *R. microplus* ticks in nine-month-old Nguni and Bonsmara heifers were also evaluated.

The molecular prevalence of *A. marginale* was similar in the Nguni (47.7 %) and local crossbred (52.3 %) cattle. Nguni cattle suffered less severe losses from and were more

resilient to *A. marginale* infection than local crossbreds. Nguni heifers had lower coat scores, hair length and tick counts than the Bonsmara heifers. The relationship between tick counts and coat score was positive and linear in the Nguni (y = 1.90x − 0.40) and quadratic in Bonsmara (y = -7.98x^2 + 12.74x - 3.12) heifers. Bonsmara cattle showed a more intense immediate reaction and no delayed hypersensitivity reaction to ULE of *Rhipicephalus* ticks. Nguni heifers presented a less intense immediate reaction and a delayed hypersensitivity reaction at 72 h post inoculation with ULE of *Rhipicephalus* ticks. Reactions to *R. decoloratus* ULE produced a more intense skin response at all time intervals in both breeds than that of *R. microplus*. Parasitized sites in Nguni heifers had higher (*P* < 0.05) counts of basophils, mast and mononuclear cells than those in the Bonsmara heifers. Conversely, parasitized sites in Bonsmara heifers had higher (*P* < 0.05) neutrophil and eosinophil counts than those in the Nguni heifers. Tick count was negatively correlated (P < 0.05) with basophil and mast cell counts. There was a positive correlation between eosinophil counts and tick counts in both breeds, and between tick counts and mononuclear cell counts in the Bonsmara breed. It was concluded that smooth and short coats, delayed type hypersensitivity and cutaneous basophil and mast cell infiltrations are responsible for increased tick resistance in the indigenous Nguni cattle breed of South Africa.

Publications

1. Marufu M.C., Qokweni L., Chimonyo M., Dzama K., 2010. Relationships between tick counts and coat characteristics in Nguni and Bonsmara cattle reared on semiarid rangelands in South Africa. Proceedings of the 30th World Veterinary Congress. 10-14 October 2010. Cape Town International Convention Centre, Republic of South Africa.

2. 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 and Tick Borne Diseases, 2: 172-177.

3. Marufu M.C., Chimonyo M., Mtshali M.S., Dzama K., 2012. Molecular prevalence of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in Nguni and local crossbred cattle under the low input production system in South Africa. Proceedings of the 10th Annual Congress of the Southern African Society For Veterinary Epidemiology and Preventive Medicine. 1-3 August 2012. Farm Inn, Republic of South Africa, pp. 57-62.

4. Marufu M.C., Chimonyo M., Dzama K., 2012. Molecular prevalence of *Anaplasma marginale* (Rickettsiales: *Anaplasmataceae*) in cattle under smallholder production in South Africa. Proceedings of the 13th International Symposium of Veterinary Epidemiology and Economics. 20-24 August 2012. Maastricht International Convenstion Centre, The Netherlands.

5. Marufu M.C., Chimonyo M., Mans B.J., Dzama K., 2013. Skin reactions to *Rhipicehalus* tick larval antigen in Nguni and Bonsmara cattle. Proceedings of the

45th South African Society for Animal Science (SASAS) Congress. 9 − 12 July 2012. East London International Convention Centre, Republic of South Africa.

6. Marufu M.C., Chimonyo M., Mans B.J., Dzama K., 2013. Cutaneous hypersensitivity responses to *Rhipicephalus* tick larval antigens in pre-sensitized cattle. Ticks and Tick Borne Diseases, 4: 311− 316.

7. Marufu M.C., Dzama K., Chimonyo M., 2013. Cellular responses to *Rhipicephalus microplus* infestations in pre-sensitised cattle with differing phenotypes of infestation. Experimental and Applied Acarology, DOI 10.1007/s10493-013-9723-5.

8. Marufu M.C., Dzama K., Chimonyo M., 2013. Comparison of the histology of *Rhipicephalus microplus* attachment sites in pre-sensitised cattle. Proceedings of the 11th World Congress on Animal Production. 15-20 October 2013. Beijing International Convention Centre, People's Republic of China.

Dedication

This thesis is dedicated to my wife Clarietta, our daughter Shamiso, my mother Colleta and my late father Christopher.

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CHAPTER 1: General Introduction

1.1 Background

More than 75 % of South Africa's land surface is used for livestock production mainly due to low precipitation which makes the land unsuitable for crop farming (Smet and Ward, 2006). Livestock farming has been identified as the agricultural enterprise with the greatest potential to improve household food security and reduce poverty in the semiarid farming areas of South Africa (Moloi, 2010). Among the different livestock enterprises, cattle production is a major contributor to the livelihoods of farmers in the semiarid areas (Musemwa et al., 2008). The smallholder sector is composed of resettled emerging and communal farmers and own more than 40 % of the 14.1 million cattle in South Africa (National Livestock Statistics, 2008). Not only are cattle a source of meat and milk for household consumption but they also provide draught power for crop production, hides, manure and cash through sales (Chimonyo et al., 1999; Palmer and Ainslie, 2006). Cattle are an inflation-free form of banking for smallholder farmers and can be sold, as a last resort, to meet household financial needs such as school fees, medical bills, village taxes and other expenses (Dovie et al., 2006).

Despite its importance, cattle herd productivity in the smallholder sector is generally low (Mapiye et al., 2009) with cattle off-take rates being as low as 2 % per annum (Ainslie et al., 2002). Among the leading causes of reduced productivity in smallholder herds is cattle mortality caused by diseases and parasites especially ticks (Hesterberg et al., 2007). Ticks and the diseases they transmit have been identified as the major cause of

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widespread morbidity and mortality in cattle kept by smallholder farmers in the semiarid areas of South Africa (Dold and Cocks, 2001; Mapiye et al, 2009). Poor cattle health management, resistance of ticks to most acaricides and the use of inappropriate cattle breeds (Dold and Cocks, 2001; Marufu et al. 2011) have increased the prevalence of ticks and tick-borne diseases (TBD) in smallholder cattle herds. Effective control of ticks and TBD may, therefore, increase cattle off-take, improve food security and livelihoods of the smallholder cattle producers.

Tick species commonly affecting cattle reared on the semiarid rangelands of South Africa are *Rhipicephalus decoloratus* and *Rhipicephalus microplus* (Marufu et al., 2010) which are known to be biological transmitters of *Anaplasma marginale* the cause of bovine anaplasmosis. It has been reported that bovine anaplasmosis is the most important cause of cattle mortalities in low-input farming areas in South Africa (Mapiye et al., 2009). *Rhipicephalus* ticks also transmit *Babesia bigemina* and *Babesia* bovis which are protozoal agents causing bovine babesiosis. A serological survey in cattle on semiarid rangelands has shown that the Nguni breed has a lower sero-prevalence of *A. marginale* and was, thus, deemed to be more resistant than the local crossbreeds (Marufu et al., 2010). Serological tests, however, do not consistently discriminate between past and present infections. Cross reactivity also occurs between *Anaplasma* species (Kocan et al., 2010). Polymerase chain reaction (PCR)-based detection methods have been developed, which are extremely sensitive and specific in the detection of *A. marginale* infections in cattle (de La Fuente et al., 2005; Molad et al. 2006). It is, therefore, important to use the more sensitive and efficient molecular techniques to generate accurate information on the

prevalence of bovine anaplasmosis which are crucial not only for developing appropriate control measures but for providing an understanding of host resistance in different cattle genotypes.

Tick control using acaricides has been the most common method of curbing the high prevalence of ticks and TBD in cattle in semiarid areas however, increasing tick resistance to these chemical is a major shortcoming (Moyo and Masika, 2009). Anti-tick vaccines have been developed, but are not completely effective in controlling the transmission of tick-borne infections in cattle (Pipano et al., 2003). At present, a major challenge of using anti-tick vaccines is that they cannot offer protection against the multiple tick species of economic importance that occur on the semiarid rangelands. Use of tick resistant cattle breeds to control ticks and TBD has been recommended as a cheap, effective, sustainable, and safe alternative to the use of acaricides (Kongsuwan et al., 2010).

In South Africa, the Nguni is a major indigenous cattle breed that is hardy, uniquely adapted to the local environment and possesses a high tolerance to ticks and TBD (Spickett et al., 1989; Mapiye et al., 2007; Muchenje et al., 2008). Past government policies to improve the productivity of smallholder farmers resulted in the stocking of imported cattle in semiarid areas of South Africa but these failed to adapt to the harsh environmental conditions which include amongst others, high temperatures, feed scarcity and disease rampancy (Musemwa et al., 2008). Uncontrolled breeding and indiscriminate crossing of these imported breeds with indigenous Nguni cattle to improve their

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adaptability has led to the production of numerous non-descript crosses (local crossbreeds) (Scholtz et al., 2008). Though the local crossbreeds are better able to cope with the harsh semiarid environments than imported breeds, their level of ticks and TBD resistance is lower than that of the indigenous Nguni cattle (Marufu et al., 2011).

The Bonsmara, a synthetic breed developed from crosses of indigenous Afrikaner, and imported Shorthorn and Hereford cattle, has gained popularity among farmers in the semiarid areas of South Africa because of its high productivity that matches that of imported breeds and adaptability to the semiarid conditions such as high temperatures (Ndlovu et al., 2008). Though it is heat tolerant, the Bonsmara is not suitable for rearing in tick-infested areas as it succumbs to tick-related illness. The Bonsmara breed is less resistant to ticks than the indigenous Nguni cattle breed (Muchenje et al., 2008). The mechanism responsible for the apparent resistance to ticks and TBD in the Nguni and susceptibility to these parasites in the local crossbred and Bonsmara cattle are yet to be established.

Resistance to ticks in cattle has been attributed to favourable coat characteristics, superior skin immunity or the abundance of tick resistance genes (Bechara et al., 2000; Verrissimo et al., 2002; Piper et al., 2008). To objectively determine the mechanism of resistance to ticks in different cattle breeds, it is important to conduct tick counts on naturally exposed animals, assess coat characteristics of the cattle, evaluate the skin hypersensitivity to tick antigen and determine the cutaneous cellular reactions to tick infestation (de Castro et al., 1991; Latif et al., 1991; Piper et al., 2010). The Nguni breed carries consistently low tick

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loads and is, thus, deemed to be resistant to ticks (Scholtz et al., 1991; Muchenje et al., 2008). However, coat characteristics such as hair length, skin thickness and coat scores which are related to tick resistance in cattle on rangelands (Verrisimo et al., 2002; Martinez et al., 2006) have not been determined in the Nguni breed. Establishing relationships between coat characteristics and tick counts in the indigenous and locally adapted Nguni breed reared on the semiarid rangelands helps to understand the mechanisms of tick resistance and to characterise these cattle breeds.

Delayed skin hypersensitivity (DTH) responses to tick antigen is an important mechanism of resistance to ticks in cattle and can be measured using an intradermal skin test to accurately predict the level of resistance to ticks in cattle (Bechara et al., 2000). Little work has been done to describe the DTH responses to tick antigen in Nguni cattle on semiarid rangelands. Thus, further work to determine the mechanism and level of skin resistance to ticks in the Nguni breed utilising the intradermal skin test is imperative. Assessing the histopathology of tick attachment sites (Latif et al., 1991) is another useful tool in objectively determine the cutaneous cellular responses to tick infestation in cattle. Its use in determining the possible mechanism of tick resistance in Nguni in the semiarid areas has not been documented and merits investigation.

1.2 Justification

The drive to restock semiarid areas with Nguni cattle for food security makes it imperative to validate the breed's resistance to ticks and TBD using more sensitive diagnostic methods before recommending its use as a tick- and TBD-resistant breed.

Cost-effective control of bovine anaplasmosis, a leading cause of cattle mortality in semiarid areas, depends on the availability of accurate prevalence data which are, however, scanty particularly in smallholder farming areas in South Africa. Data on the prevalence of bovine anaplasmosis obtained by means of molecular techniques with higher sensitivity and specificity are essential to develop control measures in target cattle populations. Various factors may influence the prevalence of *A. marginale* infection in cattle including production system, tick infestation level, cattle breed, age and nutritional performance, but, their effect on the molecular prevalence of bovine anaplasmosis in semiarid farming areas of South Africa are not fully known.

The blue ticks, *R. decoloratus* and *R. microplus* are a great impediment to cattle production on the semiarid rangelands due to their direct effects and indirectly through the transmission of bovine anaplasmosis. Elucidating the mechanisms by which resistant cattle prevent heavy blue tick infestations is potentially useful for the development of anti-tick vaccines and is a crucial step in the development of predictive biomarkers for tick resistance for use in selective breeding programmes. The information obtained from such assessments can be useful in the selection and rearing of appropriate breeds that are resistant to ticks and TBD in semiarid areas. Description of reactions at tick feeding sites enhances knowledge on the host-parasite relationships so as to understand the host defences to pathogen transmission by ticks.

1.3 Objectives

The broad objective of the study was to determine the mechanisms of resistance to ticks in Nguni cattle reared in the semiarid areas of South Africa. The specific objectives were to:

i. Determine the molecular prevalence of *A. marginale* in Nguni and local crossbred cattle reared by smallholder farmers on the semiarid rangelands of South Africa;

ii. Establish the relationships between tick count and coat characteristics such as skin thickness, hair length and coat score, in Nguni and Bonsmara cattle;

iii. Assess the skin reactions to unfed larval extracts of the ticks *R. decoloratus* and *R. microplus* in Nguni and Bonsmara cattle; and

iv. Assess the histopathology of attachment sites of *R. decoloratus* and *R. microplus* in Nguni and Bonsmara cattle.

1.4 Hypotheses

The main hypothesis tested in the present study was that morphological coat traits, skin hypersensitivity responses and cellular reactions at the tick infestation sites are the mechanisms responsible for tick resistance in Nguni cattle. The specific hypotheses tested were:

i. The prevalence of *A. marginale* is higher in local crossbred cattle than in the Nguni reared by smallholder farmers on the semiarid rangelands of South Africa;

ii. Relationships between tick count and morphological coat traits are different in Nguni and Bonsmara cattle;

iii. Unfed larval extracts of the ticks *R. decoloratus* and *R. microplus* elicit dissimilar skin reactions in Nguni and Bonsmara cattle; and

iv. Histopathology of attachment sites of *R. decoloratus* and *R. microplus* are different in Nguni and Bonsmara cattle.

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CHAPTER 2: Literature Review

2.1 Introduction

Cattle production plays an important role in the economy and in the socio-economic development of millions of households in the semiarid areas of South Africa. Despite its significant contribution to agricultural production in semiarid areas, cattle production is greatly threatened by ticks and TBD (Hesterberg et al., 2007). The use of host resistance to control ticks and TBD, and improve cattle productivity, has recently gained attention as it provides a cheap, effective, sustainable, environmentally friendly and safe alternative to the use of acaricides, which have major short-comings (Ferreira et al., 2003; Kongsuwan et al., 2010). It has been recommended that in Southern Africa, farmers should use the indigenous Nguni cattle breed in the integrated control of ticks and TBD as it is adapted to the smallholder environment and carries lower tick loads with lower prevalence of TBD compared to local crossbred cattle (Marufu et al., 2010; 2011). It is important, however, to determine the mechanism of resistance exhibited by this breed before recommending its use in the integrated control of ticks on the semiarid rangelands. This review discusses cattle production, common TBD and ticks, and their impact on cattle production in the semiarid areas of South Africa. The mechanisms of tick resistance in cattle, heritability estimates and genetic selection for tick resistance are also discussed.

2.2 Cattle production in the semiarid areas

Cattle production in the semiarid areas of South Africa is dominated by smallholder producers with a smaller contribution coming from the large scale commercial sector.

The smallholder sector owns more than 40 % of the total cattle population in the country and is composed of resettled emerging and communal farmers (National Livestock Statistics, 2008). Resettled emerging farmers are beneficiaries of the government's land reform programme, own private pieces of land and have a commercial orientation utilising some technology and external inputs (Ainslie et al., 2002). They farm on larger pieces of land on which they keep bigger herds of up to 100 cattle (Palmer and Ainslie, 2006). Some resettled farmers practise controlled breeding, provide supplementary feeding and actively market their animals.

Communal farmers, on the other hand, have no exclusive land tenure, share natural resources such as grazing land, and manage these resources collectively (Palmer and Ainslie, 2006). They are categorised as 'subsistence farmers' since they produce mainly for household consumption (van Averbeke and Mohamed, 2006). Most communal farmers farm on smaller tracts of land of up to 5 ha and keep fewer animals of mixed species (Lahiff and Cousins, 2005). In communal farming systems, farmers rarely practise controlled breeding, supplementary feeding and marketing of animals. Cattle are kept under traditional management conditions and are mostly affected by harsh environmental conditions, seasonal labour constraints, diseases, and nutritional deficiencies (Makhura and Wasike, 2003).

Cattle have various roles and contribute to the livelihoods of farmers in the semiarid areas. They are a source of meat and milk for household consumption, provide draught power for crop production, hides, manure for fertilising fields and cash through sales

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(Chimonyo et al., 2000; Palmer and Ainslie, 2006). Cattle are an inflation-free form of savings for for farmers and can be sold to meet emergency household financial needs (Mapiye et al., 2009). Despite its important contribution to smallholder farmers' livelihoods, the major challenges to cattle production in semiarid areas are diseases and parasites (Mapiye et al., 2009).

Ticks and TBD are the greatest impediment to production and profitability for the cattle enterprise in semiarid areas (Dreyer et al., 1998; Dold and Cocks, 2001). To avert production losses caused by TBD, farmers utilise various measures to reduce tick loads on cattle. Smallholder farmers mainly depend on the application of acaricides to control ticks, whose frequency of application in the communal sector is determined by the functioning of government-subsidised programmes (Rikhotso et al., 2005). Many smallholder farmers supplement acaricide application with methods such as the hand removal of ticks or application of homemade remedies (such as used engine oil, jeyes fluid or other household detergents sometimes mixed with used engine oil) and other ethnoveterinary medicines (Moyo and Masika, 2009). The use of acaricides as the principal means of tick and TBD control is expensive and results in the development of acaricide-resistant ticks along with contamination of the environment and animal products. Acaricide resistance is associated with mutations in the ticks' genes related to drug susceptibility. These consequences have led to the need to explore alternative and more sustainable, environmentally friendly strategies of tick control in the smallholder sector, such as host resistance.

Alternative tick control methods include the use of genetically resistant animals, the management of pastures and the adoption of biologic and immunological formulations such as anti-tick vaccines (Parizi et al., 2009). Marufu et al. (2011) argued that farming with high tick resistance cattle breeds is the most important alternative strategy for controlling ticks in the smallholder sector on the semiarid rangelands. The mechanism of resistance to ticks and TBD in these breeds has not been documented. For the adoption of tick resistant cattle breeds in semiarid areas, it is important to elucidate their mechanisms of resistance and adequately characterise them before recommending them to farmers.

2.3 Cattle breeds reared on the semiarid rangeland

Various cattle breeds are reared by farmers in the semiarid rangelands of South Africa chief amongst which are the indigenous Nguni, local crossbred (non-descript) and Bonsmara cattle breeds. Imported cattle breeds are also used including Hereford, Angus and Brahman albeit less commonly.

2.3.1 Indigenous Nguni cattle

Indigenous Nguni cattle (*Bos taurus africanus*) are part of the Sanga group in Southern Africa, descendants of *Bos taurus* animals that were domesticated in north-eastern Africa between 7000 and 8000 years ago. They were later crossed with zebu cattle from the Arabian Peninsula (Bester et al., 2003) that arrived in South Africa around 300 to 700 AD (Scherf, 2000). Nguni cattle have adapted to the harsh conditions in the semiarid areas, where high temperatures and the long dry season, characterized by feed shortages, are normal and diseases are rampant. Among the characteristics attributed to indigenous

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Nguni cattle, tolerance to climatic stresses, genetic adaptation to poor quality forages and increased resistance to endemic diseases and parasites (Collins-Lusweti, 2000) have been viewed as the most important. Nguni cattle have higher heat tolerance compared to European breeds and this has been attributed to a combination of factors including lower mature body weights, smaller body sizes and shorter hairs (Baker and Rege, 1994). The indigenous Nguni cattle also utilise poor quality feed resources better than imported breeds due to their high selectivity on the rangelands (Strydom, 2008).

Increased resistance to endemic diseases and parasites is a well-known attribute of indigenous cattle breeds. In many subtropical and semiarid environments in Africa, indigenous dual purpose breeds are highly resistant to ticks and suffer marginal direct losses from low infestation rates (Pegram et al., 1993). The indigenous Nguni breed of South Africa is more resistant to ticks and TBD than crossbred and imported cattle breeds (Rechav and Kostrzewski, 1991; Marufu et al., 2011). The physiological and genetic mechanisms associated with resistance to ticks in the Nguni breed are not fully understood and need to be established. Tick resistance in cattle is related to a pre-immunity to ticks often established through a continuous contact with the infectious agents from early in life (Mattioli et al., 2000). It has also been postulated that tick resistance in cattle might vary with the species of infesting tick (Ali and de Castro, 1993). It is not known whether tick resistance in the Nguni breed is associated with pre-immunity to ticks and how resistance varies with infestation by different tick species. Further investigations are, thus, warranted to determine whether acquired immunity to

tick infestation plays a part in increasing resistance to different tick species in the Nguni cattle breed.

2.3.2 Local crossbred (non-descript) cattle

Local crossbred (non-descript) cattle arose from institutional policies that promoted the use of imported beef breeds in smallholder areas to improve productivity. The resultant uncontrolled breeding and indiscriminate crossing of imported (*Bos taurus*) cattle with the Nguni created numerous local crossbred cattle (Scholtz et al., 2008). Indigenous-imported crosses are characterised by large frame sizes and appear to be less adapted to the semiarid environment than indigenous cattle (Mapiye et al., 2009). They suffer heavier losses in body condition, protein and energy during the cool dry season than indigenous Nguni cattle mainly due to their large body size. Local crossbred cattle are also thought to have comparably lower resistance to diseases and parasites compared to the indigenous Nguni cattle breed (Marufu et al., 2010).

Local crossbreds endure tick infestation for longer periods without acaricide application, and have lower tick burdens than imported breeds (Fivaz and de Waal, 1993). However, their level of resistance to ticks was comparably lower than that of pure indigenous breeds (Fivaz et al., 1992; Marufu et al., 2011). The lower tick resistance in the local crossbreds than in the Nguni cattle breed is thought to be the cause of the increased susceptibility to TBD in the former (Marufu et al., 2010). It remains to be tested, however, using highly sensitive and specific molecular techniques how the molecular

prevalence of TBD differs between the two genotypes and the associated mechanisms of this resistance.

2.3.3 Bonsmara cattle

The Bonsmara is a composite breed which originated from a 5/8 : 3/8 combination of the Afrikaner (*Bos taurus africanus*) and Shorthorn/Hereford (*Bos taurus taurus*) cattle breeds (Strydom et al., 2001). It was created to compete with European beef cattle breeds, while tolerating semiarid conditions such as high temperatures (Ndlovu et al., 2008). The Bonsmara has a well-pigmented thick skin which is tolerant to heat and radiation (Porter, 1991). The breed adapts well to rangeland and feedlot conditions in semiarid areas. Though it is heat tolerant, the Bonsmara is less adapted to diseases and parasites that are rampant in the semiarid areas.

The Bonsmara has an intermediate resistance to gastrointestinal parasites and ticks, being less susceptible than imported beef breeds such as the Angus, and less resistant than indigenous Nguni cattle (Muchenje et al., 2008; Ndlovu et al., 2009). The Bonsmara tends to have intermediate losses to tick infestation, suffering less severe losses in weight than the Hereford and more than the Nguni (Scholtz et al., 1991). The higher susceptibility of the Bonsmara than the indigenous Nguni breed to ticks has not been fully explained and requires investigation. Spickett et al. (1989) suggested that thicker hairs and longer coats could be related to increased tick infestation in the Bonsmara. Such a hypothesis and other possible immunological mechanisms, however, need to be tested.

2.4 Tick-borne diseases of cattle on the semiarid rangelands

Tick-transmitted infections cause widespread morbidity and cattle mortality in the low input cattle production system (Hesterberg et al., 2007; Mapiye et al., 2009). The three infectious agents observed to be commonly infecting cattle in the semiarid rangelands of South Africa were *Babesia bovis*, *Babesia bigemina* and *Anaplasma marginale* (Marufu et al., 2010). The babesias are apicomplexan protozoal parasites which affect erythrocytes of cattle causing an infectious anemia, while anaplasmosis is a rickettsia also affecting erythrocytes causing severe anaemia and icterus in cattle (Kocan et al., 2010). Anaplasmosis is the leading cause of cattle mortalities in the smallholder production system on the semiarid rangelands in South Africa (Mapiye et al., 2009; Ndou et al., 2010). More attention, therefore, needs to be focussed on bovine anaplasmosis as it is a leading cause of production losses in the smallholder cattle production system in the semiarid areas of South Africa.

2.4.1 Bovine anaplasmosis

Bovine anaplasmosis caused by the intraerythrocytic rickettsia *A. marginale* belonging to the order Rickettsiales, and phylum Proteobacteria (Kocan et al., 2003). Another species, *A. centrale*, causes a less severe disease than that caused by *A. marginale*, in which anaemia and mortality of up to 50 % have been recorded (Minjauw and McLeod, 2003). The disease is thought to have existed in South Africa for many years, even before the appearance of babesiosis (1870), but was commonly confused with the later disease (de Waal, 2000). Clinically bovine anaplasmosis is characterised by pyrexia, progressive anaemia and icterus particularly in imported and naïve indigenous cattle breeds

(Muhanguzi et al., 2010). It results in considerable economic losses to the cattle industry through cattle mortality, decreased productivity, lowered working efficiency of draught cattle and increased veterinary costs (Kocan et al., 2003).

Transmission of *A. marginale* to cattle is biological, through the tick vector. The tick species commonly transmitting *A. marginale* to cattle on the semiarid rangelands are *R. decoloratus* and *R. microplus*. *Hyalomma marginatum rufipes*, *Rhipicephalus evertsi evertsi* and *Rhipicephalus simus* were shown to experimentally transmit bovine anaplasmosis and hence have the potential to be natural vectors in the field (Potgieter, 1979; de Waal, 2000). The rickettsia *A. marginale* undergoes a complex developmental cycle in ticks that begins by infection of gut cells, and transmission to susceptible hosts occurs through salivary glands during tick feeding (Kocan et al., 2010). Transmission of anaplasmosis by ixodid ticks is horizontal, while transovarial transmission does not appear to occur and transstadial transmission occurs from stage to stage (de La Fuente et al., 2010). Mechanical transmission of anaplasmosis by haematophagus insects, contaminated hypodermic needles and instruments used in veterinary procedures also occurs (Kocan et al., 2010). Biological transmission by ticks was reported to be more efficient than mechanical transmission by biting flies (Scoles et al., 2005).

Bovine anaplasmosis is the most widely distributed TBD of cattle in South Africa (Figure 2.1). Losses associated with anaplasmosis are related to impaired production, mortalities and control measures (Regassa et al., 2003). Since tick transmission is an important method of spread, the control of tick infestations on cattle by regular application of

acaricides can, to a large extent, eliminate or considerably reduce the source of infection of bovine anaplasmosis. Tick control has, however, been applied blindly to control anaplasmosis and, as a result, has been largely ineffective due to lack of specific epidemiological data which forms the basis of a control regime (Ndou et al., 2010). The use of tick-resistant cattle breeds to control bovine anaplasmosis could potentially augment acaricide application (Rikhotso et al., 2005) and yet this has not been examined on the semiarid rangelands of South Africa. Innate resistance to *A. marginale* in the different cattle breeds reared on the semiarid rangelands requires further examination.

To confirm the diagnosis of anaplasmosis, laboratory tests such as light microscopic evaluation of stained blood smears, serological or molecular diagnostic procedures are required. Field diagnosis is usually based on the detection of *A. marginale* organisms in erythrocytes on thin blood smears stained with Giemsa, Wright Giemsa or Diff-Quick stain under a light (Aubry and Geale, 2010). The method, however, has the major shortcoming of failing to detect advanced or persistently infected cases (Carelli et al., 2007). The serological test that is widely used to diagnose *A. marginale* infection in cattle is a competitive enzyme-linked immunosorbent assay (cELISA) which is based on use of a monoclonal antibody (Mab) ANAF16C1 that recognizes MSP5 in *A. marginale* (Kocan et al., 2010).
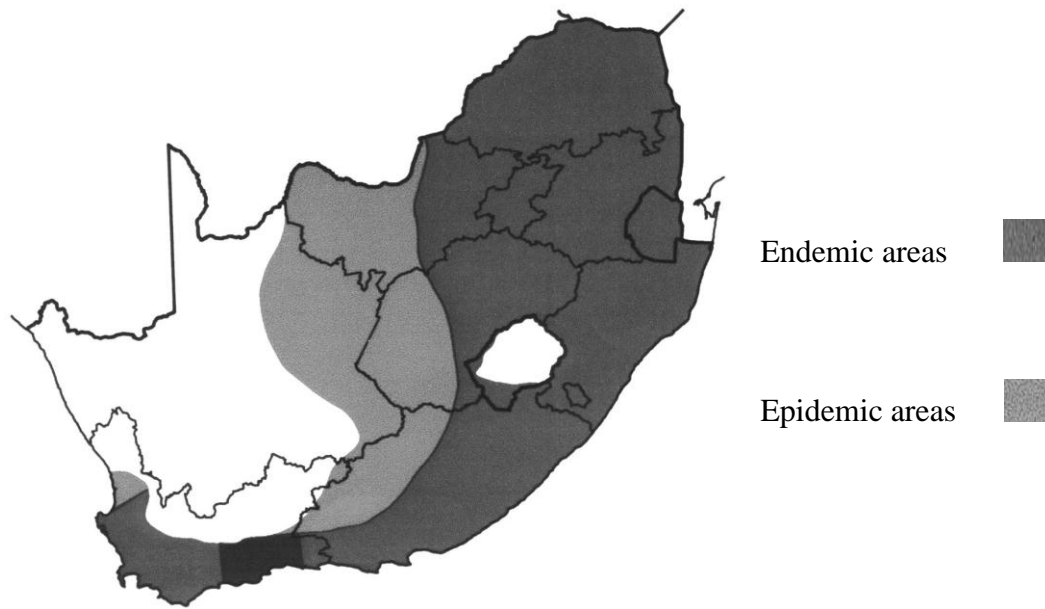
Figure 2.1: Distribution of Anaplasma marginale in South Africa (de Waal, 2000)

It should be noted, however, that serological tests do not consistently discriminate between past-exposure and present infections and also between carriers and currently infected animals as they rely on the detection of antibodies (Martins et al., 2008; Yamada et al., 2008). Cross-reactivity sometimes occurs between *Anaplasma* species because of antigenic similarity and is also a major disadvantage of the serological techniques (Buling et al., 2007; Carelli et al., 2007). Polymerase chain reaction (PCR)-based detection methods have been developed and were reported to be extremely sensitive and specific in the detection of *A. marginale* infections in cattle (de La Fuente et al., 2005; Molad et al. 2006). Some of these assays, such as the real-time PCR, were developed to enable simultaneous detection and quantification of the *A. marginale* DNA in bovine blood, which is essential in supporting the clinical diagnosis, assessing the carrier status of the cattle and evaluating the efficacy of vaccines and antirickettsial drugs (Carelli et al., 2007).

Several surveys have been conducted to provide epidemiological data on the prevalence of anaplasmosis in cattle in the semiarid areas of South Africa through blood smear analysis and serological tests (Dreyer et al., 1998; Mbati et al., 2002; Hesterberg et al., 2007). One serological survey reported that indigenous Nguni cattle have a lower prevalence for *A. marginale* than local crossbred cattle (Marufu et al., 2010) suggesting a higher resistance to bovine anaplasmosis in the indigenous cattle breed. It is crucial however, to provide accurate data on prevalence of bovine anaplasmosis obtained by the highly sensitive and specific molecular techniques. These data are crucial not only for

developing appropriate control measures but could also provide an improved understanding of host resistance to *A. marginale* in different cattle genotypes reared on the semiarid rangelands of South Africa. Cattle on the rangelands commonly suffer from mixed infections of anaplasmosis and babesiosis, which share the same tick vectors.

2.4.2 Bovine babesiosis

Blue ticks are vectors of babesiosis or redwater which is also prevalent in cattle on semiarid rangelands (Marufu et al., 2010). Bovine babesiosis is caused mainly by the tick-borne apicomplexan protozoal parasites *Babesia bigemina*, the cause of African redwater and *B. bovis*, the cause of Asiatic redwater (de Vos and Potgieter, 1994). The protozoan *B. bigemina* was probably present in Africa before the arrival of imported cattle breeds (Regassa et al., 2003). Asiatic redwater was first reported in South Africa in 1941 (Neitz, 1941) and was probably introduced during the latter part of the 19th century with *R. microplus*, the only biological vector in South Africa. Clinically, acute disease is associated with fever, hemolytic anemia, anorexia, lethargy, hemoglobinuria, tachycardia, and icterus (Suarez and Noh, 2011). In the case of *B. bovis* infection, a more severe disease occurs often resulting in cerebral babesiosis, characterized by convulsions, hyperaesthesia, and paralysis, because of the sequestration of infected erythrocytes in cerebral capillaries (Bock et al., 2004).

The distribution of babesiosis in South Africa corresponds with that of the tick vectors *R. decoloratus* and *R. microplus*. Both babesial parasites generally have the same distribution, however, *B. bigemina* is more widespread due to the wider distribution of *R.*

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decoloratus especially in colder and drier areas (de Waal and Combrink, 2006). Breeds of cattle that are indigenous to Babesia-endemic regions often have a certain degree of natural resistance to these diseases and the consequences of infection are not as serious as when exotic *Bos taurus* breeds are involved (Bock et al., 2004). Cattle that recover from the primary acute infection remain persistently infected and serve as a reservoir for transmission. Thus, control measures of bovine babesiosis require effective diagnostic tools that can detect carrier animals (Terkawi et al., 2011). Serological diagnostic assays such as the indirect immunofluorescent antibody tests (IFATs) and enzyme-linked immunosorbent assay (ELISA) have been widely utilized for epidemiological surveys of bovine babesiosis (Tonnesen et al., 2006; Goff et al., 2008). Several problems regarding their sensitivity, specificity, subjective interpretation, and low throughput have, however, limited their practical use (Terkawi et al., 2011).

Various methods have been employed to control bovine babesiosis in the smallholder production system of South Africa, including vector control, chemoprophylaxis and vaccination. Applying strict tick control to control babesiosis will require the implementation of a well managed intensive dipping programme especially in endemic areas (de Waal and Combrink, 2006). Such an intensive dipping programme carries high risk as regards the development of tick resistance to the dipping compound (Jonsson et al., 2000). Use of anti-babesial drugs such as imidocarb or dimenazene has also been effective in the control of bovine babesiosis. The inappropriate use of the babesicides may lead to emergence of drug-resistant *Babesia* strains in the field (Zintl et al., 2003).

The use of *B. bigemina* and *B. bovis* attenuated strains that have been passaged in splenectomised calves to produce live vaccines has also been implemented albeit on a small scale in smallholder farming systems in the semiarid areas of South Africa (de Waal and Combrink, 2006). Live *Babesia* vaccines are usually safely administrated in young cattle, but older animals can still be susceptible to the vaccine strain and have a higher risk of succumbing to severe acute disease upon vaccination (Suarez and Noh, 2011). Use of tick-resistant breeds, such as the Nguni, is recommended in the control of bovine babesiosis on semiarid rangelands of South Africa (Marufu et al., 2010).

2.5 Common ticks and tick-borne diseases infesting cattle in the semiarid areas

Ticks have attracted a great deal of attention primarily because of their direct effects on productivity and also because of their role as vectors of numerous pathogens of cattle (Kaufman, 2010). Two major families exist among ticks, Argasidae (soft ticks) and Ixodidae (hard ticks), the latter having more importance in cattle. Of the seven important genera of hard ticks that affect livestock throughout the world (Jongejan and Uilenberg, 2004), four are known to affect cattle on the semiarid rangelands of South Africa, namely, *Amblyomma*, *Rhipicephalus*, *Hyalomma* and the subgenus *Rhipicephalus* (formerly *Boophilus*) (Nyangiwe et al., 2011). The four most common tick species that are of major economic importance as vectors of diseases that affect domestic cattle in South Africa are *R. decoloratus*, *R. microplus*, *A. hebraeum* and *R. appendiculatus* (Marufu et al., 2011). The species *R. evertsi evertsi* was also observed to commonly affect cattle on rangelands (Nyangiwe and Horak, 2007).

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2.5.1 *Rhipicephalus decoloratus* **and** *Rhipicephalus microplus*

Rhipicephalus decoloratus (African blue tick) and *R. microplus* (pantropical blue tick), commonly called blue ticks, are one host ticks which are regarded as the most economically important *Rhipicephalus* species affecting cattle in Africa (Tonnesen et al., 2004). The former is considered to be indigenous to Africa while the latter is thought to have been introduced into Africa through cattle movements from Asia (Nyangiwe and Horak; 2007). Blue ticks are proficient vectors of bovine anaplasmosis on the semiarid rangelands of South Africa. The African blue tick is an efficient vector of *Babesia bigemina*, the cause of African redwater in cattle, while the pantropical blue tick is responsible for the transmission of *Babesia bigemina* and *Babesia bovis*, the latter causing Asiatic redwater in cattle (Norval and Horak, 1994).

The blue ticks are classified as 1-host ticks and thus complete their life cycle on the same bovine host (Jongejan and Uilenberg, 2004). While *R. decoloratus* is more tolerant to cold and drought, *R. microplus* thrives in warm and humid conditions, and has higher reproductive rates (Zeman and Lynen, 2010). They compete with each other by mutually boosting cattle's cross-protective immunity against feeding by both tick species (Rechav et al., 1991). The autochtonous blue tick, however, has the advantage of being a more catholic feeder, thus escaping from the competition by parasitizing alternative hosts, particularly wildlife, wherever available (Horak et al., 2003; Zeman and Lynen, 2010).

Walker et al. (2003) mapped the distribution of the blue ticks in Africa (Figure 2.2). In Africa, *R. decoloratus* is the more widespread of the blue tick species (Bryson et al.,

2002; Mtshali et al., 2004). The distribution of *R. decoloratus* is being reduced through displacement by *R. microplus* especially in the eastern and south eastern parts of Africa (Estrada Pena et al., 2006). The displacement is thought to be caused by the shorter life cycle of the pantropical blue tick, its tendency to assortative mating and more successful feeding on cattle (Tonnesen et al. 2004). The exact cause of competitive inferiority that is thought to be crucial for the ousting of *R. decoloratus* by *R. microplus* from the autochthon's historical ranges in southern and eastern Africa is, as yet, not fully understood (Lynen et al., 2008). Understanding of the causes of displacement of the African blue tick by the pantropical blue tick may assist in formulation of strategies that mitigate the risk of spread of the latter in to new areas and thus alleviating its impact on cattle production (Madder et al., 2011). Further investigation of the causes of the displacement of *R. decoloratus* from its traditional habitat by *R. microplus* is, thus, required.

Due to the 1-host nature of their life cycle, selection for acaricide resistance is particularly intense in the African and pantropical blue ticks, both of which spend approximately three consecutive weeks on their hosts to complete their life cycles (Ntondini et al., 2008). This can lead to heavy infestations on cattle herds, particularly those with a low degree of resistance, and cause considerable direct damage (Jongejan and Uilenberg, 2004). Although blue ticks have short mouthparts, they cause extensive damage to hides. They also transmit haemoparasites which further excercabate their economic impact on cattle production.
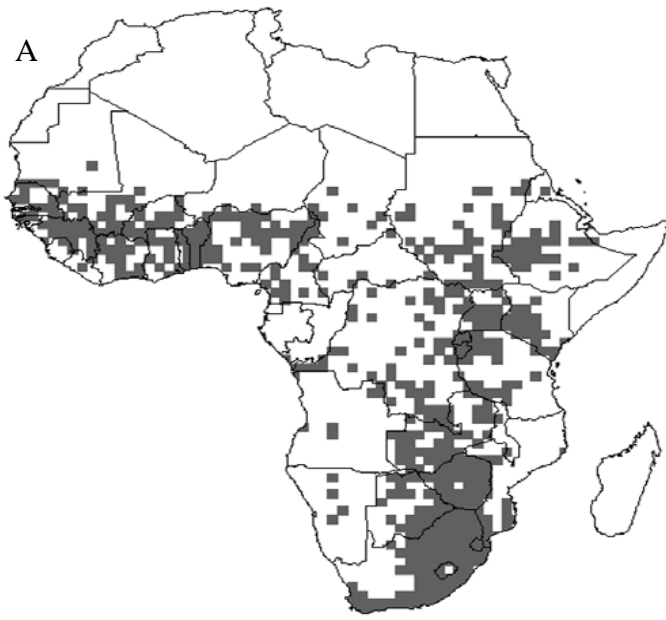
Figure 2.2: Distribution of *Rhipicephalus decoloratus* (A) and *Rhipicephalus microplus* (B) in Africa (Adapted from Walker et al., 2003)

Much research has focused on a single blue tick species, *R. microplus* at the expense of the equally important African species *R. decoloratus*. Differences in infestation rates and susceptibility have been observed for *R. decoloratus* and *R. microplus* on Nguni and Bonsmara cattle, suggesting that resistance to each tick species is differentially expressed in both breeds (Marufu et al., 2011). It is crucial however to investigate the mechanisms of resistance to both ticks in cattle to fully understand the causes of this phenomenon in cattle.

2.5.2 *Rhipicephalus appendiculatus*

Rhipicephalus appendiculatus (brown ear tick) is a 3-host tick, which occurs on a wide variety of domestic and wild ruminants (Jongejan and Uileberg, 2004) and feeds on the ears of its hosts in the adult stage of its life cycle. The brown ear tick has a wide, but patchy, distribution from the tropical regions of East Africa to the temperate regions of South Africa (Norval et al., 1992). The tick has adapted to the large seasonality which exists in the semiarid areas of South Africa by undergoing diapause, a phenomenon in which the adult tick goes through a period of quiescence thought to be controlled by varying day length (Berkvens et al., 1995; Randolph, 1997). On the semiarid rangeland of South Africa, *R. appendiculatus* has been observed to be the most prevalent in cattle reared on communal rangelands (Marufu et al., 2011). The brown ear tick is the vector of *Theileria parva* the cause of bovine theileriosis which has since been eradicated in South Africa (Lawrence et al., 1994).

2.5.3 *Amblyomma hebraeum*

The 'bont tick' is the common name given to ticks of the genus *Amblyomma* with *A. hebraeum* being the predominant species. It inhabits the southern and eastern parts of the subcontinent. *Amblyomma* ticks have a 3-host life cycle and are the only vector of *Ehrlichia ruminantium*, the cause of heartwater in ruminants (Allsopp et al., 2005). Bont ticks were reported to occur only in the warm, moist coastal areas of South Africa (Coetzer et al., 1994). It has recently been reported that *A. hebraeum*'s distribution is expanding to the inland semiarid areas of South Africa (Nyangiwe et al., 2011). The expansion in the distribution of *A. hebraeum* may be associated with more intense periods of drought especially in the inland highlands areas (Estrada Pena et al., 2008).

Ticks are less prevalent in the Nguni breed, suggesting that this breed is more resistant to ticks than imported and local crossbred cattle (Muchenje et al., 2008; Marufu et al., 2011). It is important, however, to correlate tick loads to various phenotypic and immunological parameters in Nguni cattle to establish the mechanisms associated with tick resistance. Ticks differ in the length and size of their mouth parts and in the composition of their salivary gland antigens (Mans et al., 2008). These differences are likely to cause variations in the immunological responses of cattle to ticks of different species and thus mechanisms of resistance employed by cattle against each tick species need to be elucidated.

2.6 Impact of ticks on cattle production

Around 70 % of global cattle production occurs in regions that have the highest prevalence of ticks (Porto Neto et al., 2011). The prevalence of tick infestations, diversity of ticks, and absolute numbers, is significantly greater in the tropics and subtropics compared with temperate regions (Jongejan and Uilenberg, 2004). Ticks are a significant hindrance to cattle production affecting about 800 million cattle around the world (Kaaya, 2000). Apart from causing diseases, ticks cause substantial losses in terms of reduced productivity and fertility and often death, and are economically the most important ectoparasites of cattle (Rajput et al., 2006).

The lack of accurate data on the epidemiology of ticks and TBD makes it difficult to determine their impact. The complexity of determining the direct and indirect economic impact of ticks and TBD and their control, is reflected in the fact that only rough estimates are available for the cost of some of the components (De Castro, 1997). Table 2 shows the estimated costs of ticks and TBD to cattle production in different countries. Although a fairly crude estimate, these values may help to comprehend the importance of ticks and TBD of cattle. These estimates, however, expose the need for more studies on the determination of the economic impact of ticks and TBD in the cattle industry especially in the developing world.

Table 2.1: Estimated costs of tick and tick-borne diseases to cattle production

Country	Costs (US$)	Reference
Global	13-18 billion	de Castro (1997)
South Africa	31.6 million	Minjauw et al. (1999)
Australia	4.09 million	Jonsson et al. (2001)
India	498.7 million	Minjauw and Mcleod (2003)
Australia	170 - 200 million	Playford et al. (2005), Sacket et al. (2006)
Brazil	800 million	Martinez et al. (2006)

Losses are partly due to the direct effects of ticks on cattle, such as damaged hides and skins, anaemia, reduced body weight gains and milk yield, tick toxicoses and mortalities (Gates and Wescott, 2000; Turton, 2001; Mtshali et al., 2004; Kaufman et al., 2006). The damage caused by tick bites also diminishes the value of skins and hides for the manufacture of leather. Ticks with long mouth parts may induce abscesses because of secondary bacterial infections. Depending on the site of infestation, these abscesses can lead to lameness or mastitis resulting in the drop in milk production and subsequent increase in calf mortalities.

Losses due to tick infestation are breed dependent with *Bos indicus* cattle being least affected, *Bos taurus* being severely affected and their crosses having intermediate losses proportional to the level of *Bos indicus* genes. An engorging female tick was reported to cause the loss of up to 1.62 g body weight in *Bos taurus* cattle (Jonsson, 2006). On the semiarid rangelands of South Africa, Nguni calves have been reported to suffer less severe losses in weight of about 4 kg as compared to 28 kg in Hereford calves (Scholtz et al., 1991). Each engorging tick was reported to be responsible for the loss of 1 ml of blood (Rieck, 1957), hence, large infestations could result in losses due to cattle mortalities attributable to anaemia, especially in susceptible hosts.

A large component of the economic cost of ticks in cattle is the application of control measures to reduce infestations (De Castro, 1997; Porto Neto et al., 2011). Conventional tick control is based on the application of acaricides. The practice of intensive tick control spread rapidly throughout Africa following the introduction of imported cattle

breeds and, in South Africa, it was enforced through legislation. There are few global reports on the costs involved in tick control and TBD treatments. Jonsson et al. (2001) estimated the total costs of tick control to contribute up to 49 % of the total costs of ticks and TBD on the dairy industry in Australia. Expenditures for tick control were estimated at US$ 8.43, 13.62 and 21.09 per animal per year for plunge dipping, hand spraying and pour-on, respectively (D'haese et al., 1999). The mean annual cost of ticks and TBD control per animal in pastoral and ranch herds was estimated to be US$4.54 (Ocaido et al., 2009). Improper use of acaricides has increased the incidence of acaricide-resistant ticks, environmental and food contamination (Parizi et al., 2009). The development of new acaricides is also a lengthy and costly process leading to increasing cost of the newer products. Regular dipping has also led to the loss of resistance to ticks and enzootic stability to TBD. Significant losses also arise indirectly due to the important role of ticks in the transmission of TBD.

Losses that can be directly attributed to TBD include mortalities, production effects of chronic cases, cost of veterinary diagnosis and treatment, cost of vaccines, cost of tick control, costs arising from restrictions on movement of cattle (Jonsson et al., 2008). Tick-borne disease can cause downgrading of live animals at sales, and of meat, offal and hides (Tisdell et al., 1999). The major component of economic cost of TBD, which can constitute up to 88% of total costs, is on their control (Ocaido et al., 2009). The control of TBD can be a large and regular part of the variable cost of smallholder farming, with control measures mainly involving a combination of acaricide and grazing management, together with a slowly growing interest in immunisation (Minjauw and McLeod, 2003).

Introduction of more tick-resistant cattle substantially reduces the costs associated with ticks and TBD. This is due to lowered infection rate of TBD because fewer ticks are likely to attach per day due to reduced numbers of ticks in the field and because a smaller proportion of ticks that do develop to feed on infected cattle will in turn be infected (due to lower parasitaemia) (Jonsson et al., 2008).

2.7 Mechanisms of host resistance to ticks

In cattle, the mechanisms of resistance to ticks can be broadly classified into innate, adaptive and non-adaptive immune factors. Innate mechanisms include morphological traits, such as hair length and skin thickness while the non-adaptive immune factors include increased grooming, avoidance behaviour, and reduced area of skin available for infestation (Meltzer, 1996; Kashino et al., 2005). Tick immunity in tick-resistant cattle may also be related to adaptive immune factors such as superior skin reaction to tick antigens and increased cellular and molecular responses at tick feeding sites in the skin.

2.7.1 Morphological characteristics of the coat

Tick load is affected by several innate morphological traits, most of which have a highly inherited pattern (Regitano and Prayaga, 2010). The exhibition of coat characteristics that are unfavourable for tick attachment is an important mechanism of resistance to tick infestation in cattle. Phenotypic coat characteristics such as hair length, skin thickness, coat smoothness and coat colour have an influence on tick counts and are related to tick resistance in cattle on rangelands (Martinez et al., 2006; Foster et al., 2008). Cattle with shorter hairs and smoother coats tend to have lower tick counts compared to those with

longer hairs and woolier coats (Verrisimo et al., 2002; Gasparin et al., 2007). A possible explanation is that ticks may have more difficulty in attaching to short and smooth hairs and that it is easier for short-haired animals to groom themself (Veríssimo et al., 1996). It was also postulated that short hairs expose ticks to harmful climatic conditions and to predators, such as birds, thus, reducing tick loads on cattle (Taylor, 2006).

Coat colour also influences tick resistance in cattle with whiter-coloured animals having significantly lower tick counts than those with darker-coloured coats (Martinez et al., 2006; Gasparin et al., 2007). It was postulated that since ticks are dark coloured, their colour acts as a camouflage on the skin of darker animals, thus protecting the ticks against predators, such as birds (Martinez et al., 2006). de Castro et al. (1991) asserted that cattle with thinner skins could also have a reduced susceptibility to ticks than those with thicker skins, though no plausible explanation was proffered for this assertion. The same authors, however, reported that tick burdens have no relationship with coat colour. Other authors have reported that no relationship exists between morphological coat traits, such as skin thickness and tick resistance (Spickett et al., 1989; Burns et al., 1988). There is, therefore, a need to estimate the relationship between coat traits and tick resistance in cattle.

There is dearth of information on the relationship between coat characteristics and tick resistance in the different cattle breeds reared in semiarid areas of South Africa. Although indigenous Nguni cattle in the semiarid areas are known to be tick resistant, little work has been done to relate this natural tick resistance to different morphological traits of

their coat. Establishing relationships between coat characteristics and tick counts in the indigenous and locally adapted cattle breeds reared on the semiarid rangelands will help to understand the mechanisms of tick resistance and to characterise these cattle breeds. While it is important to understand the phenotypic mechanisms responsible for tick resistance in cattle, it is also essential to determine the immunological mechanisms involved in resistance to ticks in cattle.

2.7.2 Skin hypersensitivity responses of cattle to tick antigen

When ticks attach and feed, they release molecules which stimulate innate and acquired immunological responses in the host (Wikel, 1996). The ability of the host to respond to these molecules results in different levels of resistance in different cattle breeds. Tick feeding induces host immune regulatory and effector pathways involving humoral (antibodies) and cellular immunity (T lymphocytes) (Brossard and Wikel, 1997). Immunologically acquired host resistance to tick feeding can result in decreased feeding, feeding time, and reproductive efficiency of the tick (Kashino et al., 2005).

It has been proposed that cutaneous hypersensitivity reactions to tick antigens are responsible for repelling tick infestation (Kemp et al., 1986). Evidence suggests that skin reactions responsible for repelling tick infestation are different in susceptible and in tick-resistant cattle breeds (Bechara et al., 2000; Piper et al., 2010; Prudencio et al., 2011). It has been shown that repeated tick infestation in tick resistant hosts results in the development of both, an immediate and a strong cutaneous delayed-type hypersensitivity (DTH) reaction to tick antigens which is absent in tick susceptible animals (Szabo et al.,

1995). Bechara et al. (2000), utilising an intradermal test, demonstrated an immediate type of reaction in susceptible *Bos taurus* cattle and both immediate and delayed type hypersensitivity reactions in tick-resistant *Bos indicus* cattle which were pre-sensitised to ticks by natural infestation on the rangeland.

The lack of a DTH in susceptible animals has been attributed to tick saliva introduced during infestations which reduces the ability of a susceptible animal host to respond to tick antigens that could stimulate a protective immune response (Ferreira et al., 2003; Brossard and Wikel, 2004). Piper et al. (2010), to the contrary, noted that an intense immediate type of hypersensitivity response to tick infestation was associated with increased tick resistance in *Bos taurus* cattle infested with large numbers of larvae. The same authors surmised that the resistance obtained by hypersensitive animals that are infested with large numbers of larvae may be partially due to a crowding effect. Prudencio et al. (2011) reported an intense immediate type hypersensitivity reaction which was pronounced in the tick-susceptible than the tick-resistant hosts, and a less pronounced delayed hypersensitivity reaction in the resistant than the susceptible hosts. Inconsistencies in the above reports, would therefore, suggest the need to determine skin hypersensitivity reactions in tick-susceptible and tick-resistant cattle.

Despite the above noted differences in cutaneous responses to tick antigens in hosts of differing tick resistance, little is known about the skin reaction to tick infestation in susceptible and resistant cattle breeds on semiarid rangelands in South Africa. Comparisons of the skin reactions are warranted as they could lead to improved

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understanding of the immunological mechanisms responsible for the differences in tick resistance in cattle on the semiarid rangelands. Inferences from previous studies can lead to postulations that since Nguni cattle are tick resistant, they will demonstrate a delayed type hypersensitivity response; however, the effect of pre-sensitisation or adaptation to ticks on skin responses in this breed is not known. Skin response to infestation by the different tick species that commonly occur on semiarid rangelands also warrants further exploration. Intradermal skin tests have been shown to accurately predict the level of resistance to ticks in cattle based on the size of the skin reaction (Bechara et al., 2000). There is, therefore, a need to conduct these tests in cattle on semiarid rangelands to rank them according to level of resistance. It is also important to investigate cellular responses to tick infestation in different breeds on the semiarid rangelands of South Africa.

2.7.3 Cutaneous cellular responses to tick attachment

The differences in the skin reactions to tick infestation in tick susceptible and resistant cattle hosts may be better understood if the cellullar reactions at tick feeding sites, particularly cell migration, are characterized. Histological examinations of tick infested skin sites have been conducted to elucidate the cellular responses involved in tick resistance in cattle (Gill, 1984; Latif et al., 1991). In these studies, several cell types were thought to be involved in the acquired resistance to ticks in cattle.

Detailed sequential quantitative histological analysis of tick feeding sites following primary and tertiary tick infestation in cattle revealed that the cellular infiltrate on primary infestation was dominated by neutrophils then mononuclear cells whereas the

tertiary infestation cellular infiltrate was characterized by massive degranulation of mast cells and basophils (Gill, 1984). Thus basophils and mast cells appeared to be the major effectors of acquired resistance at tick feeding sites in cattle. Other histopathological studies reported a vigorous granulocyte response especially in the earlier stages of infestations to be characteristic of the immediate type hypersensitivity reaction responsible for tick rejection in tick susceptible taurine cattle (Latif et al., 1991). This granulocytic response in non-resistant hosts was further classified to be predominantly neutrophilic (Walker and Fletcher, 1986; Szabo´ and Bechara 1999). Tatchell and Moorhouse (1970) reported that neutrophils might be responsible for paving the way for tick feeding by destroying the extracellular matrix around the tick attachment lesion allowing ticks access to tissue fluids and blood.

An abundance of mononuclear cells, basophils and eosinophils is characteristic of a delayed type hypersensitivity reaction at tick feeding sites on the skin of highly resistant hosts following repeated infestations (De Castro and Newson, 1993; Szabo´ and Bechara, 1999). Verrissimo et al. (2008) observed that the upper and deep dermis of naturally infested tick-resistant Nelore cattle carried a greater number of mast cells than those in less resistant cattle and inferred that dermal mast cells play an important role in the mechanism of resistance to the cattle tick. Mast cells, and the histamine they contain inside cytoplasmic granules, are of fundamental importance to the self-grooming mechanism, which is thought to be critical to resistance of cattle to ticks (Kousataal et al., 1976; Kemp and Bourne, 1980; Schleger et al., 1981). Carvalho et al. (2010) reported that increased resistance to adult *R. microplus* ticks was associated with high eosinophil

presence in the tick bite site of infested cattle and concluded that resistant bovines have a greater capacity than susceptible hosts to retain eosinophils in the lesion of adult tick-infested skin. Eosinophils are thought to be involved in the translocation of mast cell histamine to the tick attachment site resulting in increased grooming and tick rejection in cattle (Schleger et al. 1981, Francischetti et al., 2009). Constantinou et al. (2010) observed the presence of consistently higher numbers of T cells in the resistant *Bos indicus* cattle and suggested that these cells might have a role in resistance to infestation. This was supported by reports of Piper et al. (2009) that tick-resistant *Bos indicus* cattle develop a T-cell-mediated response to infestation which is absent in the *Bos taurus* cattle.

Histological analyses of tick feeding sites on cattle reared in semiarid areas of South Africa are yet to be conducted. Comparisons of the histopathological responses on skin feeding sites of ticks in cattle of differing resistance on semiarid rangelands will aid in improving knowledge on the cellullar reactions to ticks in the bovine host. Elucidating the cellular mechanisms by which tick resistant cattle prevent heavy infestation is also important for the comprehension of TBD transmission and can also aid the development of alternative immune-based tick control methods. The correlation between breed and cellular responses to tick infestation and its relationship to tick immunity in the different cattle breed in semiarid areas also requires investigation. Studying the histopathology of tick attachment sites in cattle to characterise the cellular reaction to ticks in this breed will aid in the establishment of the immunological mechanisms involved in resistance to ticks. The cellular responses to tick infestation could also be better understood if gene expression of tick infestation sites on cattle were evaluated.

2.7.4 Gene-expression associated with tick infestation

Gene-expression provides insight into the biological mechanisms, genes, and pathways by which cattle respond to tick infestation. It is thought that gene expression associated with tick infestation in tick resistant and tick susceptible cattle breeds differs (Wang et al., 2007; Piper et al., 2008; 2010). Wang et al. (2007) and Piper et al. (2010) attributed the differences in histopathology to tick infestations in resistant and susceptible cattle to the differential expression of the genes involved in skin responsiveness to tick infestation. Genes involved in innate inflammatory processes and immune responsiveness to tick infestation were observed to be up-regulated in tick-infested susceptible cattle, resulting in increased cellular inflammatory response which possibly led to increased tick susceptibility. The higher expression of many genes involved in innate inflammatory processes in the susceptible animals at tick attachment sites suggests a non-directed pathological response to infestation (Piper et al., 2008). In the tick resistant cattle, the genes were not up-regulated leading to a reduced cellular response and thus reduced susceptibility to ticks (Wang et al., 2007). The most notable group of genes that were detected as differentially expressed between tick-naïve and tick-infested cattle were the cytokines, chemokines and complement factors up-regulated in the tick-infested animals. The higher expression of these chemokines (that target monocytes, T cells and natural killer cells, among others), suggests that non-resident, inflammatory cell populations are being actively recruited into the dermis at the site of tick attachment (Piper et al., 2010).

Differences in the expression of genes associated with the extracellular compartment were found between tick infested and non-infested cattle, independent of breed type (Wang et al., 2007; Piper et al., 2010). Highly tick-resistant Belmont Red cattle showed greater expression of the keratins KRT5 and KRT14 and epidermal barrier catalysing enzyme transglutaminase 1 (TGM1) than low tick-resistant Belmont Red cattle in skin adjacent to the site of tick attachment (Kongsuwan et al., 2010). These studies highlight the important role of non-immune, structural components in determining tick resistance in cattle (Porto Neto et al., 2011). Variation in gene expression has been observed between outbred individuals within a population, and this variation is exacerbated when experimental groups are subjected to trauma or disease (Whitehead and Crawford, 2006). Piper et al. (2008) demonstrated relatively large degrees of variation in gene expression within animal, within breed and between breed. These major shortcomings indicate the need for large biological replication in gene expression studies.

2.8 Heritability and genetic selection for tick resistance

A genetic basis for variation in tick resistance, within and between breeds, has been recognised for many years (Wilkinson, 1955; Francis, 1966). Zebu cattle such as the Brahman (*Bos indicus*) are recognised to be generally more resistant to ticks than European cattle breeds (*Bos taurus*) such as the Angus and Hereford. African cattle breeds (*Bos taurus africanus*), such as the Nguni, have also been shown to be more resistant to ticks than imported and local crossbred cattle. The host genetic resistance to ectoparasites is thought to be as heritable as milk yield or growth, and breed resistance to ticks can be increased to very high levels by natural selection.

Estimates of heritabilities for tick infestation are shown in Table 2.2. The moderate heritability estimates for tick count indicate that genetic improvement through selection for tick resistance could be effective. It should be noted that low tick infestations in cattle, and use of tick scores instead of tick counts could result in the lowering of heritability estimates (Prayaga and Henshall, 2005; Prayaga et al., 2009). Genetic variation in tick resistance between cattle increases as natural infestation increases under extensive conditions (Budelli et al., 2009). Despite the generally moderate heritability estimates across various breeds implying a scope for selection, one difficulty lies with the trait measurement hindering application in traditional genetic evaluation systems (Regitano and Prayaga, 2010).

Factors that may contribute to variability in estimating of heritability could be related to environmental factors that affect the intensity of the natural challenge, cattle breed, production system used, immunological maturity and pre-sensitisation to tick infestation (Porto Neto et al., 2011). Given the high genetic variability among individuals and breeds (Morris, 2007), the identification of superior genes is thus important for the development of breeding programmes for tick resistance in cattle. Information on resistance status within the various breeds of cattle reared on the semiarid rangelands is needed to provide a basis for selection, by either breeding from animals with resistance, or culling cattle with low tick resistance, or both (Budeli et al., 2009). It is, however, important to be mindful of the correlated responses in other economic traits such as growth, meat quality, or milk yield as a consequence of selection for tick resistance.

Table 2.2: Heritability estimates for tick infestation reported in literature

h^2	Country	Breed	Tick trait	Challenge	Reference
0.44	Australia	Africander cross	Count	Natural	Burrow (2001)
0.37	Australia	Taurine composites	Count	Natural	Turner et al. (2010)
0.34	Australia	Brahman Zebu crosses	Count	Natural	Davies (1993)
0.21	Brazil	Gyr X Holstein F2	Count	Artificial	Machado et al. (2010)
0.15	Australia	Brahman	Score	Natural	Prayaga et al. (2009)
0.13	Australia	Cross-bred	Count	Natural	Prayaga and Henshall (2005)
0.05 – 0.17	South Africa	Bonsmara Belmont	Count	Natural	Budeli et al. (2009)

Prayega and Henshall (2005) observed moderately positive genetic correlations among tick infestation, internal parasite burdens and heat resistance, and proposed that closely-linked genes affect these adaptive traits. The same authors however reported lowly positive correlations between tick infestation and growth traits, such as birth weight and weaning weight. Lack of association between adaptive traits such as tick counts and related production traits (such as milk yield) suggests that selection for productive traits in tropical beef cattle genotypes does not adversely affect tropical adaptability of cattle (Prayega et al., 2009; Turner et al., 2010). It is important to first determine tick resistance parameters in cattle as these will enable the determination of heritability estimates of tick resistance.

2.9 Evaluation of tick resistance in cattle

There are several methods of estimating tick resistance in cattle including amongst others enumeration of ticks on cattle, skin hypersenisitivity tests and histological analysis of tick infested sites.

2.9.1 Tick counts

Enumeration of ticks on the skin of cattle has long been used as a means of estimating tick resistance in different animal species. The majority of studies that have evaluated tick burden in cattle have used multiple counts of ticks after artificial infestations with known (approximate) numbers of larvae. Tick resistance was then expressed as 100 minus the percentage yield of female tick larvae applied. Other studies have used

multiple counts of engorged adult ticks after natural tick infestation of cattle grazed on tick-infested rangelands. A valid concern regarding artificial tick infestations is that the tick-host associations studied are not those normally occurring in nature, and that acquired resistance is more prominent in these relationships than in natural infestations (Ribeiro, 1989; Boppana et al., 2005).

Tick scores have also been used such as from 0 (no ticks) to 5 (visually estimated > 150 ticks) to estimate tick burdens and thus tick resistance in cattle (Fraga et al., 2003; Prayaga et al., 2009). Tick scores are quicker to obtain and allow the phenotyping of a larger number of cattle compared with tick counts. The grouping of quantitative measure into tick scores reduces the precision of the measure. Tick counts remain a reliable indicator of tick resistance. Acquired resistance has been most often observed after infestation by female ticks. Feeding male ixodids also induce acquired resistance, but to a lesser degree than that caused by females alone or together with males (Maharana et al., 2011). When evaluating natural tick infestations, engorging adult female ticks are the easiest tick stage to identify and enumerate. Tick counts of engorging adult female ticks will be used in the present study as these are deemed a reliable proxy for tick resistance in cattle.

2.9.2 Skin hypersensitivity tests

The skin hypersensitivity test or intradermal testing has long been used to identify antigens responsible for allergic reactions. Intradermal inoculation of tick salivary antigens to elicit cutaneous hypersensitivity reactions has also been used to broadly

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evaluate immune responses to ticks in hosts and is useful in classifying them according to level of susceptibility (Bechara et al., 2000). Use of the skin test to classify cutaneous hypersensitivity responses is documented for dogs (Mukai et al., 2002; Ferreira et al., 2003), rabbits (Hlatshwayo et al., 2004), horses (Szabo et al., 2004) and bovines (Bechara et al., 2000; Prudencio et al., 2011). The extent of cutaneous reaction generally depends upon duration of exposure of the cattle to tick infestation (Hlatshwayo et al., 2004). The cutaneous hypersensitivity test is thus useful as a feasible tool to assess the tick-resistance status of previously exposed animals to ticks. Cutaneous hypersensitivity responses to tick infestation in cattle of different breeds in semiarid areas of South Africa are largely unknown.

2.9.3 Histological analyses

Various techniques have been employed to evaluate the cellular responses to tick infestation in cattle. Measurement of cellular parameters in the peripheral circulation of cattle was used to assess the cellular response of susceptible and resistant cattle breeds to tick infestation (Piper et al., 2009). It should be noted, however, that changes in cellular composition of the peripheral circulation may not necessarily reflect the changes at the tick-host interface, that is the skin, and hence cellular dynamics at the attachment sites of ticks would require evaluation. Other studies have used microscopic evaluation of cell counts utilising various staining techniques on skin biopsies taken at tick attachment sites as a reliable tool to evaluate cellular responses to ticks in different cattle breeds (Carvalho et al. 2010; Constantinoiu et al. 2010; Piper et al., 2010). Though cell counts are deemed laborious in the selection of individual animals for tick resistance, previous studies show

that they can be appropriately used to immunological test for resistance. Cellular responses of Nguni cattle need to be evaluated using histological analyses of inflammatory cell infiltrates in skin biopsies from feeding sites at tick attachment sites.

2.10 Summary

Ticks and TBD are a major constraint to cattle production especially in the semiarid areas. The molecular prevalence of bovine anaplasmosis in Nguni and local crossbred cattle reared under the smallholder production system in semiarid areas of South Africa is unknown. Though the Nguni breed has been observed to be tick resistant and adapted to the semiarid environment, there is a dearth of information on the mechanisms of resistance employed by the breed to combat ticks and TBD. Information regarding the response of Nguni cattle to infestations by the diverse range of tick species found in the semiarid areas is also limited. There is therefore a need to determine the molecular prevalence of bovine anaplasmosis and investigate the mechanisms employed by Nguni cattle to combat this disease. It is also imperative to establish the coat characteristics associated with tick infestation, and hypersensitivity and cutaneous cellular responses of Nguni cattle to infestation by *Rhipicephalus* tick species. The main objective of the present study was to determine the mechanisms of resistance to ticks and TBD in Nguni cattle.

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CHAPTER 3: Molecular prevalence of *Anaplasma marginale* in Nguni and local crossbred cattle reared in the smallholder production systems in South Africa

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Abstract

Little is known about the prevalence of *A. marginale* in the cattle breeds reared by smallholder farmers despite it being one of the leading causes of cattle mortality in the semiarid farming areas of South Africa. The objective of the current study was to establish the molecular prevalence of *A. marginale* in Nguni and local crossbred cattle in smallholder production systems in South Africa. The molecular prevalence of *A. marginale* was determined by PCR from the blood of Nguni and local crossbred cattle from different production systems, age groups and both sexes. Body condition score (BCS), packed cell volume (PCV) and tick infestation levels were also determined for each animal. Molecular prevalence of *A. marginale* in cattle reared under the smallholder farming system was moderate. Nguni and local crossbred cattle had similar prevalence of *A. marginale*. High levels of infection in calves and immunity in adult cattle, coupled with the absence of clinical disease were observed and reflective of a situation of endemic stability to bovine anaplasmosis. Calves, cattle with low BCS and those on small scale farms had higher ($P < 0.05$) odds of being infected by *A. marginale*. Nguni cattle suffered less severe losses from and were more resilient to *A. marginale* infection than local crossbreds. Further elucidation of the genotype associated resilience to anaplasmosis in indigenous Nguni cattle is required.

Keywords: Endemic stability; haemoparasite; polymerase chain reaction; production system.

3.1 Introduction

Bovine anaplasmosis, caused by the rickettsial haemoparasite *Anaplasma marginale* (*A. marginale*) and transmitted to cattle biologically by *Rhipicephalus* (*Boophilus*) ticks and mechanically by flies and fomites (Aubrey and Geale, 2010), is the most important cause of cattle mortalities in low-input farming areas in South Africa (Mapiye et al., 2009; Ndou et al., 2010). The disease results in considerable economic losses to the cattle industry through decreased productivity, lowered working efficiency of cattle, increased veterinary costs and cattle mortality (Kocan et al., 2003). Cost-effective control of bovine anaplasmosis depends on the availability of accurate prevalence data which is, however, scanty particularly in small scale (resettled emerging) and communal farming areas that constitute the smallholder production system in South Africa.

Epidemiological field studies using serological tests that detect antibodies reactive with tick borne haemoparasites in cattle in communal areas have shown that the Nguni genotype has a lower sero-prevalence of *A. marginale* than the local crossbreeds (Nguni x imported crosses) (Marufu et al., 2010). It was thus postulated that the Nguni genotype could have superior resistance to bovine anaplasmosis than local crossbreds. Serology-based techniques have a major disadvantage of cross reactivity between species and they do not differentiate current from previous infection (Kocanet al., 2010). Polymerase chain reaction (PCR)-based detection methods are extremely sensitive and specific in the

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detection of *A. marginale* infections in cattle (de La Fuente et al., 2005; Molad et al., 2006). Accurate data on prevalence of bovine anaplasmosis obtained by such highly sensitive and specific molecular techniques are crucial not only for developing appropriate control measures but for providing an understanding of host resistance in different cattle genotypes.

A strong relationship exists between nutrition and disease infections in cattle (Coop and Kyriazakis, 1999). Animals with higher levels of protein and/or energy are better able to control the establishment of new diseases and reduce fecundity of existing pathogens (Coop and Holmes, 1996). The role of nutrition and its relationship with resistance to bovine anaplasmosis in cattle of different breeds in the low input system is, however, unclear. Determining the contribution of nutrition to the resistance to *A. marginale* infection in cattle may be important in understanding the physiological mechanisms of resistance to bovine anaplasmosis. It is also essential to investigate the influence of *A. marginale* infection on nutritional performance measures such as body weight, body condition score and packed cell volume in cattle of differing resistance. Determining the associations between molecular prevalence of *A. marginale* and production parameters in the different cattle breeds would also be important for estimating production losses in each breed. The objective of the current study was to determine the molecular prevalence of *A. marginale* in Nguni and local crossbred cattle in the low input farming system. It was hypothesised that the prevalence of bovine anaplasmosis is dissimilar in Nguni and local crossbred cattle reared by smallholder farmers in the semiarid areas of South Africa.

3.2 Materials and methods

3.2.1 Study site and farmer selection

Blood samples were collected in June 2010 at communal areas and small scale farms located in the Sakhisizwe Local Municipality of Chris Hani District Municipality in the Eastern Cape Province, South Africa (Figure 3.1). The study area is located on 27° 50′ East and 31° 27′ South, and composed of a sour rangeland in which forages have low nutritive value and are largely unpalatable during the dry season (Ellery et al., 1995). The most common grass species are *Themeda triandra*, *Sporobolus africanus* and *Microchloa ciliate*. *Euryops pyroides*, *Chrysocoma ciliate* and *Dyspyrose scrabrida* are the common bush species in the areas (Lesoli, 2008). The study area lies at an altitude of between 1350 and 1900 m and receives moderate average annual rainfall of between 600 and 800 mm which mostly occurs during the wet season (November to April). Average temperature is highest in the hot wet season (20 °C) and lowest in the cool dry season (11 °C). Cattle graze on rangelands throughout the year.
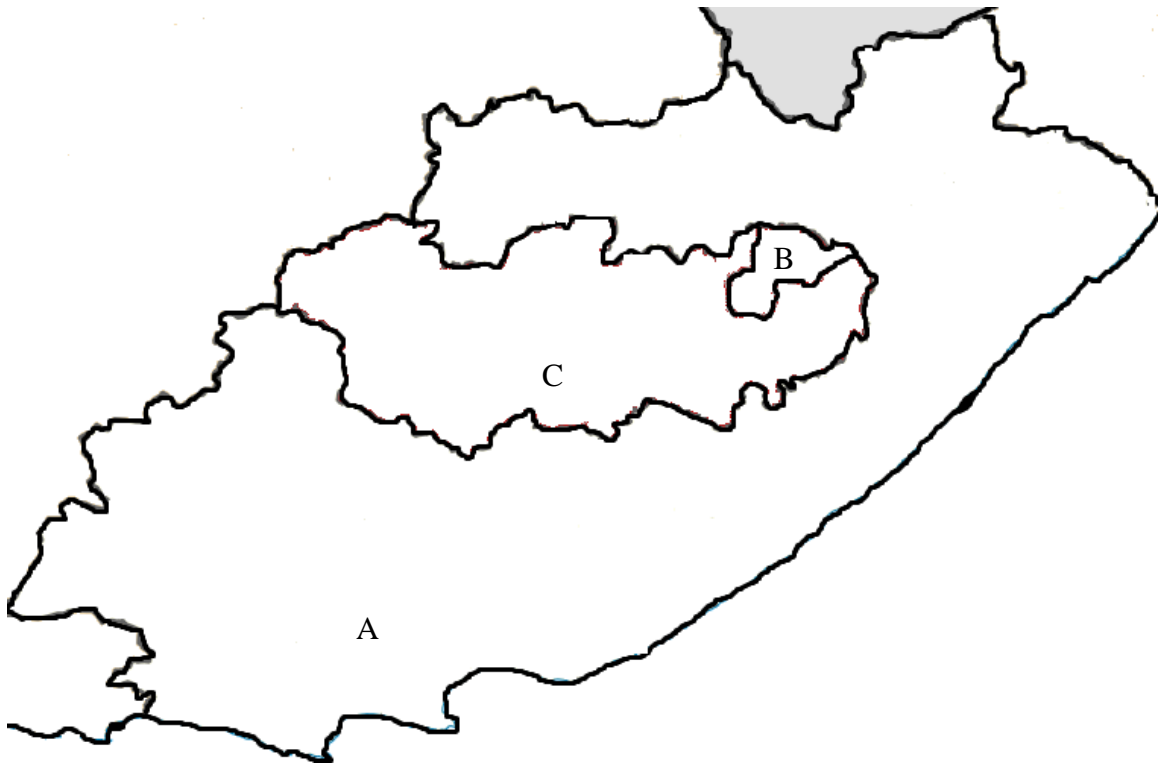
Figure 3.1: Map of the Eastern Cape Province (A) showing the study area Sakhisizwe Municipality (B) within the Chris Hani District Municipality (C)

Stratified random sampling based on the production system was used to select the three communities and three farms sampled in the study. Small scale farms that were owned by beneficiaries of the government's land restitution programme and the surrounding communal areas were chosen. Land restitution is a government-initiated programme of redistributing commercial agricultural land to benefit previously disadvantaged farmers (Mapekula et al., 2009).

3.2.2 Study animals

All experimental procedures were specifically approved for this study by the University of KwaZulu-Natal Animal Ethics Research Committee (Reference number: 097/11/Animal) (Appendix 4) and were in compliance with internationally accepted standards for animal welfare and ethics. A total of 149 clinically healthy cattle classified according to genotype (70 Nguni and 79 local crossbred), sex (68 male and 81 female), age (72 less than 2 years old and 77 older than 2 years old) and production system (75 communal and 74 small scale) were selected and sampled. The animals were selected on the basis of the owners' willingness to participate in the study. Small scale farmers dipped their cattle four times in the wet season (November to February), and twice in the dry season (May to October). Communal farmers depended on State Veterinary Services for dipping which was conducted fortnightly in the wet season (November to February) and monthly in the dry season (May to October) at the communal dipping tank. All animals were grazed on natural rangelands throughout the study period.

3.2.3 Body weights, body condition scores and tick infestation levels

Assessment of body weights, body condition scores and tick infestation levels of the study animals was conducted once in the cool dry season (June 2010). For each animal, the body weight was estimated using a cattle weigh-band whilst visual assessment of the body condition was made using the five-point scoring system (1 – very thin and 5 – obese) (Osoro and Wright, 1992). Engorged adult *Rhipicephalus* (*Boophilus*) ticks were counted from the whole body of each animal. The tick counts were classified into three infestation levels as follows: low (<30 engorged adult ticks on the whole body), moderate

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(> 30 – 50 engorged adult ticks on the whole body) and high (> 50 engorged adult ticks on the whole body).

3.2.4 Blood collection

Blood samples for packed cell volume (PCV) determination and *A. marginale* detection were collected from the 149 animals. The cattle were held in a crush while the blood samples were collected from the tail vein using an 18 gauge needle into two well labelled, blood tubes containing ethelenediaminetetraacetic acid for each animal. One blood tube was stored at 4 ^{0}C and used for PCV determination while the other was stored at -20 ^{0}C and used for deoxyribonucleic acid (DNA) extraction.

3.2.5 Determination of packed cell volume

For the determination of PCV blood which was stored at 4 ^{0}C was transferred into micro-haematocrit tubes and centrifuged in a micro-haematocrit centrifuge (Gemmy Industrial Corp., Taiwan) for 3 minutes. Reading of the PCV was performed on a Micro-haematocrit Reader Scale.

3.2.6 DNA extraction and amplification

For each blood sample, DNA was extracted from 100 μl of EDTA blood using the ZR Genomic DNATM- Tissue MiniPrep Kit (Zymo Research, California, USA) at the National Zoological Gardens Parasitology Laboratory. The DNA was re-suspended in sterile distilled water and stored at −20 °C until used in PCRs. The 1733 F: 5'-TGTGCTTATGGCAGACATTTC-3'   and   2957   R: 5'-

AAACCTTGTAGCCCCAACTTATCC-3' genes were amplified from 1 μg *A. marginale* DNA by PCR using 10 pmol of each primer (1733 F and 2957 R) in a 25 μl volume (12.5 μl DreamTaq™ Green PCR Master Mix, 10 pmol of each primer and 1 μg of Template DNA) in the BOECO Thermal cycler (Hamburg, Germany). The amplification cycles, following an initial denaturation at 94 °C for 3 minutes, consisted of 35 cycles of 1 minute at 94 °C, 1 minute at 60 °C and 1 minute at 72 °C, followed by a final cycle with a 10 minutes extension step at 72 °C. Amplified PCR products were separated in 1% TBE (89 mM Tris, 89 mM Boric acid, 2 mM EDTA) agarose gel, using GeneRuler™ 1Kb Plus DNA ladder (Fermentas Life Sciences, USA). Gel was visualized and photographed under UV illumination after Biotium GelRed acid staining.

Molecular prevalence of *A. marginale* was calculated as:

$$P = \frac{d}{n} \times 100$$

where P = prevalence; d = number of animals that test positive for DNA to *A. marginale*; and n = total number of animals tested for *A. marginale* (Thrusfield, 1995).

3.2.7 Statistical analyses

Data were analysed using Statistical Analysis System Version 9.2 (SAS, 2009). To test for normality, the data was subjected to univariate analysis. Data for BCS were not normally distributed and were subjected to square root transformation to confer normality. The effect of production system, genotype, age, sex, infection with *A. marginale* and their interactions on the body weight, BCS and PCV were determined

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using PROC GLM (SAS, 2009). Comparisons of least square means were done using the PDIFF option. The chi square test was used to determine the associations between tick infestation level or molecular prevalence of *A. marginale* and production system, genotype, age and sex. Logistic regression was used to determine the odds of infection with *A. marginale* between production systems, breeds and sexes and across seasons and age groups.

3.3 Results

3.3.1 Molecular prevalence of *A. marginale*

A sample photograph of the agarose gel plate after exposure to ultraviolet light, with some positive reactions, is shown in Figure 3.2. Of the 149 cattle sampled for molecular prevalence in the study, 88 (59.1%) were infected with *A. marginale*, 42 (47.7%) being of the Nguni genotype, while 46 (52.3%) were local crossbreeds. There was an association (P < 0.05) between molecular prevalence of *A. marginale* and production system (χ^2= 46.8; P<0.05). Cattle in the communal production system had a lower (P < 0.05) molecular prevalence of *A. marginale* than those in the small scale system (Table 3.1). There were no associations (P > 0.05) between molecular prevalence of *A. marginale* and genotype, age and sex.
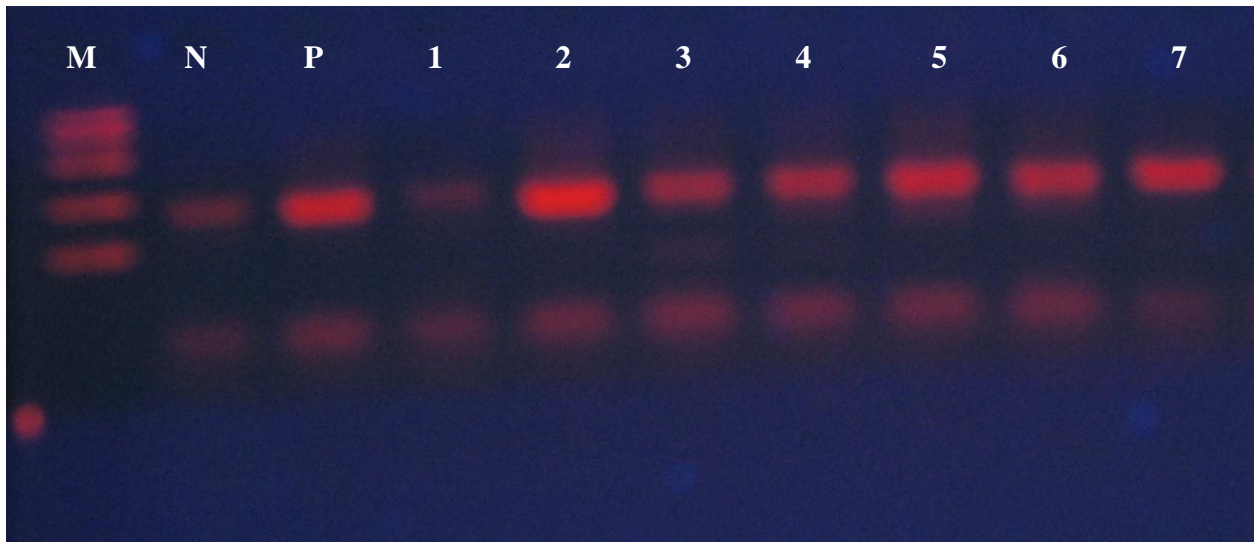
Figure 3.2: Photograph of the gel plate (A and B) showing some of the PCR products visualized under ultraviolet illumination. M: Molecular marker, N: Negative control, P: Positive control, 1-7: Test samples. P and Test sample 2 were strongly positive while samples 3-7 were weakly positive and N and Test sample 1 were negative

Table 3.1: Molecular prevalence of *Anaplasma marginale* in different production systems, genotypes, sexes and age groups of cattle in the low input farming system

Parameter	N	Prevalence (%)	χ^2	Significance
Management type				
Communal	77	30.6	46.8	*
Small scale	78	85.7		
Genotype				
Nguni	70	47.7	1.5	NS
Local crossbred	79	52.3		
Sex				
Male	68	49.4	0.6	NS
Female	81	53.2		
Age				
< 2 years old	72	47.4	2.3	NS
> 2 years old	77	36.9		

* Significant at $P < 0.05$; NS not significant

3.3.2 Probability of infection with *A. marginale*

The odds of an animal being infected by *A. marginale* were higher (P < 0.05) for cattle in the small scale farms than in the communal areas (Table 3.2). Animals below two years old had higher odds of infection by bovine anaplasmosis than older animals. Cattle with high BCS had lower odds of infection with *A. marginale* than those with low BCS.

3.3.3 Effect of *A. marginale* infection on body weights, body condition scores and packed cell volume

The interaction between infection with *A. marginale* and genotype and between infection with *A. marginale*, genotype and production system had a significant (P < 0.05) effect on the body weight of the animals. Uninfected local crossbred cattle had higher (P < 0.05) body weights than their infected counterparts in the communal and small scale areas, while both infected and uninfected Nguni cattle had similar (P > 0.05) body weights in both production systems (Table 3.3). There was a significant interaction between infection with *A. marginale* and genotype on the BCS of the animals. Infected and non-infected Nguni cattle had similar mean BCS which were higher (P < 0.05) than those of non-infected local crossbred cattle while infected local crossbred cattle had the least mean BCS (Table 3.3). The interaction between infection with *A. marginale* and age on PCV was also significant. Young infected animals had significantly lower (P < 0.05) PCV than young non-infected animals, older infected and older non infected animals (Table 3.4).

Table 3.2: Odds ratio estimates, lower and upper confidence interval (CI) of an animal being infected by *Anaplasma marginale* in the smallholder areas

Infection with *A. marginale*	Point estimate	Lower CI	Upper CI
Management type	15.52	6.41	37.59
Tick infestation level	7.45	0.83	67.07
Body condition score	2.95	1.19	7.29
Age	0.56	0.36	0.88

Table 3.3: Least square mean (± standard error) body weight, body condition score and packed cell volume of infected & non-infected Nguni and local crossbred cattle in each production system

Variable	Production system	Infected		Non-Infected	
		Nguni	Local crossbred	Nguni	Local crossbred
BCS	Communal	2.7 ± 0.17^c	2.3 ± 0.09^a	2.8 ± 0.15^c	2.4 ± 0.15^b
	Small scale	2.7 ± 0.16^c	2.2 ± 0.09^a	2.7 ± 0.24^c	2.4 ± 0.09^b
Weight	Communal	327.3 ± 33.69^b	284.9 ± 23.82^a	363.8 ± 14.59^b	388.3 ± 24.65^c
	Small scale	331.8 ± 27.74^b	321.6 ± 39.33^a	370.2 ± 14.15^c	393.4 ± 26.46^c
PCV	Communal	34.5 ± 1.62	34.2 ± 1.39	37.0 ± 1.47	32.4 ± 0.85
	Small scale	37.2 ± 1.60	34.1 ± 1.34	38.0 ± 2.50	33.0 ± 3.41

[a, b, c] Means in the same row with different superscripts are significantly different at $P < 0.05$.

Table 3.4: Least square mean (± standard error) packed cell volume of infected and non-infected young and old cattle

Age	Infected	Non-infected
Young (< 2 years)	2.35 ± 0.137^a	2.58 ± 0.147^b
Old (> 2 years)	2.69 ± 0.146^b	2.79 ± 0.188^c

[a, b, c] Means with different superscripts are significantly different at $P < 0.05$.

3.3.4 Tick infestation levels

Tick infestation levels were associated with management type ($\chi^2 = 14.2$; $P < 0.05$). Cattle on the small scale farms had higher ($P < 0.05$) tick infestation levels than those in the communal areas. Genotype, age and sex were not significantly associated with tick infestation levels in cattle.

3.4 Discussion

The overall molecular prevalence of 59.1 % observed for *A. marginale* in the current study was moderate and similar to that reported by Mtshali et al. (2007) for cattle in the Free State Province. However, it was higher than that reported previously in a sero-prevalence study for communal cattle in the Eastern Cape Province by Marufu et al. (2010). Differences in the prevalence between the two studies could be attributed to the inclusion of small scale farms in the present study which are known to have different tick and TBD control strategies to communal farmers (Bryson et al., 2002; Rikhotso et al., 2005). The differences in the specificity of the serological methods used by Marufu et al. (2010) and the molecular techniques used in the current study could also explain the disparities in the observed prevalence. Molecular diagnostic techniques used in the present study detect active infection and thus provide more accurate temporal data on disease prevalence status in the study area than serological tests used in the previous study which only detect exposure to infection which is not necessarily current.

The observed differences in molecular prevalence of *A. marginale* in the small scale and communal production systems were likely influenced by variations in the control and

infestation levels of ticks, the major biological vectors of *A. marginale*. Communal farmers rely heavily on the government subsidised dipping programme (Rikhotso et al. 2005) while small scale farmers utilise their own resources to purchase acaricides (Mapiye et al., 2009). In the present study acaricides were applied once fortnightly in the communal cattle herds and approximately once monthly and according to tick infestation levels in the small scale areas. Increased host (cattle) - vector (tick) contact times result in increased TBD transmission rates (Muhanguzi et al., 2010) which could have caused the observed higher odds of infection with *A. marginale* in the small scale production system.

Despite higher prevalence of *A. marginale* in the study area, clinical disease in cattle was not observed in the animals, which might reflect a situation of endemic stability. Endemic stability is an epidemiological state, in which clinical disease is scarce despite high levels of infection in the population (Coleman et al., 2001; Jonsson et al., 2012). Such a situation arises if the force of infection is high enough that acquisition of functional immunity occurs in the majority of the population at a relatively young age, when the disease is often mild compared with disease in older animals (Hay, 2001). In the present study, younger animals had a higher prevalence of *A. marginale* and higher odds of infection than adult cattle, which could likely have contributed to the development of endemic stability. To minimize the direct effects of ticks in cattle while conserving endemic stability, farmers have to maintain tick loads above a minimum threshold (Eisler et al., 2003). This could be achieved by dipping cattle at strategic times when infestations on rangelands are higher especially during the hot-dry and hot-wet seasons (Marufu et al., 2011). Strategic dipping has been shown to decrease the sero-prevalence of bovine

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anaplasmosis in cattle (Rikhotso et al., 2005). The minimum threshold for ticks on beef cattle populations owned by smallholder farmers in semiarid areas, however, is still vague and needs to be established.

The absence of clinical disease in young animals in the current study despite high odds of infection reinforces the view that young animals are less susceptible to clinical bovine anaplasmosis (Kocan et al., 2003). The cause of reduced susceptibility to anaplasmosis in young animals is not well understood. Variation in susceptibility to *A. marginale* between young and old cattle is thought to arise from the differences in the dominant immune response (innate or acquired) to *A. marginale* infection in the two catergories (Muhanguzi et al., 2010). Young animals however most likely become persistently infected or "carriers" for life (de La Fuente et al., 2010) despite their higher immunity to infection. Low input farmers need to be wary of persistently infected animals as they serve as reservoirs of infection for the tick vectors and sources of infection for possible sporadic outbreaks in the herd. To reduce the chances of sporadic outbreaks especially in susceptible adult cattle, smallholder farmers could vaccinate their animals using live or modified vaccines (Aubrey and Geale, 2010). It should be noted that vaccination does not prevent cattle from becoming persistently infected (Kocan et al., 2010); it does, however, reduce the economic impact of the disease.

Higher infection rate of *A. marginale* in younger animals negatively affected the nutritional status (reduced BCS and PCV) of this group in the present study. Infection with *A. marginale* causes anorexia resulting in reduction of weight and condition (Aubrey

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and Geale, 2010; Kahn, 2006). *Anaplasma marginale*, replicates inside red blood cells causing increased extravascular haemolysis and reduction in the PCV (Riond et al., 2008; Kocan et al., 2010). The risk of infection with *A. marginale* was observed to be greater in poorly conditioned animals, thus showing an important link between nutrition and disease in cattle in the present study. Both cellular and humoral components of the immune system are required to combat infection of the blood-borne haemoparasite. Adequate nutrition thus plays an important role in replenishing the molecules lost during the battle against infection. Improving the appetite and nutritional status, especially in young cattle by providing vitamin and supplementary feed, respectively, can result in increased immunity (Marufu et al., 2010) and thus improved resistance of cattle in low input areas to *A. marginale* infection.

In the present study, genotype was not associated with the molecular prevalence of *A. marginale* suggesting that there are no differences in innate immunity to anaplasmosis between the two genotypes. This finding contrasts the earlier report by Marufu et al. (2010) that genotype is associated with differences in sero-prevalence between Nguni and local crossbreds. Differences in tick loads, and hence transmission rates of the TBD, were cited as the reasons for genotype related differences in *A. marginale* prevalence in the earlier study, which was not the case in the current study. Bock and de Vos (1999) reported similarities in prevalence between *Bos indicus* and their crosses in Australia and attributed this to similarities in innate resistance to infection in the two genotypes. Mattioli et al. (2000) hypothesized that a more effective cellular immune response in addition to innate immunity leads to superior resistance to tick-borne infections in

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indigenous cattle breeds. It should be noted that all breeds of cattle may be at risk of severe disease if exposed to virulent *A. marginale* especially for the first time (Bock and de Vos, 1999).

Infection with *A. marginale* did not affect the body weight and condition scores of Nguni cattle suggesting that the Nguni breed is more resilient to the adverse effects of infection with *A. marginale* than local crossbreds, despite the similar odds of infection in the two genotypes studied presently. The Nguni genotype managed to maintain BCS despite infection with *A. marginale* serving as testimony to the genotype's reduced susceptibility to the debilitating effects of the disease. The actual mechanisms that render Nguni cattle less susceptible to the effects of *A. marginale* infection still remain unclear. Further investigations are thus warranted to elucidate the mechanisms of resistance to *A. marginale* infection in the Nguni genotype to improve knowledge on genotype related host resistance in indigenous cattle. The high tick infestation levels in cattle in small scale farming areas could result in direct losses such as tick worry, anaemia, damage to hides and skins of animals and tick toxicoses. High tick burdens could lead to immunosuppression in cattle facilitating the transmission of anaplasmosis (Jonsson, 2006). To avoid direct losses caused by high tick infestations in cattle, small scale farmers could select for and breed cattle with shorter and smoother coats as they tend to be less susceptibile to ticks (Marufu et al., 2011). The resultant reduced tick load could also play an important role in reducing challenge to cattle but maintaining endemic stability to *A. marginale* in cattle.

3.5 Conclusions

The molecular prevalence of *A. marginale* was moderate in the low input production system with cattle in the small scale farms having higher prevalence than those in the communal areas. A situation of endemic stability to bovine anaplasmosis was observed characterised by the absence of clinical disease despite high levels of infection in calves, and a high level of immunity in adult cattle. Cattle were more likely to be infected with *A. marginale* if they were young, resident on small scale farms and in poor body condition. Nguni cattle were more resilient to anaplasmosis and suffered less severe losses from A. *marginale* infection than local crossbreds. Further elucidation of the role of coat characteristics in tick resistance and their association with resilience to anaplasmosis in indigenous Nguni cattle is required.

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CHAPTER 4: Relationships between tick counts and coat characteristics in Nguni and Bonsmara cattle reared on semiarid rangelands in South Africa

(Published in *Ticks and Tick-borne Diseases*, see Appendix 2)

Abstract

Indigenous Nguni cattle are adapted to the semiarid rangeland and appear to be resistant to ticks; however, the mechanism for tick resistance is yet to be established. To understand tick resistance in cattle, relationships among skin thickness, hair length, coat score and tick counts were evaluated in Nguni (n = 12) and Bonsmara (n = 12) heifers on semiarid rangelands of South Africa. The tick species that were observed to infest the heifers were *R. decoloratus* (frequency = 76 %), *R. microplus* (9 %), *A. hebraeum* (5 %), *R. appendiculatus* (5 %), *R. evertsi evertsi* (3%) and *H. marginatum rufipes* (2%). Nguni heifers had lower (P<0.05) $\log_{10} (x + 1)$ transformed coat scores (0.6 ± 0.01), hair length (1.4 ± 0.01) and tick counts (1.4 ± 0.03) than Bonsmara heifers whose $\log_{10} (x + 1)$ transformed coat score, hair length and tick count values were 0.7 ± 0.01; 1.5 ± 0.01 and 1.8 ± 0.02, respectively. The skin thickness between the two breeds were similar (P>0.05). There was a positive linear (P<0.05) relationship between $\log_{10} (x + 1)$ tick counts and $\log_{10} (x + 1)$ coat score in the Nguni (y = 1.90x − 0.40) and a quadratic relationship in the Bonsmara (y = -7.98x^2 + 12.74x - 3.12) breed. It was concluded that the smooth coats may be one of the important mechanisms of tick resistance in the indigenous Nguni breed. Determination of immunologic responses to ticks in the Nguni breed is recommended as this will give more specific indication to the resistance mechanism in this breed.

4.1 Introduction

Selecting for and rearing cattle breeds that are resistant to ticks is a more sustainable way of controlling ticks and tick-borne diseases in rangeland-based beef farming enterprises (Kongsuwan et al., 2010). Farmers are opting to use the Nguni and Bonsmara breeds on the semiarid rangelands of South Africa, because they are able to withstand the harsh environmental conditions, such as high temperatures, long dry periods, diseases and parasites (Ndlovu et al., 2008). In Chapter 3, the Nguni genotype was observed to be more resilient to the debilitating effects of *A. marginale* infection than local crossbreds. Resistance to *A. marginale* could be related to the Nguni breeds' resistance to ticks. It has been reported in earlier studies that the indigenous Nguni breed harboured significantly fewer ticks during periods of peak abundance than either Bonsmara or Hereford cattle (Spickett et al., 1989; Scholtz et al., 1991). Other authors have more recently shown that Nguni cattle carry lower tick loads and therefore appear to be more tick resistant than Angus and Bonsmara cattle (Muchenje et al., 2008). Although the Nguni breed appears to be resistant to ticks, the mechanism for tick resistance is yet to be established. Resistance to ticks in the Nguni breed could be related to favourable coat characteristics, superior skin immunity or the abundance of tick resistance genes.

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Evidence suggests that morphological coat traits such as hair length, skin thickness and coat scores influence tick counts and are significantly related to tick resistance in cattle on rangelands (Verrisimo et al., 2002; Martinez et al., 2006; Foster et al., 2008). It has been reported that animals with shorter hairs and smoother coats tend to have lower tick counts compared to those with longer hairs and woolier coats (Martinez et al., 2006), and those with thinner skins could also have a reduced susceptibility to ticks than those with thicker skins (de Castro et al., 1991; Foster et al., 2008). While indigenous cattle in the semiarid areas are known to carry low tick loads, little work has been done to relate these to coat characteristics in Nguni and Bonsmara cattle. Although correlations have been reported between tick count and coat characteristics, there is no information on the nature of the relationships between tick count and coat characteristics in Nguni cattle. There is a need to establish relationships between coat characteristics and tick count in the indigenous and locally adapted cattle breeds reared on the semiarid rangelands so as to understand the mechanisms of tick resistance and thus characterise these cattle breeds.

Cattle herds owned by smallholder farmers in the semiarid areas are mainly composed of heifers and cows which due to their vulnerability to poor nutrition suffer greater stress and have increased susceptibility to diseases and parasites (Mapiye et al., 2009). As tick resistance is of moderate heritability (Norris et al., 2009), it is important to identify and select tick resistant females that remain in the herd for longer periods, so as to confer resistance to their offspring. Coat characteristics, if well understood, could be easily used to select for tick resistant animals. The identification, selection and rearing of tick resistant breeds is one of the cheap, effective and sustainable methods of controlling ticks

(Latif, 1992; Mattioli et al., 2000) in the cattle enterprises. Selecting tick resistant cattle benefits the farmer by reducing costs on ticks and TBD control while increasing productivity and profitability in their enterprise. In the current study, relationships among skin thickness, hair length, coat score and tick count were determined in Nguni and Bonsmara cattle on semiarid rangelands. The hypothesis tested was that relationships between tick count and morphological coat traits are different in Nguni and Bonsmara cattle.

4.2 Materials and methods

4.2.1 Study site

The study was conducted at Fort Cox College of Agriculture and Forestry farm which is located on 27° 01 East and 32° 46 South in the False Thornveld of the Eastern Cape. The vegetation is composed of several trees, shrubs, and grass species. *Acacia karroo*, *Themeda triandra*, *Panicum maximum*, *Digitaria erientha*, *Eragrostis* species and *Cynodon dactylon* are dominant. The topography of the area is generally flat with a few steep slopes. The climate is semiarid with the average annual rainfall of about 480 mm most of which occurs in the hot wet season. Temperature ranges from 7°C in the cool dry season to 35°C in the hot dry season. The major tick species are *Rhipicephalus* species, *Hyalomma* and *Amblyomma* species. Only two cattle breeds are kept on the farm, Nguni and Bonsmara, and managed as separate herds with similar breeding programs. The farm keeps heifers and cows and sells all steers and bulls to the beef feedlots and surrounding communal farmers.

4.2.2 Experimental design

Twenty four heifers aged between seven and nine months each of Nguni (n = 12) and Bonsmara (n = 12) breeds were used in the study. The heifers were ear tagged for easy identification and grazed on natural pasture throughout the 6-week experimental period during the hot wet season (November 2010 to December 2010). The average initial body weights and $\log_{10} (x + 1)$ transformed body condition scores of the heifers were 219.5 ± 4.49 and 0.6 ± 0.01 for the Bonsmara and 209.3 ± 4.53 and 0.6 ± 0.01 for the Nguni breed. The rangeland forage biomass was estimated every week by random sampling of natural pasture using a disc meter. The heifers did not receive acaricide treatment three months prior to and during the period of data collection to enable natural tick infestation. Only those animals that became anaemic and debilitated (based on the pallor of mucous membranes, decreased body weight and body condition), due to heavy tick infestation were treated. The trial was stopped when three Bonsmara heifers that became anaemic and debilitated due to heavy tick loads were treated after a period of six weeks. All experimental procedures were approved as described in section 3.2.2 and were in compliance with internationally accepted standards for welfare and ethics in animals (Austin et al., 2004).

4.2.3 Measurement of the body weights and body condition score

Body weights were measured weekly using a cattle scale (LS4, Taltec, South Africa). Body condition was visually appraised weekly, by the same independent assessor throughout the experimental period. A 5-point scale was used to score the heifers with score 1 being very thin and a score of 5 being very fat/obese (Osoro and Wright, 1992).

4.2.4 Coat scores, skin thickness and hair length

Coat scores were assessed visually by the same independent assessor throughout the experimental period. The coat of each animal was scored using a 1 to 5 scale based on the level of smoothness of the coat, with 1= excessively smooth, 2= fairly smooth, 3= long coat, 4= woolly and 5= excessively woolly coat (Taylor et al., 1995).

Measurement of the skin thickness was conducted at the same time as visual appraisal of the coat. Skin thickness was determined using a pair of tuberculin calipers. The skin thickness was measured on the midside area (just caudal to the thirteenth rib about 20 cm below the dorsal line) since skin thickness on this part is relatively uniform (Wesonga et al., 2006; Foster et al., 2008). A double fold of skin was measured with the tuberculin calipers placed in an anterior to posterior direction relative to the body of the animal. The skin thickness was measured in millimeters.

Hair samples were collected from the skin of the midside area using a shaving stick adapted in such a way that all hairs within a 200 mm^2 area could be plucked out. The samples were stored in plastic bottles with screw on caps and sent to the laboratory for the measurement of hair length. The hair length (mm) was taken as the average length of the 10 longest hairs of the sample, according to Machado et al. (2010) and Foster et al. (2008).

4.2.5 Tick counts

Two trained enumerators, one on either side of the animal, were used to carefully examine the animal which was restrained in a crush pen, identifying and recording all visible engorged adult ticks on the skin of the cattle. The ticks were not removed from the skin of animals during the process of enumeration.

4.2.6 Statistical analyses

The data for body condition score (BCS), skin thickness (ST), coat score (CS), hair length (HL) and tick count (TC) were not normally distributed and were transformed using $\log_{10} (x + 1)$ to confer normality. The mixed model procedures for repeated measurements (SAS, 2006) was used to determine the effect of breed and week of sampling on body weight (BW), and the $\log_{10} (x+1)$ transformed BCS, ST, CS, HL and TC. First-order autoregressive correlation (AR [1]) was fitted to the model on the measurement of interest. Comparisons of least square means were done using the PDIFF option of SAS (2006). Correlations among the $\log_{10} (x + 1)$ transformed ST, CS, HL and TC were determined using the PROC CORR (SAS, 2006). Those skin parameters that were observed to be significantly related to the $\log_{10} (x+ 1)$ TC on the correlations analysis were regressed on the $\log_{10} (x + 1)$ TC using PROC REG (SAS, 2006) to determine the nature of the relationships. The tick count data was grouped into low (0-30), moderate (31-60) and high (>61) categories to enable the determination of frequencies. Frequencies for coat score and tick count were obtained using the PROC FREQ (SAS, 2006).

4.3 Results

4.3.1 Breed and week effects on tick count and skin parameters

The tick species that were observed to infest the study animals were *R. decoloratus*, *R. microplus*, *A. hebraeum*, *R. appendiculatus*, *R. evertsi evertsi* and *H. marginatum*, with the following relative frequencies 76, 9, 5, 5, 3 and 2 %, respectively. Since *R. decoloratus* ticks were observed to be the predominant tick species, only counts for this tick were used for the data analysis. Significant breed differences (P<0.05) were observed for $\log_{10} (x + 1)$ transformed CS, HL and TC (Table 4.1). Bonsmara heifers had higher (P<0.05) CS, HL and TC values compared to the Nguni heifers. There were no significant breed effects on BW, ST and BCS. The week of sampling had a significant (P<0.05) effect on the BW, BCS, ST and TC in both breeds. The BW and BCS were unchanged from the first to the third week followed by increases (P<0.05) from the fourth to the sixth week of sampling in both breeds (Figure 4.1). The skin thickness was unchanged in the first two weeks, followed by a decrease (P<0.05) in the third week, succeeded by a slight increase in the fourth week (P<0.05) and became unchanged from the fourth to the sixth week as shown in Figure 4.1. There was a general increase (P<0.05) in TC from the first through to the last week of sampling in both breeds (Figure 4.1).

4.3.2 Correlations among skin parameters and tick count

Significant (P<0.05) positive correlations were observed between TC and CS for Nguni (r = 0.60) and Bonsmara (r = 0.64) breeds (Table 4.2). The TC was significantly (P < 0.05) correlated to HL (r = 0.30) in the Bonsmara breed. It was also observed that CS was

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significantly positively correlated (P < 0.05) to HL (r = 0.48) in the Bonsmara breed. No relationships were observed between TC, ST and HL in the Nguni breed and between TC and ST in the Bonsmara breed.

4.3.3 Relationships between tick count and skin parameters

There was a significant linear relationship (P<0.05) between TC and CS in the Nguni breed while a significant quadratic relationship (P<0.05) was observed between the two parameters in the Bonsmara breed (Figure 4.2). As the CS increased, TC increased in a linear fashion in the Nguni breed. In the Bonsmara breed, initially, TC increased markedly with a slight increase in the CS but later remained unchanged at higher CS. A significant linear relationship (P < 0.05) was also observed between TC and HL in the Bonsmara breed. The variations in tick counts and coat scores in the Nguni and Bonsmara heifers are shown in Tables 4.3 and 4.4, respectively. All the Nguni heifers had an initially low tick count, as time progressed, more Nguni heifers became moderately infested with ticks and very few had high tick counts by the sixth week. Few Bonsmara heifers had low initial tick counts while the majority had moderate to high tick infestations. As time progressed, all Bonsmara heifers had high tick counts by the sixth week. The majority of Nguni heifers were observed to have lower coat scores in the first three weeks, becoming moderate in last three weeks. Most Bonsmara heifers had moderate coat scores in the first two weeks, and in the last four weeks all the Bonsmara heifers had high coat scores.

Table 4.1: Mean (± standard error) of the body weight (BW), body condition score (BCS), skin thickness (ST), coat score (CS), hair length (HL), and tick count (TC) in the Nguni and Bonsmara heifers

	Bonsmara		Nguni	
Variable	mean±se	Range	mean±se	range
BW (kg)	219.5±4.49	117.00 - 306.00	209.3±4.53	117.00 - 296.00
BCS[*]	0.6±0.01	0.48 - 0.65	0.6±0.01	0.48 - 0.65
ST[*] (mm)	1.1±0.01	1.00 - 1.23	1.1±0.01	0.95 - 1.20
CS[*]	0.7±0.01[b]	0.48 - 0.78	0.6±0.01[a]	0.30 - 0.70
HL[*] (mm)	1.5±0.01[b]	1.29 - 1.63	1.4±0.01[a]	1.24 - 1.49
TC[*]	1.8±0.02[b]	0.48 - 2.23	1.4±0.03[a]	0.30 - 1.82

[ab] Means in the same row with different superscripts are significantly different ($P<0.05$).

[*] Indicates values that are $\log_{10}(x + 1)$ transformed

se = standard error
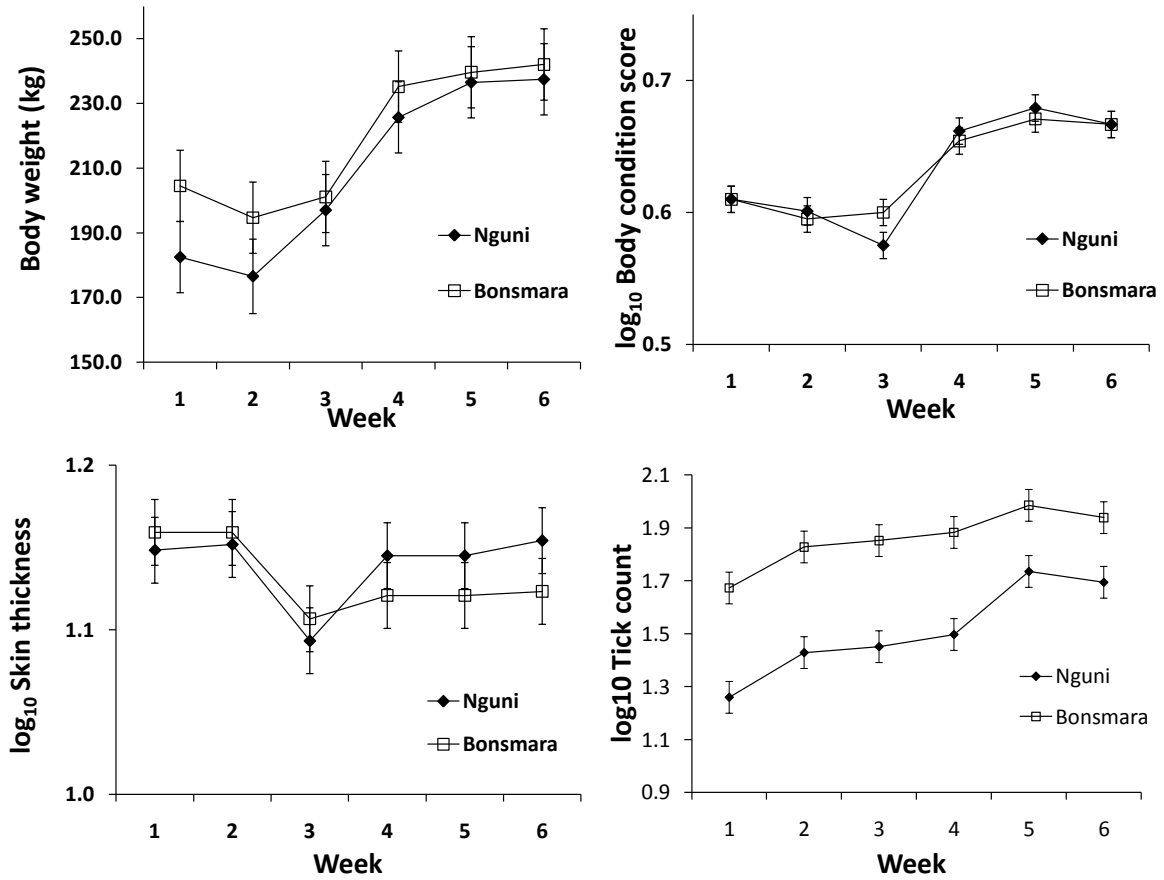
Figure 4.1: Weekly changes in the body weight, and body condition score, skin thickness and tick count in the Nguni and Bonsmara heifers

Table 4.2: Correlations of the coat characteristics with tick count (TC) in the Nguni and Bonsmara heifers

	Nguni TC	Bonsmara TC	Overall TC
Weight	0.31[**]	0.16	0.26[**]
BCS	0.44	0.21	0.29[***]
Skin thickness	0.11	0.13	0.07
Coat score	0.6[***]	0.64[***]	0.71[***]
Hair length	0.05	0.3[**]	0.35[***]

[*]P < 0.05, [**]P < 0.01, [***]P < 0.001
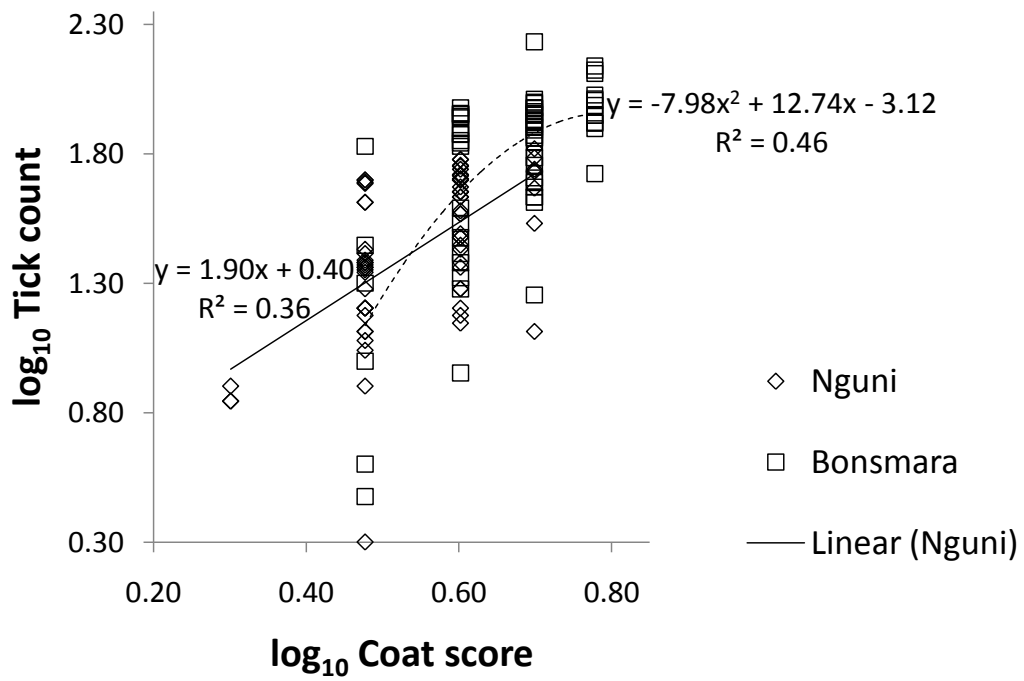
Figure 4.2: Relationship between tick count and coat score in Nguni and Bonsmara heifers

Table 4.3: Weekly variations in the numbers of Nguni and Bonsmara heifers at different tick infestation levels

| Week | Breed | Tick infestation level | | | n |
		Low	Moderate	High	
1	Nguni	12	0	0	12
	Bonsmara	3	5	4	12
2	Nguni	10	2	0	12
	Bonsmara	1	3	8	12
3	Nguni	9	3	0	12
	Bonsmara	1	3	8	12
4	Nguni	7	5	0	12
	Bonsmara	0	2	10	12
5	Nguni	6	4	2	12
	Bonsmara	0	0	12	12
6	Nguni	6	3	3	12
	Bonsmara	0	1	11	12

Table 4.4: Weekly variations in the numbers of Nguni and Bonsmara heifers at different coat scores

Week	Breed	Coat score					n
		1	2	3	4	5	
1	Nguni	1	6	5	0	0	12
	Bonsmara	0	1	2	7	2	12
2	Nguni	2	5	5	0	0	12
	Bonsmara	0	1	2	7	2	12
3	Nguni	1	5	5	1	0	12
	Bonsmara	0	3	5	2	2	12
4	Nguni	0	5	4	3	0	12
	Bonsmara	0	1	4	4	3	12
5	Nguni	0	3	7	2	0	12
	Bonsmara	0	0	5	4	3	12
6	Nguni	0	3	7	2	0	12
	Bonsmara	0	0	3	8	1	12

4.4 Discussion

Although indigenous Nguni cattle appear to be adapted to the semiarid areas of South Africa and resistant to ticks, their mechanism for tick resistance is yet to be established. Tick resistance in cattle may be related to coat characteristics including skin thickness, hair length and coat score (Verrisimo et al., 2002); and can be ranked through the use of tick counts (de Castro et al. 1991). In the current study, coat characteristics and tick counts were determined in the indigenous Nguni and the synthetic Bonsmara breeds to determine the mechanism of resistance in the two breeds. The hypothesis tested was that there are no relationships between coat characteristics and tick count in Nguni and Bonsmara cattle.

Tick count was observed to be lower in the Nguni compared to the Bonsmara heifers suggesting a higher tick resistance in the Nguni breed. These findings agree with Spickett et al. (1989) and Muchenje et al. (2008) who found Nguni cattle to have lower tick loads than Bonsmara cattle. The lower tick count in the Nguni cattle may be related to the breed's smoother coat and shorter hairs that tend to discourage tick attachment (Bonsma, 1981). Webb and David (2002) reported lower tick count on positions which have short hairs in Tswana, Simmentaler and Brahman cattle suggesting that cattle breeds with short hairs have decreased susceptibility to tick infestation. Tick avoidance behaviour, skin sensitivity and increased grooming activity may also play a role in reducing tick numbers on the skin of this indigenous Sanga cattle breed as proposed by Meltzer (1996). Further studies are, however, required to evaluate tick avoidance, skin sensitivity and grooming activity in the Nguni breed to fully understand the mechanism(s) of tick resistance.

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The lack of breed differences in the skin thickness concurs with the findings of Spickett et al. (1989). However the current study's findings are in contrast to earlier reports by Verrisimo et al. (2002) who observed significant breed differences in skin thickness between indigenous and crossbred cattle. In their study, Verissimo and colleagues (2002) measured skin thickness on the scapular region while in the present study double fold skin thickness was measured over the midside area which is more uniform in thickness. The skin thickness is influenced by the amount of subcutaneous fat and site on which it is measured (Brown et al., 2008), thus likely accounting for the different findings in the two studies. Further work needs to be done on the consistent and correct method of measuring skin thickness so as to accurately relate it to tick resistance in cattle.

The initial decrease in body weight and BCS from the first to the third week of the study period could be attributed to poor herbage quality and rangeland condition during this period. As new growth occurred on the rangeland, the animals responded positively with increases in the body weight and BCS from the fourth to the sixth week. It was reported that BCS has an influence on skin thickness (Ayresa et al., 2009). The fluctuations in the skin thickness observed in the current study followed a similar trend to that of the BCS, dropping in the first three weeks and then increasing from the fourth to the sixth week. The general increase in tick count from the first to the sixth week was expected as the animals were not being dipped and hence carried progressively heavier tick loads. It was noted that Nguni cattle had consistently lower tick loads throughout the study period which may be further testimony of their higher resistance to ticks. Three Bonsmara

heifers succumbed to the increasing tick loads and had to be dipped and treated to prevent mortalities.

The observation that tick count was positively correlated to coat score in both the Bonsmara and Nguni heifers agrees with the findings of Verrisimo et al. (2002) who observed positive correlations between the two parameters in cattle. Heifers that had shorter and smoother coats were also observed to have lower tick counts. This finding further validates earlier assertions by Foster et al. (2008) that animals with smoother coats similarly carried lower tick counts than those with woolly coats. The observed relationship between tick count and hair length in the Bonsmara breed supports earlier assertions by Taylor et al. (1995) that hair length has a role in tick susceptibility in cattle. Animals with shorter hairs tend to have lower tick counts compared to those with longer hairs, since long hairs create favourable conditions for tick survival (Taylor et al., 1995). It is also believed that cattle breeds with short hairs expose ticks to harmful climatic conditions and to predators such as birds (Taylor, 2006). In addition, longer coats may protect ticks from the animal's self-grooming that helps remove attached ticks from the coat (Machado et al., 2010).

The observed relationship between tick count and coat score which was linear in the Nguni and quadratic in the Bonsmara breed suggest that coat score is an important determinant of tick count in these cattle breeds. These findings conforms to the findings of Martinez et al. (2006) and Machado et al. (2010) who reported significant positive relationships between tick infestation and coat score in cattle. Smoother coats are thought

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to secrete more sebum, which acts as a deterrent to tick attachment (Bonsma, 1981; Taylor, 2006). A woolier coat however, creates a microclimate that helps keep the ticks attached to the surface of the animal. It was noted from the regression analysis that, at lower coat scores, both breeds carried similarly low tick loads. However, as the coat score increased, the Bonsmara breed carried higher tick loads than the Nguni breed. Selecting cattle with lower coat scores may thus reduce the level of tick susceptibility and increase tick resistance in the herd. A greater majority of Nguni heifers had low tick counts while exhibiting moderate to high coat scores while a larger number of Bonsamara heifers had high tick loads despite exhibiting moderate coat scores. The higher numbers of Nguni cattle with low tick counts even at high coat scores may attest to the Nguni breed's superior ability to resist tick infestation which may not be dependent on coat score alone.

Indigenous cattle breeds are known to have adaptive mechanisms of tick resistance (Latif, 1992) making them more resistant to ticks than imported or crossbred cattle (Mattioli et al., 2000). The current study highlights the important role that coat characteristics play in increasing the resistance of indigenous cattle to ticks. The breed differences in tick counts observed in this study also point to differences in genetics playing a part in tick resistance in these cattle breeds. Genetic mechanisms such as cutaneous hypersensitivity reactions to tick antigens and cellular responses to ticks may have developed in the Nguni breed over a long period and contributed to the development of a breed with superior genetic resistance to ticks. Little is known however, about the genetic mechanisms involved in the resistance of indigenous Nguni cattle to ticks. Genetic resistance may contribute to

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the biological control of ticks, since the use of resistant animals is one of the most effective solutions to control this parasite (Nascimento et al., 2010). It is thus imperative to study skin immunity to ticks in the Nguni breed as this will give specific indicators to the mechanisms of host resistance in this breed.

Farmers in semiarid areas should utilize cattle breeds like the Nguni that have smoother coats and shorter hairs as these are less susceptible to tick infestation. Since coat score have been shown to be significantly related to tick count, it is important for farmers in semiarid areas to augment visual enumeration of ticks with assessment of the coat score so as to accurately identify cattle with higher tick resistance in their herds. By selecting Nguni cattle that have consistently low tick counts, shorter hairs and smoother coats, farmers in semiarid areas will generally improve their herds' resistance to ticks.

4.5 Conclusions

Tick count had a positive linear relationship with coat score in the Nguni and a quadratic relationship with coat score in the Bonsmara breed. Nguni heifers had smoother coats and shorter hairs, adaptive mechanisms, which made them less susceptible to ticks than Bonsmara heifers. Selection and rearing of the Nguni cattle with smoother and shorter coats and hence increased resistance to ticks could increase profitability and productivity in cattle enterprises on semiarid rangelands. Determination of skin immunity to ticks in the Nguni breed is recommended as it gives specific indicators to the mechanism of host resistance.

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CHAPTER 5: Cutaneous hypersensitivity responses to *Rhipicephalus* tick larval antigens in pre-sensitised cattle

(Published in *Ticks and Tick-borne Diseases*, see Appendix 3)

Abstract

Nguni cattle are known to be more resistant to ticks than Bonsmara cattle yet the immunological mechanisms responsible for this phenomenon are not fully understood. Cutaneous hypersensitivity responses to unfed larval extracts (ULE) of the ticks *Rhipicephalus decoloratus* and *Rhipicephalus microplus* were investigated in Nguni and Bonsmara cattle to improve knowledge on immunity to ticks. Hypersensitivity reactions were induced by intradermal inoculation of 0.1 ml of ULE of *R. decoloratus* and *R. microplus* ticks (50 µg protein) in the right and left ear, respectively of 8 to 9 months old Nguni (n = 11) and Bonsmara (n = 9) heifers. Ear thickness was measured using callipers before and 0.5, 1, 6, 24, 48 and 72 hours post-inoculation (PI). Bonsmara cattle showed a more intense immediate reaction with maximum response at one hour PI and no delayed hypersensitivity reaction. Nguni heifers, conversely, presented a less intense immediate reaction with maximum response at one hour PI, and a delayed hypersensitivity reaction at 72 hours PI. Reactions to *R. decoloratus* ULE produced a more intense skin response at all time intervals in both breeds than that of *R. microplus*. Nguni cattle showed lower tick infestation indicating higher tick resistance than Bonsmara cattle. Delayed hypersensitivity reaction could be associated with superior tick resistance in the Nguni breed, while immediate hypersensitivity reaction could be associated with increased tick susceptibility in the Bonsmara breed. This study indicates the need for further

investigations into the correlation of tick resistance and cellular immune responses to tick infestation in Nguni cattle.

5.1 Introduction

Large variation in resistance to ticks exists in different cattle breeds (Mattioloi et al., 2000). In Chapter 4, tick resistance in the Nguni breed was largely attributed to smoother and shorter coats which act as a deterrent for tick attachment. Resistance to ticks in cattle has also been ascribed to other non-adaptive immune factors such as grooming activity, skin colour and thickness, and area of skin available for infestation (Meltzer et al., 1996; Machado et al., 2010). Adaptive immune factors involving humoral and cellular responses to tick attachment also contribute to tick resistance in cattle (Brossard and Wikel, 2004). Despite numerous studies on host resistance, the mechanisms of naturally acquired immunity to ticks in cattle are still poorly understood.

It has been proposed that cutaneous hypersensitivity reactions to tick antigens are responsible for repelling tick infestation in cattle (Kemp et al., 1986). An intradermal skin test was, subsequently, developed to measure skin hypersensitivity responses to tick antigens and rank cattle according to their level of tick resistance (Bechara et al., 2000). Studies on skin hypersensitivity responses of tick-infested cattle have, however, yielded varied and sometimes conflicting results. Bechara et al. (2000) demonstrated an

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immediate type reaction in susceptible *Bos taurus* cattle and both immediate and delayed type hypersensitivity reactions in tick-resistant *Bos indicus* cattle. In contrast, Piper et al. (2010) noted that an intense immediate type hypersensitivity response to tick infestation was associated with increased tick resistance in *Bos taurus* cattle. Prudencio et al. (2011) reported an intense immediate type hypersensitivity reaction and a slight delayed hypersensitivity reaction in both susceptible and resistant cattle. Inconsistencies in the above reports, therefore, suggest the need for further evaluation of skin hypersensitivity reaction in tick susceptible and resistant cattle.

In Chapter 4, *R. decoloratus* and *R. microplus* were the tick species shown to have the highest frequencies on the semiarid rangelands. The two tick species are known to be the most important biological transmitters of *A. marginale*, a leading cause of cattle mortalities in the smallholder farming system on the semiarid rangelands of South Africa. Although antigenicity differs between tick species (Steen et al., 2006; Mans et al., 2008), research has focused on the skin hypersensitivity responses to a single blue tick species, *R. microplus* while ignoring the other equally important African species *R. decoloratus*. Differences in infestation rates and susceptibility were reported for *R. decoloratus* and *R. microplus* on Nguni and Bonsmara cattle in the previous chapter. The repertoire of antigenic molecules exhibited by *R. decoloratus* and *R. microplus* are, therefore, expected to differ.

Skin hypersensitivity reactions to *Rhipicephalus* tick antigens remain uncharacterised in the indigenous Nguni cattle breed despite evidence of its tick resistance status. It remains

unknown whether the Nguni breed has similar cutaneous hypersensitivity responses to those of other tick resistant breeds. It is also unclear whether cutaneous hypersensitivity in Nguni cattle differs with the less tick-resistant Bonsmara breed. Comprehension of skin hypersensitivity reactions will aid in the development of anti-tick vaccines which should be designed to promote an appropriate immune response to infestation (Piper et al., 2008). In the current study, cutaneous hypersensitivity reactions to unfed larval extracts of *R. decoloratus* and *R. microplus* were compared in Nguni and Bonsmara cattle. It was hypothesised that ULE of the ticks *R. decoloratus* and *R. microplus* elicit dissimilar skin reactions in Nguni and Bonsmara cattle.

5.2 Materials and methods

5.2.1 Study site

The study was conducted at Fort Cox College Farm as described in section 4.2.1.

5.2.2 Study animals

Nguni (n = 11) and Bonsmara (n = 9) heifers aged between 7 to 9 months were used in the study. Selection and description of the heifers has been described in Chapter 4. The heifers were ear-tagged for easy identification. To enable natural tick infestation, the heifers were grazed on natural pasture known to be infested with *R. decoloratus* and *R. microplus* tick larvae for at least a month before the experiment. All experimental procedures were approved by the University of KwaZulu-Natal Animal Ethics Research Committee (Reference Number: 097/11/Animal) and were in compliance with internationally accepted standards for welfare and ethics in animals (Austin et al., 2004).

5.2.3 Preparation of unfed larval extract

Two-months-old unfed larvae from laboratory colonies of the *R. decoloratus* and *R. microplus* ticks were prepared separately into unfed larval extract (ULE). In brief, the larvae were ground up in liquid nitrogen using a motor and pestle before suspension in phosphate buffered saline (PBS, pH 7.4) that contained a cocktail of protease inhibitors (Sigma-Aldrich). The homogenate was sonicated for 60 seconds (20 MHz) to produce a crude larval extract. Crude larval extract was centrifuged at 3 000 g (4°C) for 30 minutes, after which the supernatant ULE was removed. Protein concentration of the ULE was determined using the bicinchoninic (BCA) dye bioassay (Biorad) and the ULE was stored at − 40°C until use.

5.2.4 Delayed hypersensitivity skin test

Rhipicephalus ULE was used to induce a delayed local cutaneous hypersensitivity reaction in the heifers. Each heifer received an intradermal injection of 0.1 ml (50 µg of protein) *R. decoloratus* ULE in a shaved area of the left ear, and a similar injection of *R. microplus* ULE on the outer surface of the contralateral ear. Ear thickness was used in the current study as it has been reported to give a more suitable and precise measurement since it is measured without folding (Prudencio et al., 2011). An equal volume of PBS was inoculated 50 mm from the ULE site in both ears to provide a control measurement. The ear thickness was measured in triplicate with the aid of callipers just before the injection, and 0.5, 1, 6, 24, 48 and 72 hours post-ULE or PBS inoculation. The response

was expressed as the mean percentage change in ear thickness in relation to pre-inoculation values.

5.2.5 Measurement of the body weight, body condition score and tick counts

Body weights, body condition scores and tick counts were determined as described in sections 4.2.3 and 4.2.5. The heifers were also examined for tick-associated dermatitis and tick bite wounds.

5.2.6 Statistical analyses

All analyses were conducted in Statistical Analysis System version 9.2 (SAS, 2009). The General Linear Model Procedure for repeated measurements (SAS, 2009) was used to determine the effects of breed, tick species, time of measurement and their interactions on the ear thickness measurements. First-order autoregressive correlation (AR [1]) was fitted to the model on the measurement of interest. Least square means were compared using the PDIFF procedure (SAS, 2009). A χ^2 test was performed to determine the associations between tick infestation and development of secondary (delayed hypersensitivity) response in ear thickness as well as between tick infestation and the development of dermatitis and tick bite wounds in cattle.

5.3 Results

5.3.1 Differences in body weight, body condition score and tick count

Body weight and BCS were similar ($P > 0.05$) in the Nguni and Bonsmara cattle. Tick counts were lower ($P < 0.05$) in the Nguni than in the Bonsmara heifers (Table 5.1).

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5.3.2 Response to *R. decoloratus* and *R. microplus* ULE

The interaction between breed and time of inoculation of skin antigen and between breed, tick species and time of inoculation of skin antigen had a significant ($P < 0.05$) effect on the cutaneous hypersensitivity responses (ear thickness) of the heifers. Bonsmara heifers showed a more intense immediate type reaction with maximum response at 30 minutes to one hour post inoculation (PI) followed by a gradual decline from 6 hours PI until the end of the experimental period (72 hours) (Figures 5.1 and 5.2). Nguni cattle showed a less intense immediate hypersensitivity reaction which peaked at 1 hour PI and declined after 6 hours. Subsequently, a second response was observed in the Nguni from 24 hours peaking at 72 hours PI (Figure 5. 1 and 5.2).

5.3.3 Associations between tick infestation level, delayed skin hypersensitivity and tick induced dermatitis

There was an association ($P < 0.05$) between tick count and presence of a delayed hypersensitivity reaction and between tick count and presence of dermatitis in the heifers (Table 5.2). Heifers that had lower tick loads exhibited a delayed hypersensitivity response while those with high tick loads did not exhibit a delayed hypersensitivity response ($P < 0.05$) across the breeds. Similarly, heifers that had lower tick loads did not exhibit tick induced dermatitis whereas in the heifers with high tick loads, tick bite wounds and dermatitis were observed ($P < 0.05$) across the breeds.

Table 5.1: Mean (± standard error) body weight, body condition score and tick counts in the Nguni and Bonsmara heifers

Variable	Bonsmara Mean ± s.e.	Nguni Mean ± s.e.	Significance
BW	254.2 ± 8.03	232.1 ± 6.69	NS
BCS	3.2 ± 0.11	3.1 ± 0.09	NS
TC	88.6 ± 3.82	48.8 ± 1.79	*

BW: body weight; BCS: body condition score; TC: tick count; s.e.: standard error

NS: not significant ($P > 0.05$), *: significant ($P < 0.05$)
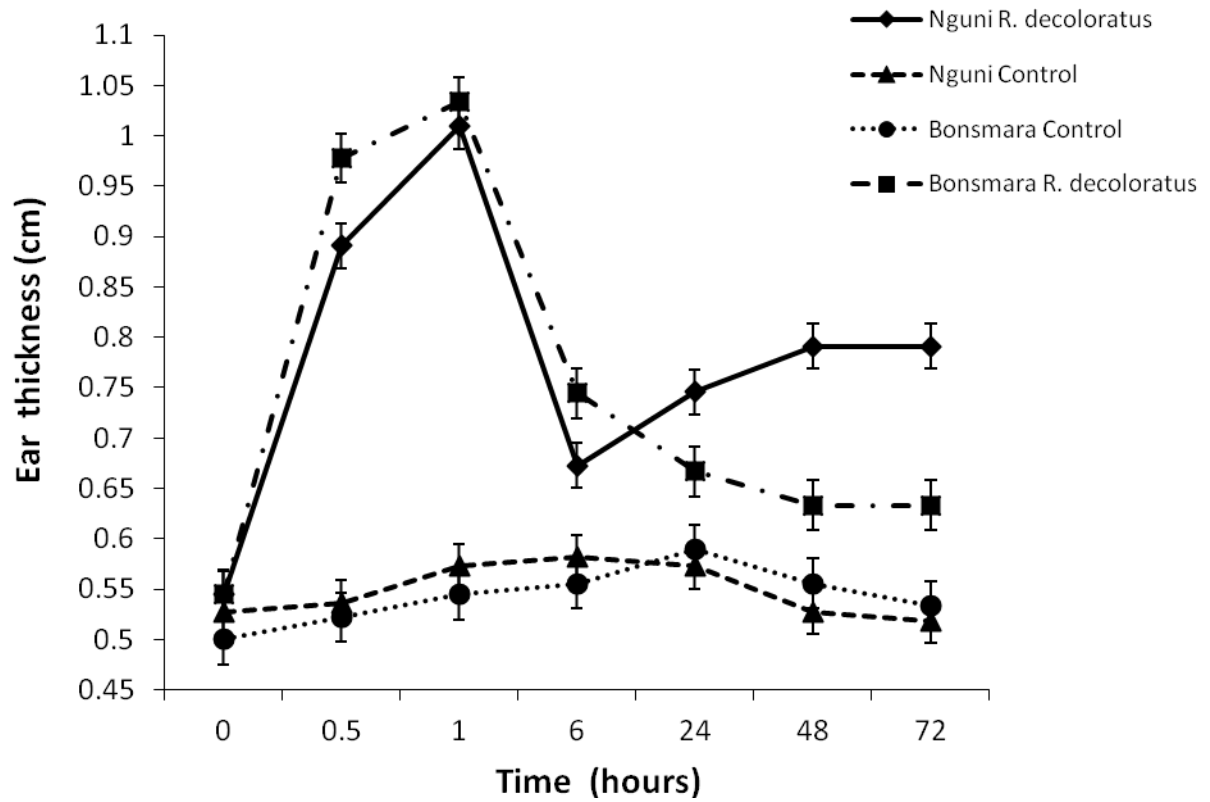
Figure 5.1: Changes in the ear thickness of Nguni and Bonsmara heifers following inoculation of *Rhipicephalus decoloratus* unfed larval extract
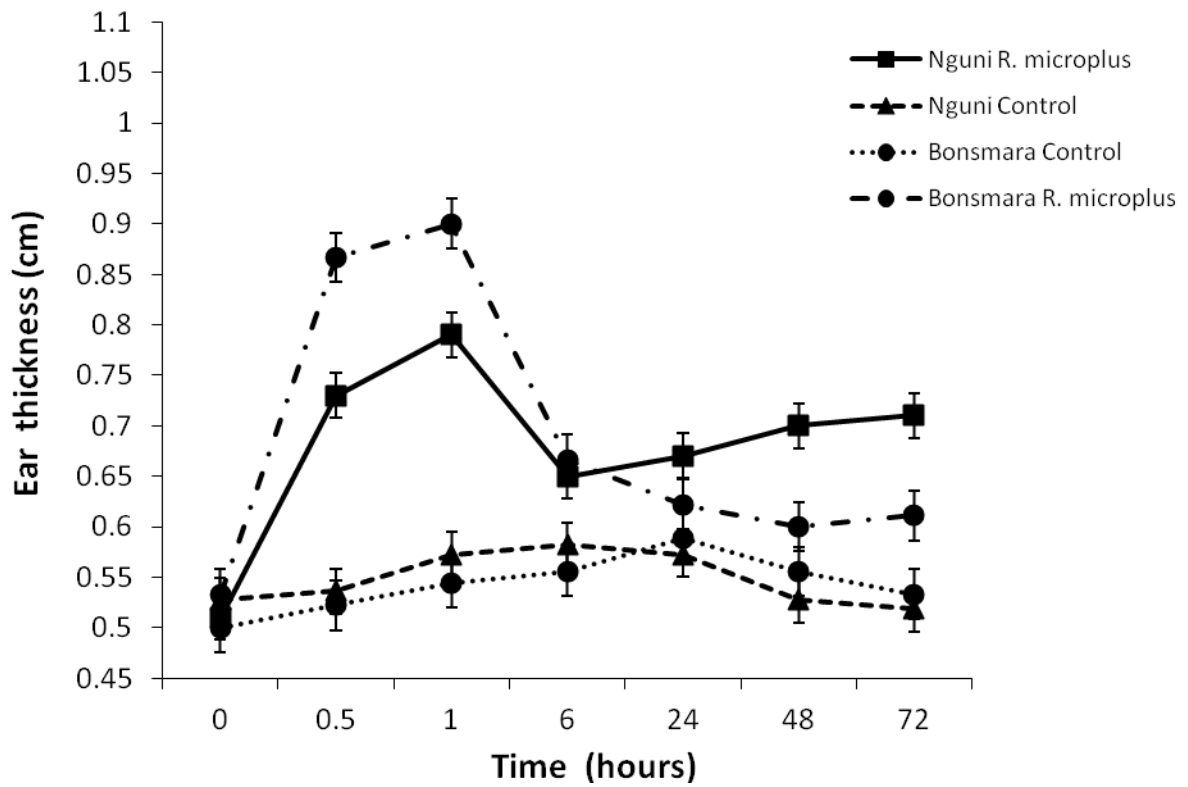
Figure 5.2: Change in ear thickness following inoculation of *Rhipicephalus microplus* unfed larval extract in Nguni and Bonsmara heifers

Table 5.2: Associations between tick count and dermatitis and delayed hypersensitivity in the Nguni and Bonsmara heifers

Tick count	High	Low	χ^2	*P*
Dermatitis				
Present	7	1	4.2	0.043
Absent	2	10		
Delayed hypersensitivity				
Present	1	11	7.1	0.008
Absent	8	0		

5.4. Discussion

Smooth coats and short hairs have been given as the cause of superior tick resistance in the Nguni cattle breed (Chapter 4). However, low tick infestation in Nguni cattle with rough coats and long hairs suggested that tick resistance in the Nguni may be dependent on non-innate immunological mechanisms (Chapter 4). To improve knowledge on the immunological mechanisms responsible for tick resistance in cattle, the current study investigated skin hypersensitivity reactions to unfed larval extracts of *R. decoloratus* and *R. microplus* in Nguni and Bonsmara heifers.

Nguni heifers were observed to have lower mean counts of engorged adult ticks than their Bonsmara counterparts, confirming the assertions of Spicket et al. (1989), Muchenje et al. (2008) and Marufu et al. (2010) that the Nguni breed has superior tick resistance. The long continuous contact between Nguni cattle and the blue ticks has most likely resulted in the development of greater resistance in this breed (Marufu et al., 2011). Tick infestation was not associated with tick sore lesions and dermatitis in the Nguni heifers, suggesting that the Nguni breed has most likely developed superior immunological defences that limit tick damage on their skin. It should be noted that there were two Bonsmara heifers which had low mean tick counts similar to some Nguni heifers and if such animals were selected and used in breeding programmes, it could improve tick resistance in the Bonsmara breed. Resistance to natural tick infestation in cattle is a trait that has been reported to have a moderate heritability averaging 0.37 (Turner et al., 2010) which is sufficient to result in the successful selection for tick resistance. Given the high genetic variability among individuals and breeds (Morris, 2007), the identification of

superior genes is thus important for the development of breeding programmes for tick resistance in cattle. It is, however, important to be mindful of the correlated responses in other economic traits such as growth, meat quality or milk yield as a consequence of selection for tick resistance.

Bonsmara heifers showed only an immediate type hypersensitivity response to ULE of both blue tick species characterised by soft swelling at the inoculation site further strengthening the view that this is the only type of hypersensitivity response mounted in tick susceptible cattle. The present findings agree with those of Bechara et al. (2000) and Prudencio et al. (2011) who reported only a non-specific immediate type hypersensitivity response to tick antigen in previously sensitised tick susceptible cattle. Immediate type hypersensitivity reactions also called Type I hypersensitivity reactions are initiated by antigen binding to immunoglobulin E (IgE) pre-attached to mast cells or basophils leading to inflammatory mediator release (Szabo et al., 2004). The resultant increased vascular permeability causes oedema and this could explain the subsequent swelling at the injection site in Bonsmara cattle. Nguni heifers however had a less pronounced immediate type hypersensitivity response to ULE of both ticks used in the present study which most likely led to less evident oedema.

In the Nguni heifers, a delayed type hypersensitivity response was observed over and above the immediate type response and was most likely the cause of tick resistance in this breed. Several authors have reported similar development of a delayed type hypersensitivity response and linked it to the expression of acquired resistance to ticks in

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a variety of tick resistant hosts (Bechara et al. 2000; Szabo et al., 2004; Prudencio et al., 2011). *Rhipicephalus* tick larvae take at least four days after initial attachment to reach engorgement and hence host rejection of tick attachment within the first 24 hours is critical for prevention of tick engorgement (Porto Neto et al., 2011). The larvae continuously change the repertoire of salivary molecules that they secrete to inhibit humoral and cellular defences of the host thus enabling early evasion of host defences resulting in engorgement. It would seem therefore that due to their long contact with ticks, Nguni cattle have developed the delayed hypersensitivity response as a key mechanism of protection against these immune evasive tactics by the ticks and thus limiting tick engorgement and fecundity. This view is supported by the low numbers of engorged adult ticks observed on the Nguni heifers in the present study. It should be noted that the results of the χ^2 test (Table 5.3) were important for showing the breed effect on the delayed hypersensitivity responses. The χ^2 test is not the most suitable test however especially when frequencies are below five it tends to inflate the values of the test.

The presence of only an immediate type response and lack of a delayed type hypersensitivity reaction to tick antigens in the Bonsmara heifers could point to a deficit response in this breed which most likely led to increased tick susceptibility. Immunomodulatory molecules in tick saliva have been described (Brossard and Wikel, 2004; Steen et al., 2006; Mans et al., 2008), which reduce the host's ability to respond to tick antigens that could stimulate a protective immune response. Kovář et al. (2001) reported an inhibitory effect of these immunomodulatory molecules on T helper 1 and a

stimulatory effect on T helper 2 cytokine elaboration. Helper 2 T cells assist B cells to develop into antibody producing cells thus promoting humoral immunity. In addition, T helper 2 cytokines recruit eosinophils which are the most important source of indoleamine 2,3 deoxygenase (IDO), an enzyme that inhibits the T helper 1 lymphocytes (Odemuyiwa et al., 2004), thus inhibiting cellular responses to ticks. The T helper 2-type response which is associated with immediate hypersensitivity reactions is thus linked to the lack of development of cellular immunity and, hence, tick resistance in susceptible hosts (Ferreira et al., 2003). Though cellular responses were not the focus of the present study, it can be surmised from the present results of cutaneous hypersensitivity test that *Rhipicephalus* ticks induce, a predominantly T helper 2 response which inhibits local cellular immunity and increases tick susceptibility in the less resistant Bonsmara breed.

The observed delayed hypersensitivity in heifers with low tick loads and its absence in those with high tick loads could be indicative of differences in cellular responses between the tick resistant and susceptible cattle. Delayed type hypersensitivity reactions are thought to occur due to a cellular infiltration and triggering of cellular immunity in resistant hosts (Hlatshwayo et al., 2004). The T helper 1 cells are implicated in the mediation of delayed hypersensitivity leading to cutaneous basophil infiltrations (Ferreira et al., 2003). Basophils have long been recognized as important effectors in tick rejection in cattle and two possible ways in which they accomplish this have been proposed. Wada et al. (2010) observed that basophils cluster close to tick mouth parts in the skin and so assumed that they function as direct effectors of the anti-tick reaction mounted in response to tick antigens. The same authors also suggested that antigen/antibody-

stimulated basophils function as activators of mast cells that in turn produce effector molecules against ticks. The cutaneous basophil hypersensitivity that is associated with delayed type hypersensitivity reaction may likely have caused the reduction of tick infestation in the tick resistant Nguni heifers. Further studies are, however, required on the characterisation of the cellular infiltrations associated with delayed hypersensitivity responses to ticks in Nguni cattle to confirm the present findings.

Skin response to *R. decoloratus* ULE was observed to be more intense than that to *R. microplus* ULE suggesting higher antigenicity in the former tick species. In the current study area, *R. decoloratus* is the more dominant species though it co-exists with *R. microplus* (Chapter 4). It should be noted that development of immunity to *R. microplus* does not necessarily confer cross protection against *R. decoloratus* (de Vos et al., 2001). When investigating the antigenicity and control of *Rhipicephalus* ticks, it is thus not sufficient to extrapolate results from studies on one tick species. Considerations should be made of the distribution and population dynamics of both tick species in a particular area. The present study's findings thus highlight the need for identifying mechanisms of host resistance to specified tick species of economic importance. This could also have significant ramification in the search for tick protective antigens which can be used as vaccine candidates in the control of ticks.

A crude larval extract was used to induce cutaneous hypersensitivity responses in cattle in the present study because it was difficult to extract larval saliva. The present results should be interpreted with caution as crude larval extract contains many bio-active

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components including metabolites and larval body proteins, which the host is not normally exposed to during tick feeding. Tick saliva, which is primarily injected into the host during tick feeding (Steen et al. 2006), would thus have been preferable, and is recommended for similar future studies. The inclusion of protease inhibitors in the ULE could have also affected the observed inflammatory response in the study heifers, by inhibiting the host enzymes involved in combating tick salivary antigens. Alternatively, the protease inhibitors could have affected the proteases in the tick saliva hence assisting the host's defence mechanisms. Given that the inhibitors were used in both control and test sites, and that Nguni heifers exhibited greater immune responses than the Bonsmaras, it may thus be concluded that the inhibitory effect of protease inhibitors could have been minor.

Intradermal inoculation of tick salivary antigens to elicit cutaneous hypersensitivity reactions has been used to broadly evaluate immune responses to ticks in hosts and is useful in classifying them according to level of susceptibility. The skin test has been used classify cutaneous hypersensitivity responses for different species including dogs (Ferreira et al., 2003), bovines (Bechara et al., 2000; Prudencio et al., 2011) and horses (Szabo et al., 2004). The present results show that the intradermal inoculation of 50 μg of ULE of *R. microplus* and *R. decoloratus* elicited immediate local inflammatory reaction in the ears of pre-sensitised Bonsmara heifers and delayed hypersensitivity responses in Nguni heifers. In view of these observations, investigations are needed to characterize the immune reactions in greater detail to measure the immune response in skin or lymph nodes biopsies. The intradermal test could be used in conjunction with the assessment of

coat characteristics and evaluation of skin or lymph node biopsies to aid in accurate characterisation of the level of tick resistance in cattle.

5.5 Conclusions

Unfed larval extracts of *R. decoloratus* and *R. microplus* induced an immediate type hypersensitivity reaction, which was associated with tick susceptibility in previously sensitised Bonsmara cattle. Immediate followed by delayed hypersensitivity reaction to ULE of *R. decoloratus* and *R. microplus* were observed and associated with tick resistance in previously sensitised Nguni cattle. The *R. decoloratus* ULE had higher antigenicity and elicited a more intense skin hypersensitivity response in both breeds than the *R. microplus* ULE. Intradermal testing of tick immune status in cattle can be used in selective breeding programmes for tick resistance. Further investigations into the associated cellular responses to tick infestation in Nguni and Bonsmara cattle were recommended.

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CHAPTER 6: Cellular responses to *Rhipicephalus* infestations in pre-sensitised cattle with differing phenotypes of infestation

(In Press in *Experimental and Applied Acarology*, see Appendix 4)

Abstract

Blue ticks, *R. decoloratus* and *R. microplus* threaten cattle production in most tropical and subtropical areas of the world. Delayed skin hypersensitivity reactions are thought to cause Nguni cattle to be more resistant to *R. microplus* than Bonsmara cattle yet the cellular mechanisms responsible for these differences have not been classified. Tick counts and inflammatory cell infiltrates in skin biopsies from feeding sites of adult *R. microplus* ticks were determined in nine-month-old Nguni and Bonsmara heifers to determine the cellular mechanisms responsible for tick immunity. Nguni heifers (1.7 ± 0.03) had lower ($P < 0.05$) tick counts than the Bonsmaras (2.0 ± 0.03). Parasitized sites in Nguni heifers had higher ($P < 0.05$) counts of basophils, mast and mononuclear cells than those in the Bonsmara heifers. Conversely, parasitized sites in Nguni heifers had lower ($P < 0.05$) neutrophil and eosinophil counts than those in the Bonsmara heifers. Tick count was negatively correlated ($P < 0.05$) with basophil and mast cell counts and positively correlated with eosinophil counts in both breeds. In the Bonsmara breed, tick count was positively correlated with mononuclear cell counts. Cellular responses to adult *R. microplus* infestations were different and correlated with differences in tick resistance in Nguni and Bonsmara cattle breeds. It is essential to further characterise the molecular composition of the inflammatory infiltrate elicited by adult *R. microplus* infestation to fully comprehend immunity to ticks in cattle.

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Keywords: Bonsmara, cutaneous basophil hypersensitivity; mast cells; Nguni cattle, *Rhipicephalus microplus*

6.1 Introduction

The immunological mechanisms responsible for the high levels of acquired resistance to ticks in Nguni cattle are still unclear. Acquired resistance to tick infestation involves humoral and cellular immune-regulatory and effector pathways (Camargo Mathias et al., 2011). In Chapter 5, cutaneous hypersensitivity responses to *Rhipicephalus* larval antigen were evaluated and strongly suggested that delayed hypersensitivity reactions could be associated with superior tick resistance in the Nguni cattle breed. The absence of a delayed hypersensitivity response coupled with the presence of an intense immediate hypersensitivity reaction was then linked to increased tick susceptibility in the less resistant Bonsmara breed. The differences in hypersensitivity reactions to ticks in Nguni and Bonsmara cattle could be better understood if cellular responses at tick feeding sites were characterised in these breeds.

Studies on the histology of tick attachment sites in cattle have revealed that mast cells, eosinophils, basophils and lymphocytes play some part in resistance to artificial tick infestations in cattle, with varying importance and roles (Verrisimo et al., 2008; Carvalho et al., 2010; Constantinoiu et al., 2010). Ribeiro (1989) and Boppana et al. (2005) pointed out however, that the disadvantage of artificially induced animal–tick associations is that they are generally characterised by more intense expression of acquired resistance than

naturally occurring tick–host relationships. Few studies have focused on comparing the immunological mechanisms of resistance to natural infestation with *Rhipicephalus* ticks in cattle of differing phenotypes of infestation. In addition, little attempts have been made to describe the relationships between the various cellular immune components to tick counts and therefore resistance to *Rhipicephalus* ticks in naturally infested cattle. Elucidating the cellular mechanisms by which tick resistant cattle prevent heavy infestation can be important for the comprehension of TBD transmission and can also aid the development of alternative immune-based tick control methods.

Differences in cellular responses to *R. microplus* infestation and their relationship with tick immunity in Nguni and Bonsmara cattle have not been studied, despite long standing evidence of differences in tick resistance between the two breeds. The correlation between breed and cellular responses to tick infestation and its relationship to tick immunity in Nguni and Bonsmara cattle also requires investigation. The objective of the current study was, therefore, to determine the cellular responses at the attachment sites of *R. microplus* ticks in Nguni and Bonsmara heifers reared on a tick infested rangeland. The alternate hypothesis tested was that the histopathology of attachment sites of *R. decoloratus* and *R. microplus* are different in Nguni and Bonsmara cattle.

6.2 Materials and methods

6.2.1 Study site

Sampling for the histopathology of tick attachment sites was conducted at Fort Cox College Farm whose climatic and edaphic information is detailed in section 4.2.1.

6.2.2 Study animals

Nine-months-old Nguni (n = 12) and Bonsmara (n = 12) heifers were randomly selected and used for this study. The heifers were ear tagged for easy identification and grazed on the same natural tick-infested rangeland for six months prior to sampling to enable natural *R. microplus* infestation. All experimental procedures on the heifers were compliant with internationally accepted standards for welfare and ethics in animals (Austin et al., 2004) and were approved by the University of KwaZulu-Natal Animal Ethics Research Committee (Reference number: 097/11/Animal).

6.2.3 Tick load evaluation

Whole body tick counts were conducted on the experimental animals exposed naturally to ticks once just before skin biopsy sampling in January 2012 as described in section 4.2.5. Briefly, each heifer was restrained in a crush pen while two trained enumerators, one on either side of the heifer, counted and recorded all visible engorged adult *R. microplus* ticks on the whole body of the heifer. Since the study heifers were subjected to natural tick infestations, adult female *R. microplus* ticks were the easiest tick stage to identify and enumerate, and as these cause the most severe losses in cattle, hence their attachment sites were selected for biopsy sampling.

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6.2.4 Skin biopsy sampling

The heifers were heavily sedated with 0.2 mg/kg body weight xylazine (Rompun®, Bayer, South Africa) administered intramuscularly in the rump. Four punch biopsies were taken from each animal, two normal skin biopsies from non-parasitized sites, and two parasitized skin biopsies from the feeding sites (parasitized) of fully engorged (4 – 6 mm diameter) adult *R. microplus* ticks respectively, using a 5 mm punch biopsy needle (Kyron Technologies, South Africa). Care was taken to ensure uniform sample collection method and depth. The biopsies were of full skin thickness, 5 mm diameter and 10 mm deep. Skin samples were immediately immersed in buffered formalin (pH 7.0) pending processing.

6.2.5 Histological processing

The skin samples were kept for 24 hours in the fixative, embedded in paraffin and processed according to routine histological techniques. Each biopsy was serially sectioned at a thickness of 4 μm and stained with Haematoxylin-Eosin and May Grünwald Giemsa, to enable general histological evaluation and differential cell counting respectively (van der Heijden et al., 2005).

6.2.6 Section analysis

Sections were analysed under light microscopy. General features were evaluated on Haematoxylin-Eosin stained sections. Total cell counts were made on sections stained by May-Grünwald Giemsa. For this purpose, cells from two areas of 0.0052 mm^2 of the

dermis, immediately below the epidermis and the cement cone, were counted. Means of each area were used for further analysis. The counting was limited by a Reichart integrating graticle (PK6 3X mn, Austria) on oil immersion fields (objective 100×). Differential cell counts (neutrophils, eosinophils, basophils, mononuclear cells and mast cells) were performed on the same sections and areas used for total cell counts. In the presence of a feeding cavity (area of liquefative necrosis in the dermis of the host which sometimes occurs under the tick attachment site) cells surrounding this cavity were counted.

6.2.7 Statistical analyses

All analyses were conducted in Statistical Analysis System version 9.2 (SAS, 2009). The count data were checked for normality using PROC UNIVARIATE (SAS, 2009) and observed to be not normally distributed. Log_{10} $(x + 1)$ transformation was then performed and conferred normality to the count data. The Generalised Linear Model Procedure (SAS, 2009) was used to determine the effects of breed and tick feeding on the cell counts and tick counts. The following statistical model was used:

$Y_{ijk} = \mu + A_i + B_j + AB_{ij} + \varepsilon_{ijk};$

where, Y_{ijk} = cell count; tick count

A_i = effect of breed (i = Nguni and Bonsmara)

B_j = effect of tick feeding (normal skin site and tick bite site)

AB_{ij} = the effect of the interaction of breed and tick feeding

ε_{ijk} = residual error.

Least square means were compared using the PDIFF option (SAS, 2009). Correlations between cell and tick counts were determined using the PROC CORR (SAS, 2009). The χ^2 test was used to determine associations between breed and histologic characteristics of the skin biopsies.

6.3 Results

6.3.1 Tick counts and differential cell counts

Breed had a significant effect ($P < 0.05$) on the $\log_{10} (x + 1)$ tick counts of the study animals, with Nguni heifers (1.7 ± 0.03) having lower ($P < 0.05$) counts than their Bonsmara counterparts (2.0 ± 0.03). Breed, parasitisation and the interaction between breed and parasitisation had a significant effect ($P < 0.05$) on the differential and total cell counts of the study heifers (Table 6.1). The non-parasitized sites in both Bonsmara and Nguni heifers had the least number ($P < 0.05$) of neutrophil and eosinophil counts followed by the parasitized sites in Nguni heifers while parasitized sites in the Bonsmara heifers had the highest counts ($P < 0.05$). Conversely, the parasitized sites in Nguni heifers had the highest basophil, mononuclear and mast cell counts followed by parasitized sites in Bonsmara heifers, non-parasitized sites in Nguni heifers and non-parasitized sites in Bonsmara heifers in descending order ($P < 0.05$). Total cell counts were highest in parasitized sites of the Bonsmara heifers followed by parasitized sites in the Nguni heifers, non-parasitized sites in the Bonsmara heifers and non-parasitised sites in the Nguni heifers in descending order ($P < 0.05$).

Table 6.1: Differential $\log_{10}$ (x+1) cell counts at normal and infested skin sites of Nguni and Bonsmara heifers

Cell type	Bonsmara		Nguni	
	Normal	**Infested**	**Normal**	**Infested**
Neutrophils	0.3 ± 0.03^a	2.3 ± 0.03^c	0.3 ± 0.03^a	1.4 ± 0.03^b
Eosinophils	1.0 ± 0.02^a	1.6 ± 0.02^c	1.0 ± 0.02^a	1.4 ± 0.02^b
Basophils	0.3 ± 0.02^a	0.8 ± 0.02^c	0.6 ± 0.02^b	1.3 ± 0.02^d
Mononuclear Cells	1.3 ± 0.03^a	1.6 ± 0.03^b	1.3 ± 0.03^a	1.8 ± 0.03^c
Mast cells	0.4 ± 0.02^a	1.3 ± 0.02^c	0.8 ± 0.02^b	1.9 ± 0.02^d
Total cell count	2.3 ± 0.08^b	3.1 ± 0.08^d	2.0 ± 0.08^a	$2.9 + 0.08^c$

[a, b, c, d] Means with different superscripts within the same row differ at $P < 0.05$.

6.3.2 Correlations between tick and cell counts

Tick count was correlated ($P < 0.05$) to the differential counts of eosinophils, basophils, mononuclear and mast cells on parasitized sites in the study heifers (Table 6.2). There were significant positive correlations ($P < 0.05$) between tick count and eosinophil counts in both breeds and between tick count and mononuclear cell counts in the Bonsmara breed. Tick count was, however, negatively correlated ($P < 0.05$) to basophil and mast cell counts of parasitized sites in both breeds. In the Nguni breed, tick count had a significant negative correlation ($P < 0.05$) with mononuclear cell counts of parasitized sites. Generally, tick count had significant positive correlations ($P < 0.05$) with eosinophil counts, significant negative relationships ($P < 0.05$) with basophil, mononuclear and mast cell counts and was not significantly correlated to neutrophil and total cell counts. No significant correlations ($P > 0.05$) were observed between tick count and differential cell counts in non-parasitised sites in both breeds.

Table 6.2: Correlations between $\log_{10} (x + 1)$ tick count and differential $\log_{10} (x + 1)$ cell counts in Bonsmara and Nguni heifers

Parameter	Bonsmara	Nguni	Overall
Neutrophils	0.14	0.12	0.14
Eosinophils	0.61[*]	0.89[**]	0.39[*]
Basophils	-0.78[**]	-0.96[**]	-0.48
Mononuclear cells	0.68[*]	-0.79[*]	-0.52[**]
Mast cells	-0.45[*]	-0.94[**]	-0.28[*]
Total cell count	0.27	0.35	0.39

[*] Indicates significant correlations at $P < 0.05$.

[**] Indicates significant correlations at $P < 0.001$.

6.3.3 General features of parasitized skin biopsies

A summary of the categorisation of the histopathological changes on the parasitized skin biopsies in the study heifers is given in Table 6.3. As shown in Figure 6.1, histopathological changes in the epidermis and dermis were more pronounced ($P < 0.05$) in parasitized skin samples from Bonsmara than Nguni heifers. In most of the parasitized skin samples from both Bonsmara and Nguni heifers, there was epidermal fracture associated with the penetration of tick mouth parts as far down as the upper dermis. Parasitized skin samples obtained from Bonsmara heifers had severe basal cell hyperplasia, epidermal necrosis accompanied by acantholysis and oedema while fewer ($P < 0.05$) parasitized samples from Nguni heifers exhibited these changes (Figure 6.1 A, B, C and D). More ($P < 0.05$) Bonsmara heifers exhibited severe pustule-like lesions in the epidermis and moderate to severe inflammatory infiltrates into the dermis. The infiltrates consisted predominantly of neutrophils with few eosinophils, basophils and mast cells. Most Nguni heifers exhibited few epidermal pustule-like lesions and the dermal inflammatory infiltrates were dominated by basophils, eosinophils and some mast cells.

Table 6.3: Histologic characteristics of parasitized skin sites in Nguni and Bonsmara heifers

Parameter	Severity	Nguni (n =12)	Bonsmara (n = 12)	χ^2	Significance
Epidermal hyperplasia	Absent	0	0	0	
	Mild	0	0	0	
	Moderate	8	0	4.8	*
	Severe	4	12		
Epidermal necrosis	Absent	10	0	9.3	*
	Mild	2	3		
	Moderate	0	3		
	Severe	0	6		
Dermal oedema	Absent	0	0		
	Mild	7	0	8.0	*
	Moderate	5	4		
	Severe	0	8		
Vascular reaction	Absent	0	0		
	Mild	8	0	6.9	*
	Moderate	3	6		
	Severe	1	6		
Hyperemia	Absent	0	0		
	Mild	6	2	5.7	NS
	Moderate	5	4		
	Severe	1	6		
Pustules	Absent	9	0	15.0	*
	Mild	2	1		
	Moderate	1	6		
	Severe	0	5		

* Significant at $P < 0.05$; NS not significant
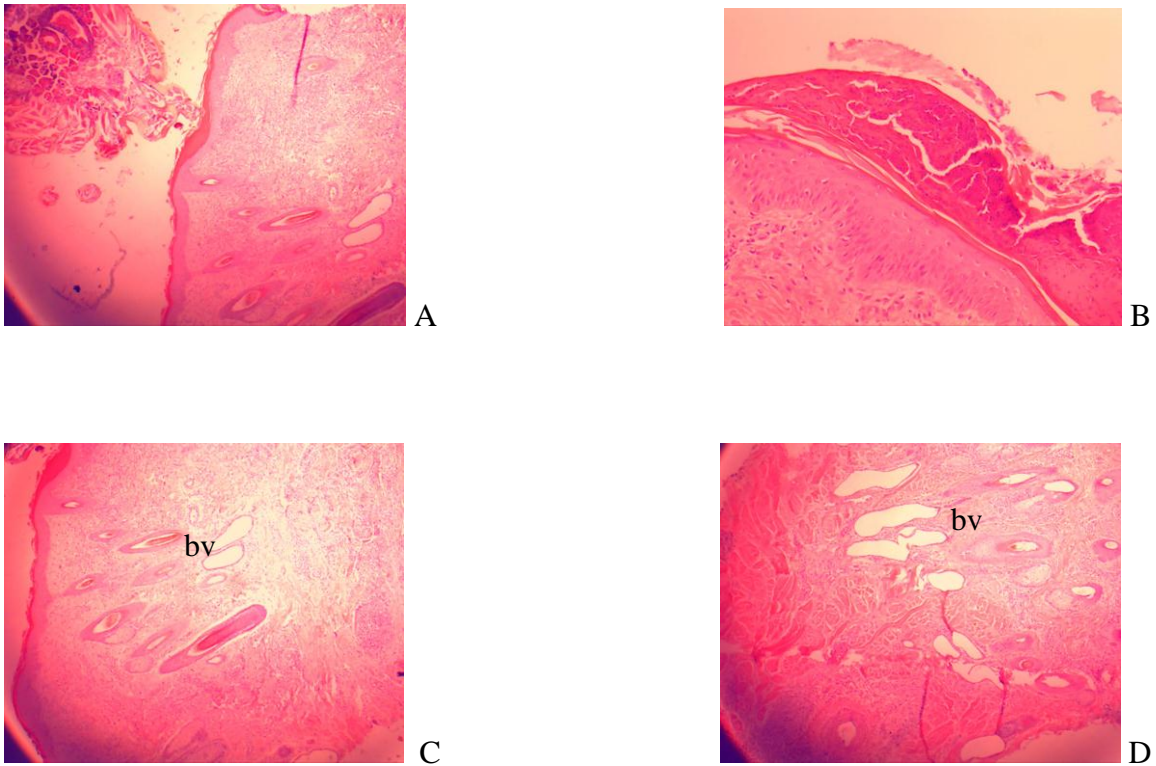
Figure 6.1: Pictures of skin sections (stained with Haematoxylin and Eosin) taken from representative tick-infested Nguni (A and C) and representative tick-infested Bonsmara heifers (B and D). Bonsmara heifers (B) had epidermal fracture, severe basal cell hyperplasia, epidermal necrosis accompanied by acantholysis and oedema while Nguni heifers (A) did not exhibit these changes. Nguni heifers (C) had fewer blood vessels (bv) in the dermis than the Bonsmara heifers (D)

6.4 Discussion

The observed lower tick counts in the Nguni heifers, may likely confirm assertions by Muchenje et al. (2008) and Marufu et al. (2010; 2011) that the Nguni has superior tick resistance than the Bonsmara breed. It was suggested in Chapter 4 that the development of greater resistance in the Nguni breed has most likely resulted from its long continuous contact with the blue ticks. High tick infestation in the tropics and subtropics (Porto Neto et al., 2011) could have placed strong selection pressure on Nguni cattle enabling them to develop heightened immune responsiveness to ticks, a trait which may have allowed them to survive in this environment. Resistance to *R. microplus* could also be related to the portion of *Bos taurus africanus* genes in the cattle, however this requires further investigation. Tick counts are a reliable proxy for tick resistance (Verissimo et al., 2008); however, tick biological parameters such as engorgement weight, feeding period, moulting period and tick yield (Wikel, 1999; Camago Mathias et al., 2011) may provide more accurate information on tick resistance. Further investigations into these tick biological parameters and their correlations with immunological parameters associated with tick resistance could be essential in providing comprehensive information on the Nguni breed's tick resistance status and immune responsiveness to ticks.

Epidermal hyperplasia, acantholysis, oedema and necrosis observed in all the parasitized biopsies in the current study were expected as these are common non-specific changes induced by noxious stimuli in the host's skin (Szabo and Bechara, 1999; Monteiro and Bechara, 2008). The more severe oedema and necrosis in the Bonsmara heifers could likely be a result of a chronic allergic-type (Type I Hypersensitivity) response to ticks

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which may have led to tick susceptibility. This chronic Type I hypersensitivity reaction was reported to be advantageous to the tick (Tatchell and Moorhouse, 1968; Piper et al., 2010) and could likely be linked to the lack of development of cellular immunity and hence decreased tick resistance in susceptible hosts. Reduced inflammatory response in the Nguni cattle, on the other hand, may likely be due to an evolutionary acquired ability to respond less vigorously to bioactive molecules in the *R. microplus* tick saliva as postulated by Piper et al. (2010) for tick resistant Brahman cattle.

Higher neutrophil and eosinophil counts on parasitized sites in the Bonsmara heifers could likely be associated with their lower tick resistance. The present findings are in accordance with those of Wada et al. (2010) who observed increased neutrophil and eosinophil infiltrations in parasitized sites of less resistant hosts and surmised that recruitment of these cells was insufficient or dispensable for the manifestation of tick resistance. Neutrophils are highly motile phagocytic cells that constitute the first line of defence of the innate immune system (Francischetti et al., 2009). Increased neutrophilic infiltration may be due to molecules present in tick saliva or to chemokines secreted by degenerating epidermal cells in the inflammatory focus. Constantinoiu et al. (2010) suggested that neutrophils do not play a major role in resistance to *R. microplus* ticks in cattle. In the present study, neutrophils were associated with increased breakdown of extracellular matrix and necrosis around tick mouth parts in parasitized sites of tick susceptible Bonsmara cattle. The present findings support the reports of Tatchell and Moorhouse (1970) that neutrophils might be responsible for paving the way for tick

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feeding by destroying the extracellular matrix around the tick attachment lesion allowing ticks access to tissue fluids and blood.

Eosinophils are predominant in body surfaces that interact with the external environment, such as the skin, and are generally associated with parasitic infestation or allergic reactions (Francischetti et al., 2009). Earlier researchers proposed that eosinophils are involved in the translocation of mast cell histamine to the tick attachment site resulting in increased grooming and tick rejection in cattle (Schleger et al., 1981). In the present study, high eosinophil presence in tick attachment sites of tick susceptible Bonsmara cattle seems to suggest that eosinophils are associated with reduced *R. microplus* resistance in cattle. Eosinophils have been linked to allergic-type reactions under the influence of immunoglobulin E and this chronic allergic type reaction may have led to increased tick susceptibility in Bonsmara cattle. The positive correlation between tick count and eosinophil count in the study heifers suggests that infiltration of the bite site by high numbers of eosinophils leads to reduced resistance to *R. microplus* ticks. This finding contrasts that of Carvalho et al. (2010) who reported that increased resistance to adult *R. microplus* ticks was associated with high eosinophil presence in the tick bite site of infested cattle. These authors concluded from their study that resistant bovines have a greater capacity than susceptible hosts to retain eosinophils in the lesion of adult tick-infested skin. The present study therefore highlights the need to further investigate the role of eosinophils in resistance to ticks in cattle.

In the indigenous Nguni heifers, the observed higher basophil and mast cell counts suggests superior tick resistance in this breed. The present findings are in agreement with Monteiro and Bechara (2008) and Carvalho et al. (2010) who reported that basophil accumulations in tick attachment sites are significantly associated with tick resistance in caprine and bovine hosts respectively. Basophils have long been reported to be responsible for the development of acquired tick resistance in cattle (Brown et al., 1984). Direct anti-tick reaction and antigen/antibody-mediated activation of mast cells to effect tick rejection, are the two ways in which basophils are thought to accomplish tick rejection (Wada et al., 2010). In the former instance, basophils are thought to migrate and cluster close to tick mouth parts in the skin, de-granulate and release local mediators which cause immune skin rejection of blood-feeding ticks, the so called cutaneous basophil hypersensitivity (CBH) reaction (Francischetti et al., 2009; Wada et al., 2010). The CBH is a form of delayed type hypersensitivity which is thought to be mediated by T helper 1 lymphocytes (Brossard and Wikel, 2004). The local mediators released by basophils that are involved in the manifestation of tick resistance, however, remain to be fully identified.

Mast cells, and the histamine they contain inside cytoplasmic granules, are of fundamental importance to the self-grooming mechanism, which is thought to be critical to resistance of cattle to the *R. microplus* tick (Koudstaal et al., 1978; Kemp and Bourne, 1980; Schleger et al., 1981). Self-grooming caused by histamine from degranulated mast cells is an important factor in reducing tick burdens (Maharanaa et al., 2011) and could have led to the lower tick loads in Nguni cattle in the present study. Mast cells also

contribute to the expression of acquired immunity to ticks through the release of other bioactive molecules such as leukotrienes, prostaglandins and enzymes at the bite site (Wikel, 1999). The observed higher mast cell counts in the Nguni cattle may thus have led to the breed's superior tick resistance compared with the Bonsmara breed. The present findings agree with those of Moraes et al. (1992) and Verrisimo et al. (2008) who also reported higher dermal mast cell counts in highly tick resistant indicine cattle breeds than in the tick susceptible taurine breeds.

The negative correlations observed between tick count and basophil and mast cell counts in the Nguni and Bonsmara heifers suggest that these cells have an important role in conferring tick immunity in cattle. The present findings support the reports of Wada et al. (2010) that both basophils and mast cells synergistically contribute to the manifestation of tick resistance in animals. Tick infestation has been thought to cause a modification in the skin of parasitized hosts leading to massive migration of basophils and mast cells to tick attachment sites thus effecting tick rejection in the highly resistant bovine hosts (Engracia Filho et al., 2006; Monteiro and Bechara, 2008). The chemical mediators released by degranulated mast cells and basophils are thought to play an important role in the resistance mechanism of cattle to ticks (Verrisimo et al., 2008). Further elucidation of the roles of each inflammatory mediator released by basophils and mast cells may lead to better understanding of their roles in tick immunity in cattle. From the present findings however, it can be concluded that the higher the infiltration of basophils and mast cells in the tick attachment site of cattle the more resistant they are likely to be to *R. microplus* infestation.

The negative correlation between mononuclear cell counts and tick count in the Nguni breed and positive correlation between the two parameters in the Bonsmara breed could signify specific differences in cellular response to *R. microplus* infestation in tick resistant and susceptible cattle. Mononuclear cells include macrophages which process and present tick antigens to T-cells which in turn stimulate cellular and humoral (antibody production) specific immune responses (Francischetti et al., 2009). Tick saliva is known to contain molecules that inhibit lymphocyte and macrophage function thus affecting cellular immune responses in susceptible hosts (Castagnolli et al., 2008). The higher *R. microplus* tick loads in Bonsmara heifers in the present study could likely have led to tick-induced suppression of lymphocyte and macrophage function, despite their infiltration of tick attachment sites. It is possible that Nguni cattle produced humoral antibodies to neutralise immunosuppressive molecules secreted by the ticks and hence lymphocyte and macrophage function was not affected in this breed. Studies on the characterisation of molecular responses at the tick–host interface in Nguni and Bonsmara cattle could give clarity on this postulation.

The present study shows the important role that mast cells, basophils and mononuclear cells play in the resistance of cattle to the *R. microplus* tick. It should be noted, however, that the present data represent a small number of biological replicates and thus caution should be taken when interpreting these results. Cellular responses to adult *R. microplus* tick bites were shown to differ in hosts of differing tick resistance and were correlated with variations in acquired resistance to tick infestation. The acquisition of tick resistance

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is associated with reduced pathogen transmission from infected ticks (Wikel, 1999; Marufu et al., 2010). The current findings may, therefore, provide avenues toward the development of novel control strategies such as the development of anti-tick vaccines which can also be used to control TBD. Considering the wide geographical distribution of *R. microplus* and its increasing range, its effect on the molecular immune responses in cattle of differing resistance need further detailing. Genetic polymorphisms originating in the host may have several effects on tick resistance and these need to be profiled in Nguni and Bonsmara cattle to elucidate the genetic mechanisms involved in tick resistance.

6.5 Conclusions

Cutaneous reactions to bites of adult *R. microplus* ticks in the Nguni breed differed significantly with those of the Bonsmara breed. The tick resistant Nguni heifers had more basophils, mononuclear cells and mast cells in their inflammatory infiltrates, while infiltrates in the less resistant Bonsmara heifers had more neutrophils and eosinophils. Tick resistance in the Nguni breed was correlated with cutaneous basophil hypersensitivity while tick susceptibility in the Bonsmara breed was associated with chronic allergic-type reaction on the tick bite sites. Further characterisation of the molecular composition of the inflammatory infiltrate elicited by adult *R. microplus* infestations remains essential for the comprehension of immunity to ticks in cattle.

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CHAPTER 7: General Discussion, Conclusions and Recommendations

7.1 General Discussion

Ticks and TBD are major challenges to cattle production for smallholder farmers in the semiarid areas of South Africa. Nguni cattle have been reported to be resistant to ticks and TBD (Muchenje et al., 2008; Marufu et al., 2010) however the mechanisms responsible for this important trait are not fully understood. The main hypothesis tested in the present study was that morphological coat traits, skin hypersensitivity responses and cellular reactions at the tick infestation sites are the mechanisms responsible for tick resistance in Nguni cattle. Prior to determining the tick resistance mechanisms in Nguni cattle, however, it is important to begin by determining the prevalence of tick-transmitted infections, such as bovine anaplasmosis, the most economically impacting TBD of cattle in semiarid areas of South Africa, and its possible associations with tick vector infestations in smallholder production systems.

Bovine anaplasmosis caused by *A. marginale* is one of the leading causes of cattle mortalities particularly in the smallholder farming system in the semiarid areas of South Africa. Various factors may influence the prevalence of *A. marginale* infection in cattle including production system, tick infestation level, cattle breed, age and nutritional performance, but, their effect on the molecular prevalence of bovine anaplasmosis in semiarid farming areas of South Africa are not fully known. In Chapter 3, the molecular prevalence of bovine anaplasmosis and subsequent production losses were determined in Nguni and local crossbred cattle reared under small scale and communal farming

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systems. It was hypothesised that the prevalence of bovine anaplasmosis is different in Nguni and local crossbred cattle reared by smallholder farmers in the semiarid areas of South Africa. Cattle owned by small scale farmers had higher prevalence of *A. marginale* than those owned by communal farmers. Small scale farmers dipped their cattle less frequently than communal farmers leading to increased tick exposure in the former's herds which could have increased the transmission of *A. marginale* to their cattle. Younger animals had a higher prevalence of *A. marginale* and higher odds of infection than adult cattle, which could likely have contributed to the development of endemic stability in the study area. Tick counts and the molecular prevalence of *A. marginale* were similar in Nguni and local crossbred cattle. Nguni cattle, nonetheless, suffered less severe losses from *A. marginale* infection than local crossbreds. Infection with *A. marginale* did not affect the body weight and condition scores of Nguni cattle suggesting that the Nguni breed is more resilient to the adverse effects of infection with *A. marginale* than local crossbreds. Resilience to *A. marginale* in Nguni cattle could be associated with the superior tick resistance in this breed however the mechanisms responsible for tick resistance still remained unclear.

Resilience to *A. marginale* infection in Nguni cattle could be linked to the breeds' resistance to ticks, which may be associated with facourable coat characteristics that deter tick attachment. In Chapter 4, the relationship between coat characteristics and tick counts (a measure of tick resistance) were determined in the Nguni and Bonsmara heifers to understand the possible mechanisms of tick resistance in cattle. The hypothesis tested was that the relationships between tick count and morphological coat traits are different

in Nguni and Bonsmara cattle. The Nguni cattle had lower tick counts, smoother coats and shorter hairs than the Bonsmara heifers. The relationship between tick count and coat score was linear in the Nguni breed and quadratic in the Bonsmara breed. This suggested that at lower coat scores, both breeds carried similarly low tick loads, however, as the coat score increased, the Bonsmara breed carried higher tick loads than the Nguni breed. These findings suggest that smoother coats and shorter hairs are responsible for higher tick resistance in Nguni cattle than in their Bonsmara counterparts. Low tick infestations were observed in Nguni cattle with rough coats and long hairs and suggested that tick resistance in the Nguni may be dependent on non-innate immunological mechanisms. Specific indicators to the mechanism of host resistance could be obtained by studying skin immunity to ticks in the Nguni breed. It was, therefore, essential to assess skin hypersensitivity responses to tick antigens in Nguni and Bonsmara cattle.

To improve knowledge on the immunological mechanisms responsible for tick resistance in cattle, skin hypersensitivity reactions to unfed larval extracts of *R. decoloratus* and *R. microplus* in Nguni and Bonsmara heifers were evaluated in Chapter 5. It was hypothesised that Nguni and Bonsmara cattle have dissimilar cutaneous hypersensitivity reactions and resistance to both *Rhipicephalus* tick species. Bonsmara heifers showed only an immediate type hypersensitivity response to ULE of both blue tick species characterised by soft swelling at the inoculation site suggesting that this is the only type of hypersensitivity response mounted in tick susceptible cattle. The presence of only an immediate type response and lack of a delayed type hypersensitivity reaction to tick antigens in the Bonsmara heifers could point to a deficit response in this breed which

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most likely led to increased tick susceptibility. In the Nguni heifers, a delayed type hypersensitivity response was observed over and above the immediate type response and was most likely the cause of tick resistance in this breed. The development of a delayed type hypersensitivity response has been linked to the expression of acquired resistance to ticks in cattle (Bechara et al., 2000; Prudencio et al., 2011). The *R. decoloratus* ULE elicited a more intense skin hypersensitivity response in both breeds than the *R. microplus* ULE signifying higher antigenicity in the former tick species. The differences in cutaneous hypersensitivity responses to *Rhiphicephalus* ticks observed in Chapter 5 could be better understood if cellular reactions at the tick attachment sites were also characterised.

In Chapter 6, inflammatory cell infiltrates in skin biopsies from feeding sites of adult female *R. microplus* ticks were evaluated in nine-month-old Nguni and Bonsmara heifers with the purpose of determining the cellular mechanisms responsible for tick immunity. The alternate hypothesis tested was that the histopathology of attachment sites of *R. decoloratus* and *R. microplus* are different in Nguni and Bonsmara cattle. Parasitized sites in Nguni heifers had higher counts of basophils, mast and mononuclear cells than those in the Bonsmara heifers. The high basophil infiltration of parasitized sites (cutaneous basophil infiltration) in Nguni heifers may be linked with the delayed hypersensitivity reaction that was reported for this breed in Chapter 5, and most likely resulted in increased tick resistance. Increased mast cells presence could have likely lead to increased self-grooming in Nguni cattle, thus reducing tick burdens in this breed. Parasitized sites in the Bonsmara heifers had higher neutrophil and eosinophil counts than

those in the Nguni heifers. High neutrophil presence in parasitized sites were associated with increased breakdown of extracellular matrix and necrosis around tick mouth parts enabling tick feeding and thus increased tick susceptibility in the Bonsmara breed. Tick count was negatively correlated with basophil and mast cell counts and positively correlated with eosinophil counts in both breeds. The negative correlations between tick count and basophil and mast cell counts in the Nguni and Bonsmara heifers could point to the synergistic contributions of these cells in conferring tick immunity in cattle. In the Bonsmara breed, tick count was positively correlated with mononuclear cell counts.

7.2 Conclusions

Molecular prevalence of *A. marginale* was similar in Nguni and local crosbred cattle. Nguni cattle were more resilient to anaplasmosis and suffered less severe losses from *A. marginale* infection than local crossbreds. Tick count had a positive linear relationship with coat score in the Nguni and a quadratic relationship with coat score in the Bonsmara breed. Nguni heifers had smoother coats and shorter hairs, adaptive mechanisms, which made them less susceptible to ticks than Bonsmara heifers. Unfed larval extracts of *R. decoloratus* and *R. microplus* induced an immediate type hypersensitivity reaction, which was associated with tick susceptibility in previously sensitised Bonsmara cattle. In previously sensitised Nguni cattle, ULE of *R. decoloratus* and *R. microplus* elicited immediate followed by delayed hypersensitivity reactions and these were associated with tick resistance. The *R. decoloratus* ULE had higher antigenicity and elicited a more intense skin hypersensitivity response in both breeds than the *R. microplus* ULE. Inflammatory infiltrates of adult female *R. microplus* cutaneous attachment sites had

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more basophils, mononuclear cells and mast cells in the tick resistant Nguni breed, and more neutrophils and eosinophils in the less resistant Bonsmara breed. Tick resistance in the Nguni breed was correlated with cutaneous basophil hypersensitivity while tick susceptibility in the Bonsmara breed was associated with chronic allergic-type hypersensitivity on the attachment sites of *R. microplus* ticks. The current study's findings highlight the important role of morphological coat traits, hypersensitivity and cellular immunity in tick resistance in the Nguni cattle breed.

7.3 Recommendations

It is recommended that small scale and communal farmers improve the levels of immunity to *A. marginale* and thus improve resistance to bovine anaplasmosis in their herds. This can be achieved by providing supplementary feeding especially to young cattle which are mostly vulnerable to *A. marginale* infection. Smallholder farmers are advised to select, utilising coat characteristics and tick counts, and breed tick resistant cattle to minimize the direct effects of ticks while conserving endemic stability to bovine anaplasmosis in their herds. It can be recommended that farmers augment visual enumeration of ticks with assessment of the coat score when identifying and selecting cattle for higher tick resistance in their herds. The intradermal test should be used in conjunction with the assessment of coat characteristics and evaluation of skin biopsies to aid in accurate characterisation of the level of tick resistance in cattle.

The following aspects require further research:

1. Determination of the minimum threshold of ticks necessary for the development of endemic stability to bovine anaplasmosis on Nguni and local crossbred cattle populations in smallholder farming areas in semiarid regions. This is important for the enhancement of protective immunity in older age cattle, thus, reducing the molecular prevalence of bovine anaplasmosis in smallholder cattle herds.

2. Elucidation of the genotype associated mechanisms of resistance to anaplasmosis in indigenous Nguni cattle. Understanding the genotype associated resistance to bovine anaplasmosis in Nguni cattle may open up avenues towards the genetic control of the disease in cattle.

3. Investigation of the role of eosinophils in tick resistance in cattle. Information from such investigations would lead to improved understanding of immediate-type hypersensitivity reactions to tick infestations in tick susceptible cattle where eosinophils are thought to play a part.

4. Elucidation of the roles of each inflammatory mediator released by basophils and mast cells in tick immunity in cattle. Understanding the functions of the molecular responses to tick infestation will increase knowledge on the DTH responses of tick-resistant cattle and augment existing information obtained from the cellular response studies.

5. Profiling genetic polymorphisms in cattle and determining their associations with tick resistance.

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7.5 Appendices

Appendix 1: Relationships between tick counts and coat characteristics in Nguni and Bonsmara cattle reared on semiarid rangelands in South Africa (Published in *Ticks and Tick-borne Diseases*)

Original article

Relationships between tick counts and coat characteristics in Nguni and Bonsmara cattle reared on semiarid rangelands in South Africa

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ABSTRACT

Indigenous Nguni cattle are adapted to the semiarid rangeland and appear to be resistant to ticks; however, the mechanism for tick resistance is yet to be established. To understand tick resistance in cattle, relationships among skin thickness, hair length, coat score, and tick counts were estimated in Nguni (n = 12) and Bonsmara (n = 12) heifers on semiarid rangelands of South Africa. The tick species observed to infest the heifers were *Rhipicephalus (Boophilus) decoloratus* (frequency: 76%), *Rhipicephalus (Boophilus) microplus* (9%), *Amblyomma hebraeum* (5%), *Rhipicephalus appendiculatus* (5%), *Rhipicephalus evertsi evertsi* (3%), and *Hyalomma marginatum* (2%). Nguni heifers had lower ($P < 0.05$) $\log_{10} (x + 1)$-transformed coat scores (0.6 ± 0.01), hair length (1.4 ± 0.01), and tick counts (1.4 ± 0.03) than Bonsmara heifers whose $\log_{10} (x + 1)$-transformed coat score, hair length, and tick count values were 0.7 ± 0.01, 1.5 ± 0.01, and 1.8 ± 0.02, respectively. The skin thickness between the two breeds were similar ($P > 0.05$). There was a positive linear ($P < 0.05$) relationship between $\log_{10} (x + 1)$ tick counts and $\log_{10} (x + 1)$ coat score in the Nguni ($y = 1.90x - 0.40$) and a quadratic relationship in the Bonsmara ($y = -7.98x^2 + 12.74x - 3.12$) breed. It was concluded that the smooth coats may be one of the important mechanisms of tick resistance in the indigenous Nguni breed. Determination of genetic resistance to ticks in the Nguni breed is recommended as this will give more specific indication to the mechanism of host resistance in this breed.

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Introduction

On semiarid rangelands, cattle are exposed to external parasites, such as ticks, and to diseases which reduce cattle performance, production, and profitability (Foster et al., 2008). Ticks and tick-borne diseases are, arguably, one of the biggest challenges to cattle productivity in semiarid areas (Mapiye et al., 2009) due to poor cattle health management, lack of knowledge on drug usage, and the use of inappropriate cattle breeds (Dold and Cocks, 2001; Marufu et al., 2010, 2011). Ticks reduce live weight gain, meat quality, milk production, fertility, and often cause death in cattle (de la Fuente et al., 2007; Muchenje et al., 2008). At present, tick control in cattle reared on semiarid rangelands in South Africa depends on the use of acaricides (Moyo and Masika, 2009). Inappropriate and prolonged use of the same chemicals without rotation leads to the development of acaricide resistance in ticks. The prolonged use of acaricides will also result in the contamination of meat, milk, and the environment (Machado et al., 2010). Selecting for and rearing cattle breeds that are resistant to ticks is a more sustainable way of controlling ticks

and tick-borne diseases on the semiarid rangelands (Marufu et al., 2010).

Currently, on the semiarid rangelands of South Africa, farmers are opting to use the Nguni and Bonsmara breeds because they are able to withstand the harsh environmental conditions such as high temperatures, long dry periods, diseases, and parasites (Muchenje et al., 2008; Ndlovu et al., 2008). The Nguni is an indigenous cattle breed that has been under natural selection pressure from many disease agents. It performs well under harsh pedoclimatic and socio-economic conditions which prevail in the semiarid areas (Mapiye et al., 2007). Imported breeds, which have been developed under relatively benign conditions, are susceptible to heat stress, nutritional stress, and perform poorly under these harsh conditions and extensive management in the semiarid areas. The Bonsmara, a synthetic breed, was developed from crosses between indigenous Afrikaner, and exotic Shorthorn and Hereford cattle to compete with European beef cattle breeds, while tolerating semiarid conditions such as high temperatures (Ndlovu et al., 2008). Though it is heat tolerant, the Bonsmara is not suitable for rearing in tick-infested areas as it succumbs to tick-related illness. Recent studies have shown that Nguni cattle carry lower tick loads and therefore appear to be more resistant to ticks than Angus and Bonsmara cattle (Muchenje et al., 2008). Although the Nguni breed appears to

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be resistant to ticks, the mechanism for tick resistance is yet to be established. Resistance to ticks in the Nguni breed could be related to favourable coat characteristics, superior skin immunity, or the abundance of tick resistance genes.

Evidence suggests that coat characteristics such as hair length, skin thickness, and coat scores influence tick counts and are significantly related to tick resistance in cattle on rangelands (Verissimo et al., 2002; Foster et al., 2008; Martinez et al., 2006). It has been reported that animals with shorter hairs and smoother coats tend to have lower tick counts compared to those with longer hairs and woollier coats (Martinez et al., 2006), and also those with thinner skins could have a reduced susceptibility to ticks compared to those with thicker skins (de Castro et al., 1991). While indigenous cattle in the semiarid areas are known to carry low tick loads, little work has been done to relate these to coat characteristics in Nguni and Bonsmara cattle. Although correlations have been reported between tick count and coat characteristics, there is no information on the nature of the relationships between tick count and coat characteristics in Nguni cattle. There is a need to establish relationships between coat characteristics and tick count in the indigenous and locally adapted cattle breeds reared on the semiarid rangelands to understand the mechanisms of tick resistance and thus characterise these cattle breeds.

Cattle herds owned by smallholder farmers in the semiarid areas are mainly composed of heifers and cows which due to their vulnerability to poor nutrition suffer greater stress and have increased susceptibility to diseases and parasites (Mapiye et al., 2009). As tick resistance is of moderate heritability (Budeli et al., 2009), it is important to identify and select tick-resistant females that remain in the herd for longer periods, so as to confer resistance to their offspring. Coat characteristics, if well understood, could be easily used to select for tick-resistant animals. The identification, selection, and rearing of tick-resistant breeds is one of the cheap, effective, and sustainable methods of controlling ticks (Latif, 1992; Mattioli et al., 2000) in the cattle enterprises. Selecting tick-resistant cattle benefits the farmer by reducing costs on ticks and tick-borne disease control while increasing productivity and profitability in their enterprise. In the current study, relationships among skin thickness, hair length, coat score, and tick count were determined in Nguni and Bonsmara cattle on semiarid rangelands.

Materials and methods

Study site

The study was conducted at Fort Cox College of Agriculture and Forestry farm which is located on 27° 01 East and 32° 46 South in the False Thorn veld. The vegetation is composed of several trees, shrubs, and grass species. *Acacia karroo, Themeda triandra, Panicum maximum, Digitaria erientha, Eragrostis* species, and *Cynodon dactylon* are dominant. The topography of the area is generally flat with a few steep slopes. The climate is semiarid with the average annual rainfall of about 480 mm most of which occurs in the hot wet season. Temperature ranges from 7 °C in the cool dry season to 35 °C in the hot dry season. The major tick species are *Rhipicephalus* (*Boophilus*) species, *Hyalomma* and *Amblyomma* species. Only two cattle breeds are kept on the farm, Nguni and Bonsmara, and managed as separate herds with similar breeding programs. The farm keeps heifers and cows and sells all steers and bulls to the beef feedlots and surrounding communal farmers.

Experimental design

Twenty-four heifers aged between 7 and 9 months each of Nguni (n = 12) and Bonsmara (n = 12) breeds were used in the study. The heifers were ear-tagged for easy identification and grazed on natural pasture throughout the 6-week experimental period during the hot wet season (November–December 2010). The average initial body weights and $\log_{10} (x + 1)$-transformed body condition scores of the heifers were 219.5 ± 4.49 and 0.6 ± 0.01 for the Bonsmara and 209.3 ± 4.53 and 0.6 ± 0.01 for the Nguni breed, respectively. The rangeland forage biomass was estimated every week by random sampling of natural pasture using a disc meter. The heifers did not receive acaricide treatment 3 months prior to and during the period of data collection to enable natural tick infestation. Only those animals that became anaemic and debilitated (based on the pallor of mucous membranes, decreased body weight and body condition), due to heavy tick infestation were treated. The trial was stopped when 3 Bonsmara heifers that became anaemic and debilitated due to heavy tick loads were treated after a period of 6 weeks. All experimental procedures were specifically approved for this study and were in compliance with internationally accepted standards for animal welfare and ethics.

Measurement of the body weights and body condition score

Body weights were measured weekly using a cattle scale (LS4, Taltec, South Africa). Body condition was visually appraised weekly, by the same independent assessor throughout the experimental period. A 5-point scale was used to score the heifers with score 1 being very thin and a score of 5 being very fat/obese (Osoro and Wright, 1992).

Coat scores, skin thickness, and hair length

Coat scores were assessed visually by the same independent assessor throughout the experimental period. The coat of each animal was scored using a 1–5 scale based on the level of smoothness of the coat, with 1: excessively smooth, 2: fairly smooth, 3: long coat, 4: woolly, and 5: excessively woolly coat (Taylor et al., 1995).

Measurement of the skin thickness was conducted at the same time as visual appraisal of the coat. Skin thickness was determined using a pair of tuberculin calipers. The skin thickness was measured on the midside area (just caudal to the 13th rib about 20 cm below the dorsal line) since skin thickness on this part is relatively uniform (Wesonga et al., 2006; Foster et al., 2008). A double-fold of skin was measured with the tuberculin calipers placed in an anterior to posterior direction relative to the body of the animal. The skin thickness was measured in millimeters.

Hair samples were collected from the skin of the mid-side area using a shaving stick adapted in such a way that all hairs within a 200-mm² area could be plucked out. The samples were stored in plastic bottles with screw-on caps and sent to the laboratory for the measurement of hair length. Hair length (mm) was taken as the average length of the 10 longest hairs of the sample, according to Machado et al. (2010) and Foster et al. (2008).

Tick counts

Two trained enumerators, one on either side of the animal, were used to carefully examine the animal which was restrained in a crush pen, identifying and recording all visible engorged adult ticks on the skin of the cattle. The ticks were not removed from the skin of animals during the process of enumeration.

Statistical analyses

The data for body condition score (BCS), skin thickness (ST), coat score (CS), hair length (HL), and tick count (TC) were not normally distributed and were transformed using $\log_{10} (x + 1)$ to confer normality. The mixed model procedures for repeated measurements

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(SAS, 2006) were used to determine the effect of breed and week of sampling on body weight (BW), and the $\log_{10} (x + 1)$-transformed BCS, ST, CS, HL, and TC. First-order autoregressive correlation (AR [1]) was fitted to the model on the measurement of interest. Comparisons of least square means were done using the PDIFF option of SAS (2006). Correlations among the $\log_{10} (x + 1)$-transformed ST, CS, HL, and TC were determined using the PROC CORR (SAS, 2006). Those skin parameters that were observed to be significantly related to the $\log_{10} (x + 1)$ TC on the correlations analysis were regressed on the $\log_{10} (x + 1)$ TC using PROC REG (SAS, 2006) to determine the nature of the relationships. The tick count data were grouped into low (0–30), moderate (31–60), and high (>61) categories to enable the determination of frequencies. Frequencies for coat score and tick count were obtained using the PROC FREQ (SAS, 2006).

Results

Breed and week effects on tick count and skin parameters

The tick species that were observed to infest the study animals were *Rhipicephalus (Boophilus) decoloratus*, *Rhipicephalus (Boophilus) microplus*, *Amblyomma hebraeum*, *Rhipicephalus appendiculatus*, *Rhipicephalus evertsi evertsi*, and *Hyalomma marginatum* with the following relative frequencies 76, 9, 5, 5, 3, and 2%, respectively. Since *Rhipicephalus (Boophilus) decoloratus* ticks were observed to be the predominant tick species, only counts for this tick were used for the data analysis. Significant breed differences ($P < 0.05$) were observed for $\log_{10} (x + 1)$-transformed CS, HL, and TC (Table 1). Bonsmara heifers had higher ($P < 0.05$) CS, HL, and TC values compared to the Nguni heifers. There were no significant breed effects on BW, ST, and BCS. The week of sampling had a significant ($P < 0.05$) effect on the BW, BCS, ST, and TC in both breeds.

Table 1
Mean (± standard deviation) of the body weight (BW) and body condition score (BCS), skin thickness (ST), coat score (CS), hair length (HL) (mm), and tick count (TC) in the Nguni and Bonsmara heifers.

Variable	Bonsmara		Nguni	
	Mean ± SE	Range	Mean ± SE	Range
BW (kg)	219.5 ± 4.49	117.00–306.00	209.3 ± 4.53	117.00–296.00
BCS[c]	0.6 ± 0.01	0.48–0.65	0.6 ± 0.01	0.48–0.65
ST[c] (mm)	1.1 ± 0.01	1.00–1.23	1.1 ± 0.01	0.95–1.20
CS[c]	0.7 ± 0.01[b]	0.48–0.78	0.6 ± 0.01[a]	0.30–0.70
HL[c] (mm)	1.5 ± 0.01[b]	1.29–1.63	1.4 ± 0.01[a]	1.24–1.49
TC[c]	1.8 ± 0.02[b]	0.48–2.23	1.4 ± 0.03[a]	0.30–1.82

[a,b]Means in the same row with different superscripts are significantly different ($P < 0.05$).
[c]This indicates values that are $\log_{10} (x + 1)$-transformed.

The BW and BCS were unchanged from the first to the third week followed by increases ($P < 0.05$) from the fourth to the sixth week of sampling in both breeds (Fig. 1). The skin thickness was unchanged in the first 2 weeks, followed by a decrease ($P < 0.05$) in the third week, succeeded by a slight increase in the fourth week ($P < 0.05$), and became unchanged from the fourth to the sixth week as shown in Fig. 1. There was a general increase ($P < 0.05$) in TC from the first to the last week of sampling in both breeds (Fig. 1).

Correlations among skin parameters and tick count

Significant ($P < 0.05$) positive correlations were observed between TC and CS and for Nguni ($r = 0.60$) and Bonsmara ($r = 0.64$) breeds (Table 2). The TC was significantly ($P < 0.05$) correlated to HL ($r = 0.30$) in the Bonsmara breed. It was also observed that CS was correlated ($P < 0.05$) to HL ($r = 0.48$) in the Bonsmara breed. No
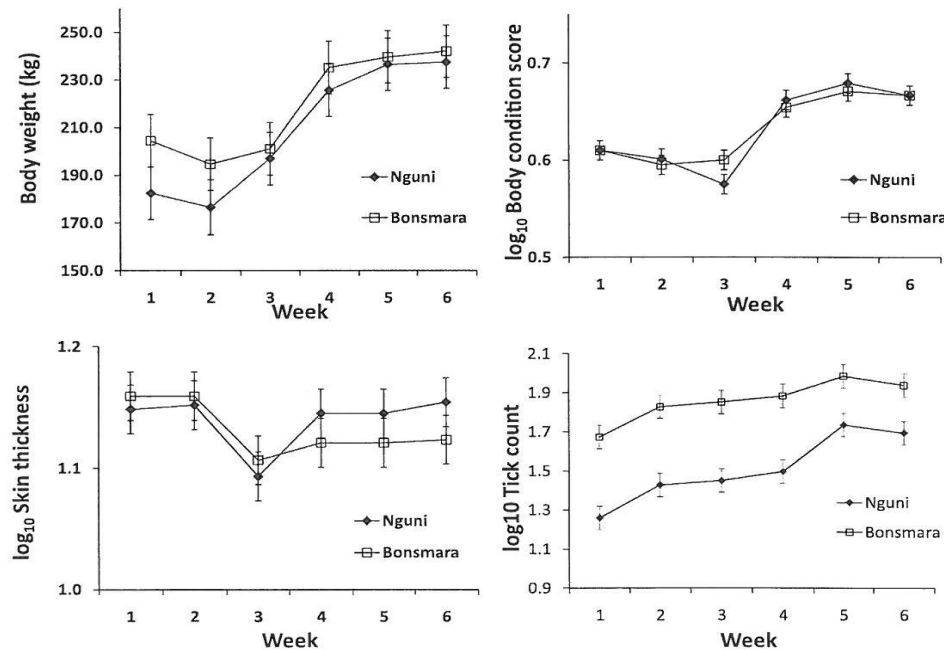

Fig. 1. Weekly changes in the body weight and body condition score, skin thickness and tick count in the Nguni and Bonsmara heifers.

Table 2
Correlations of coat characteristics with tick count (TC) in the Nguni and Bonsmara heifers.

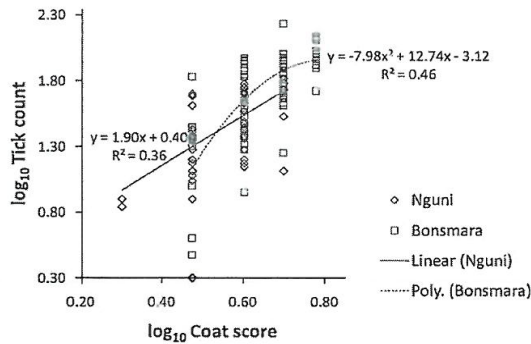|	Nguni TC	Bonsmara TC	Overall TC
Weight	0.31**	0.16	0.26**
BCS	0.44	0.21	0.29***
Skin thickness	0.11	0.13	0.07
Coat score	0.6***	0.64***	0.71***
Hair length	0.05	0.3**	0.35***

** $P < 0.01$.
*** $P < 0.001$.

Fig. 2. Relationship between tick count and coat score in Nguni and Bonsmara heifers.

relationships were observed between TC, ST, and HL in the Nguni breed and between TC and ST in the Bonsmara breed.

Relationships between tick count and skin parameters

There was a significant linear relationship ($P < 0.05$) between TC and CS in the Nguni breed while a significant quadratic relationship ($P < 0.05$) was observed between the two parameters in the Bonsmara breed (Fig. 2). As the CS increased, TC increased in a linear fashion in the Nguni breed. In the Bonsmara breed, initially, TC increased markedly with a slight increase in the CS, but later remained unchanged at higher CS. A significant linear relationship ($P < 0.05$) was also observed between TC and HL in the Bonsmara breed. The variations in tick counts and coat scores in the Nguni and Bonsmara heifers are shown in Tables 3 and 4, respectively. All the Nguni heifers had an initially low tick count, but as time progressed, more Nguni heifers became moderately infested with ticks, and very few had high tick counts by the sixth week. Few Bon-

Table 3
Weekly variations in the numbers of Nguni and Bonsmara heifers at different tick infestation levels.

Week	Breed	Tick infestation level			n
		Low	Moderate	High	
1	Nguni	12	0	0	12
	Bonsmara	3	5	4	12
2	Nguni	10	2	0	12
	Bonsmara	1	3	8	12
3	Nguni	9	3	0	12
	Bonsmara	1	3	8	12
4	Nguni	7	5	0	12
	Bonsmara	0	2	10	12
5	Nguni	6	4	2	12
	Bonsmara	0	0	12	12
6	Nguni	6	3	3	12
	Bonsmara	0	1	11	12

Table 4
Weekly variations in the numbers of Nguni and Bonsmara heifers at different coat scores.

Week	Breed	Coat score					n
		1	2	3	4	5	
1	Nguni	1	6	5	0	0	12
	Bonsmara	0	1	2	7	2	12
2	Nguni	1	5	5	0	0	11
	Bonsmara	0	1	2	7	2	12
3	Nguni	1	5	5	1	0	12
	Bonsmara	0	3	5	2	2	12
4	Nguni	0	5	4	3	0	12
	Bonsmara	0	1	4	4	3	12
5	Nguni	0	3	7	2	0	12
	Bonsmara	0	0	5	4	3	12
6	Nguni	0	3	7	2	0	12
	Bonsmara	0	0	3	8	1	12

smara heifers had low initial tick counts while the majority had moderate to high tick infestations. As time progressed, all Bonsmara heifers had high tick counts by the sixth week. The majority of Nguni heifers were observed to have lower coat scores in the first 3 weeks, becoming moderate in last 3 weeks. Most Bonsmara heifers had moderate coat scores in the first 2 weeks, and in the last 4 weeks, all the Bonsmara heifers had high coat scores.

Discussion

Although indigenous Nguni cattle appear to be adapted to the semiarid areas of South Africa and resistant to ticks, their mechanism for tick resistance has yet to be established. Tick resistance in cattle may be related to coat characteristics including skin thickness, hair length, and coat score (Verissimo et al., 2002) and can be ranked through the use of tick counts (de Castro et al., 1991). In the current study, coat characteristics and tick counts were determined in the indigenous Nguni and the synthetic Bonsmara breeds to determine the mechanism of resistance in the two breeds. The hypothesis tested was that there are no relationships between coat characteristics and tick count in Nguni and Bonsmara cattle.

Tick count was observed to be lower in the Nguni compared to the Bonsmara heifers suggesting a higher tick resistance in the Nguni breed. These findings agree with Spicket et al. (1989) and Muchenje et al. (2008) who found Nguni cattle to have lower tick loads than Bonsmara cattle. The lower tick count in the Nguni cattle may be related to the breed's smoother coat and shorter hairs that tend to discourage tick attachment (Bonsma, 1981). Webb and David (2002) reported lower tick count on positions which have short hairs in Tswana, Simmentaler, and Brahman cattle suggesting that cattle breeds with short hairs have a decreased susceptibility to tick infestation. Tick avoidance behaviour, skin sensitivity, and increased grooming activity may also play a role in reducing tick numbers on the skin of this indigenous Sanga cattle breed as proposed by Meltzer (1996). Further studies are, however, required to evaluate tick avoidance, skin sensitivity, and grooming activity in the Nguni breed to fully understand the mechanism(s) of tick resistance.

The lack of breed differences in the skin thickness concurs with the findings of Spicket et al. (1989). However, the current study's findings are in contrast to earlier reports by Verissimo et al. (2002) who observed significant breed differences in skin thickness between indigenous and crossbred cattle. In their study, Verissimo and colleagues (2002) measured skin thickness on the scapular region while in the present study double-fold skin thickness was measured over the mid-side area which is more uniform in thickness. Skin thickness is influenced by the amount of subcutaneous fat and the site on which it is measured (Brown

et al., 2008), thus likely accounting for the different findings in the two studies. Further work needs to be done on a consistent and correct method of measuring skin thickness so as to accurately relate it to tick resistance in cattle.

The initial decrease in body weight and BCS from the first to the third week of the study period could be attributed to poor herbage quality and rangeland condition during this period. As new growth occurred on the rangeland, the animals responded positively with increases in body weight and BCS from the fourth to the sixth week. It was reported that BCS has an influence on skin thickness (Ayresa et al., 2009). The fluctuations in skin thickness observed in the current study followed a similar trend to that of the BCS, dropping in the first 3 weeks and then increasing from the fourth to the sixth week. The general increase in tick count from the first to the sixth week was expected as the animals were not being dipped and hence carried progressively heavier tick loads. It was noted that Nguni cattle had consistently lower tick loads throughout the study period which may be further testimony of their higher resistance to ticks. Three Bonsmara heifers suffered from the increasing tick loads and had to be dipped and treated to prevent mortalities.

The observation that tick count was positively correlated to coat score in both the Bonsmara and Nguni heifers agrees with the findings of Verissimo et al. (2002) who observed positive correlations between the two parameters in cattle. Heifers that had shorter and smoother coats were also observed to have lower tick counts. This finding further validates earlier assertions by Foster et al. (2008) that animals with smoother coats similarly carried lower tick counts than those with woolly coats. The observed relationship between tick count and hair length in the Bonsmara breed supports earlier assertions by Taylor et al. (1995) that hair length has a role in tick susceptibility in cattle. Animals with shorter hairs tend to have lower tick counts compared to those with longer hairs, since long hairs create favourable conditions for tick survival (Taylor et al., 1995). It is also believed that cattle breeds with short hairs expose ticks to harmful climatic conditions and to predators such as birds (Taylor, 2006). In addition, longer coats may protect ticks from the animal's self-grooming that helps remove attached ticks from the coat (Machado et al., 2010).

The observed relationship between tick count and coat score which was linear in the Nguni and quadratic in the Bonsmara breed suggests that coat score is an important determinant of tick count in these cattle breeds. These findings conform to the findings of Martinez et al. (2006) and Machado et al. (2010) who reported significant positive relationships between tick infestation and coat score in cattle. Smoother coats are thought to secrete more sebum, which acts as a deterrent to tick attachment (Bonsma, 1981; Taylor, 2006). A woollier coat however, creates a microclimate that helps keep the ticks attached to the surface of the animal. It was noted from the regression analysis that, at lower coat scores, both breeds carried similarly low tick loads. However, as the coat score increased, the Bonsmara breed carried higher tick loads than the Nguni breed. Selecting cattle with lower coat scores may thus reduce the level of tick susceptibility and increase tick resistance in the herd. A greater majority of Nguni heifers had low tick counts while exhibiting moderate to high coat scores while a larger number of Bonsmara heifers had high tick loads despite exhibiting only moderate coat scores. The higher numbers of Nguni cattle with low tick counts even at high coat scores may attest to the Nguni breed's superior ability to resist tick infestation which may not be dependent on coat score alone.

Indigenous cattle breeds are known to have adapted mechanisms of tick resistance (Latif, 1992) making them more resistant to ticks than exotic or crossbred cattle (Mattioli et al., 2000). The current study highlights the important role that coat characteristics play in increasing the resistance of indigenous cattle to ticks. The breed differences in tick counts observed in this study also point

to differences in genetics playing a part in tick resistance in these cattle breeds. Genetic mechanisms such as cutaneous hypersensitivity reactions to tick antigens and cellular responses to ticks may have developed in the Nguni breed over a long period and contributed to the development of a breed with superior genetic resistance to ticks. Little is known however, about the genetic mechanisms involved in the resistance of indigenous Nguni cattle to ticks. Genetic resistance may contribute to the biological control of ticks, since the use of resistant animals is one of the most effective solutions to control these parasites (Nascimento et al., 2010). It is thus imperative to study skin immunity to ticks in the Nguni breed as this will give specific indicators to the mechanisms of host resistance in this breed.

Farmers in semiarid areas should utilize cattle breeds like the Nguni that have smoother coats and shorter hairs as these are less susceptible to tick infestation. Since coat score has been shown to be significantly related to tick count, it is important for farmers in semiarid areas to augment visual enumeration of ticks with assessment of the coat score to accurately identify cattle with higher tick resistance in their herds. By selecting those cattle within the Nguni cattle breeds that have consistently a low tick count, shorter hairs, and smoother coats (and hence less susceptibility to tick infestations), farmers in semiarid areas will generally improve their herds' resistance to ticks.

Conclusions

Tick count had a positive linear relationship with coats score in the Nguni and a quadratic relationship with coat score in the Bonsmara breed. Nguni heifers had smoother coats and shorter hairs, adaptive mechanisms, which made them less susceptible to ticks than Bonsmara heifers. Selection and rearing of the Nguni cattle with smoother coats and an increased resistance to ticks could increase profitability and productivity in cattle enterprises on semiarid rangelands. Determination of skin immunity to ticks in the Nguni breed is recommended as it gives specific indicators to the mechanism of host resistance.

Acknowledgements

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Appendix 2: Cutaneous hypersensitivity responses to *Rhipicephalus* tick larval antigens

in pre-sensitized cattle (Published in *Ticks and Tick-borne Diseases*)

Original article

Cutaneous hypersensitivity responses to *Rhipicephalus* tick larval antigens in pre-sensitized cattle

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ABSTRACT

Nguni cattle are known to be more resistant to ticks than Bonsmara cattle, even if the immunological mechanisms responsible for this phenomenon are not fully understood. Cutaneous hypersensitivity responses to unfed larval extracts (ULE) of the ticks *Rhipicephalus decoloratus* and *Rhipicephalus microplus* were investigated in Nguni and Bonsmara cattle to improve knowledge on the immunity to ticks. Hypersensitivity reactions were induced by intradermal inoculation of 0.1 ml of ULE of *R. decoloratus* and *R. microplus* ticks (50 µg protein) in the right and left ear, respectively, of 8–9-month-old Nguni ($n = 11$) and Bonsmara ($n = 9$) heifers. Ear thickness was measured using callipers before and 0.5, 1, 6, 24, 48, and 72 h post inoculation (PI). Bonsmara cattle showed a more intense immediate reaction with maximum response at 1 h PI and no delayed hypersensitivity reaction. Nguni heifers, conversely, presented a less intense immediate reaction with maximum response at 1 h PI, and a delayed hypersensitivity reaction at 72 h PI. Reactions to *R. decoloratus* ULE produced a more intense skin response than to *R. microplus* in both breeds at all time intervals. Nguni cattle showed lower tick infestation indicating higher tick resistance than Bonsmara cattle. Delayed hypersensitivity reaction could be associated with superior tick resistance in the Nguni breed, while immediate hypersensitivity reaction could be associated with increased tick susceptibility in the Bonsmara breed. This study indicates the need for further investigations on the correlation of tick resistance and cellular immune responses to tick infestation in Nguni cattle.

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Introduction

Blue ticks, also known as *Rhipicephalus* (formerly known as *Boophilus*) ticks, severely constrain cattle production in the tropical and subtropical regions of the world (Jongejan and Uilenberg, 2004). The species *R. decoloratus* transmits *Babesia bigemina* and *Anaplasma marginale*, whilst *R. microplus* in addition to those 2 pathogens also transmits *Babesia bovis*. These pathogens cause diseases that economically impact cattle productivity (Jonsson et al., 2008). Blue ticks also affect cattle production directly through debilitation, anaemia, weight loss, and death (Jonsson, 2006), making their control imperative. To satisfy consumer demands for high-quality products while maintaining a clean environment, selection for host resistance has become the method of choice for non-chemical control of ticks (Kongsuwan et al., 2010). An understanding of mechanisms of resistance to ticks in different hosts is, however, a prerequisite for the successful development of host resistance as a tick control method in cattle (Ibelli et al., 2012). The

knowledge thus obtained is also important for reducing transmission of tick-borne diseases (Wikel, 1999; Castagnolli et al., 2003).

Large variation in resistance to ticks exists in different cattle breeds (Mattioli et al., 2000). For example, in the semiarid areas of South Africa where tick infestation levels are moderate to high all year round, the indigenous Nguni cattle has been shown to be more resistant to ticks than the synthetic Bonsmara breed (Muchenje et al., 2008). Tick resistance in the Nguni breed is likely to be of innate origin and has been largely attributed to smoother and shorter coats which act as a deterrent for tick attachment (Marufu et al., 2011a). Resistance to ticks in cattle has also been ascribed to other non-adaptive immune factors such as grooming activity, skin colour and thickness, and area of skin available for infestation (Meltzer, 1996; Machado et al., 2010). Adaptive immune factors involving humoral and cellular responses to tick attachment also contribute to tick resistance in cattle (Brossard and Wikel, 2004). Despite the existence of numerous studies on host resistance, the mechanisms of naturally acquired immunity to ticks in cattle are still poorly understood.

It has been proposed that cutaneous hypersensitivity reactions to tick antigens are responsible for repelling tick infestation (Kemp et al., 1986). An intradermal skin test was, subsequently, developed

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to measure skin hypersensitivity responses to tick antigens and rank cattle according to their level of tick resistance (Bechara et al., 2000). Studies on skin hypersensitivity responses of tick-infested cattle have, however, yielded varied and sometimes conflicting results. Bechara et al. (2000) demonstrated an immediate type of reaction in susceptible *Bos taurus* cattle and both immediate and delayed type hypersensitivity reactions in tick-resistant *Bos indicus* cattle. In contrast, Piper et al. (2010) noted that an intense immediate type of hypersensitivity response to tick infestation was associated with increased tick resistance in *Bos taurus* cattle. Prudencio et al. (2011) reported an intense immediate type hypersensitivity reaction and a slightly delayed hypersensitivity reaction in both susceptible and resistant cattle. Inconsistencies in the above reports, therefore, suggest the need for further evaluation of skin hypersensitivity reaction in tick-susceptible and -resistant cattle.

Although antigenicity differs between tick species (Steen et al., 2006; Mans et al., 2008), research has focused on the skin hypersensitivity responses to a single blue tick species, *R. microplus* while ignoring the other equally important African species *R. decoloratus*. Differences in infestation rates and susceptibility have been observed for *R. decoloratus* and *R. microplus* on Nguni and Bonsmara cattle (Marufu et al., 2011a). The repertoire of antigenic molecules exhibited by *R. decoloratus* and *R. microplus* are, therefore, expected to differ. Skin hypersensitivity reactions to *Rhipicephalus* tick antigens remain uncharacterised in the indigenous Nguni cattle breed despite evidence of its tick resistance status. It remains unknown whether the Nguni breed has similar cutaneous hypersensitivity responses to those of other tick resistant breeds. It is also unclear whether cutaneous hypersensitivity in Nguni cattle differs from the less tick-resistant Bonsmara breed. Comprehension of skin hypersensitivity reactions will aid the development of anti-tick vaccines which should be designed to promote an appropriate immune response to infestation (Piper et al., 2008). In the current study, cutaneous hypersensitivity reactions to unfed larval extracts of *R. decoloratus* and *R. microplus* were compared in Nguni and Bonsmara cattle. It was hypothesised that Nguni and Bonsmara cattle have dissimilar cutaneous hypersensitivity reactions and resistance to both *Rhipicephalus* tick species.

Materials and methods

Study site

The study was conducted at Fort Cox College Farm (27°01′E 32°46′S) in the False Thorn veld of the Eastern Cape in January 2012. Vegetation at the farm was composed of several trees, shrubs, and grass species and dominated by *Acacia karroo*, *Themeda triandra*, *Panicum maximum*, *Digitaria erientha*, *Eragrostis* species, and *Cynodon dactylon*. It is situated at an altitude of 547 m a.s.l. and receives an average annual rainfall of about 480 mm, most of which occurs in the hot wet season (November–February). Mean maximum temperature ranges from 7 °C in the cool dry season (May–August) to 35 °C in the hot dry season (September–October). The major tick species occurring at the farm are *R. decoloratus*, *R. microplus*, *Hyaloma marginatum*, and *Amblyomma hebraeum* (Marufu et al., 2011a). Nguni and Bonsmara cattle are reared and managed as separate herds with similar breeding programmes on the farm.

Study animals

Nguni ($n = 11$) and Bonsmara ($n = 9$) heifers aged 7–9 months were used in the study. Selection and description of the heifers was detailed in a previous study (Marufu et al., 2011a). The heifers were ear-tagged for easy identification. To enable natural tick infestation, the heifers grazed on natural pasture known to be infested

with *Rhipicephalus* tick larvae for at least a month before the experiment. All experimental procedures were approved by the University of KwaZulu-Natal Animal Ethics Research Committee (Reference Number: 097/11/Animal) and were in compliance with internationally accepted standards for welfare and ethics in animals (Austin et al., 2004).

Preparation of unfed larval extract

Two-month-old unfed larvae from laboratory colonies of the *R. decoloratus* and *R. microplus* ticks were prepared separately into unfed larval extract (ULE). In brief, the larvae were ground up in liquid nitrogen using a motor and pestle before suspension in phosphate buffered saline (PBS, pH 7.4) that contained a cocktail of protease inhibitors (Sigma–Aldrich). The homogenate was sonicated for 60 s (20 MHz) to produce a crude larval extract. Crude larval extract was centrifuged at $3000 \times g$ (4 °C) for 30 min, after which the supernatant ULE was removed. The protein concentration of the ULE was determined using the bicinchoninic (BCA) dye bioassay (Biorad), and the ULE was stored at −40 °C until use.

Delayed hypersensitivity skin test

Rhipicephalus ULE was used to induce a delayed local cutaneous hypersensitivity reaction in the heifers. Each heifer received an intradermal injection of 0.1 ml (50 µg of protein) *R. decoloratus* ULE in a shaved area of the left ear, and a similar injection of *R. microplus* ULE on the outer surface of the contralateral ear. Ear thickness was used in the current study as it has been reported to give a more suitable and precise measurement since it is measured without folding (Prudencio et al., 2011). An equal volume of PBS was inoculated 50 mm from the ULE site in both ears to provide a control measurement. Ear thickness was measured in triplicate with the aid of callipers just before the injection, and 0.5, 1, 6, 24, 48, and 72 h post-ULE or PBS inoculation. The response was expressed as the mean percentage change in ear thickness in relation to pre-inoculation values.

Measurement of the body weight, body condition score, and tick counts

Body weights were measured using a cattle scale (LS4, Taltec, South Africa) while body conditions (BCS) were visually appraised by the same assessor and rated on a 5-point scale with score 1 being very thin and a score of 5 being very fat/obese (Osoro and Wright, 1992). Two trained enumerators, one on either side of the animal, were used to carefully examine the animal which was restrained in a crush pen, identifying and recording all visible engorged adult ticks on the skin of the cattle. Ticks were not removed from the skin of animals during the enumeration process. The heifers were also examined for tick-associated dermatitis and tick bite wounds.

Statistical analyses

All analyses were conducted in Statistical Analysis System version 9.2 (SAS, 2009). The General Linear Model Procedure for repeated measurements (SAS, 2009) was used to determine the effects of breed, tick species, time of measurement, and their interactions on the ear thickness measurements. First-order autoregressive correlation (AR [1]) was fitted to the model on the measurement of interest. Least square means were compared using the PDIFF procedure (SAS, 2009). A χ^2 test was performed to determine the associations between tick infestation and development of secondary (delayed hypersensitivity) response in ear thickness as well as between tick infestation and the development of dermatitis and tick bite wounds in cattle.

Table 1
Mean (± standard error) body weight, body condition score, and tick counts in the Nguni and Bonsmara heifers.

Variable	Bonsmara Mean ± s.e.	Nguni Mean ± s.e.	Significance
BW	254.2 ± 8.03	232.1 ± 6.69	NS
BCS	3.2 ± 0.11	3.1 ± 0.09	NS
TC	88.6 ± 3.82	48.8 ± 1.79	*

BW, body weight; BCS, body condition score; TC, tick count; s.e., standard error.
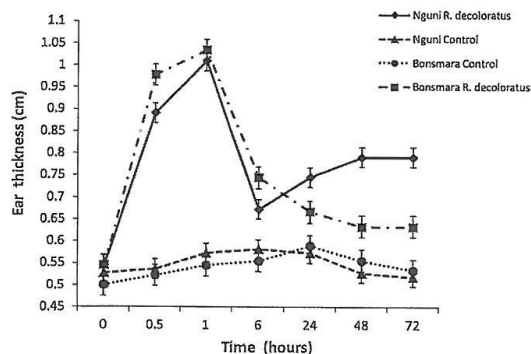NS, not significant ($P > 0.05$).
* Significant ($P < 0.05$).

Fig. 1. Change in ear thickness of Nguni and Bonsmara heifers following inoculation of *Rhipicephalus decoloratus* unfed larval extract.

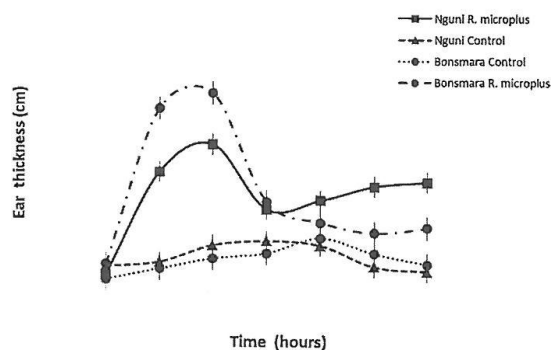Fig. 2. Change in ear thickness following inoculation of *Rhipicephalus microplus* unfed larval extract in Nguni and Bonsmara heifers.

Results

Differences in body weight, body condition score, and tick count

Body weight and BCS were similar ($P > 0.05$) in the Nguni and Bonsmara cattle. Tick counts were lower ($P < 0.05$) in the Nguni than in the Bonsmara heifers (Tables 1 and 2).

Response to Rhipicephalus decoloratus and R. microplus ULE

The interaction between breed and time of inoculation of skin antigen and between breed, tick species, and time of inoculation of skin antigen had a significant ($P < 0.05$) effect on the cutaneous hypersensitivity responses (ear thickness) of the heifers. Bonsmara heifers showed a more intense immediate type reaction with maximum response at 30 min to one h post inoculation (PI) followed by a gradual decline from 6 h PI until the end of the experimental period (72 h PI) (Figs. 1 and 2). Nguni cattle showed a less intense immediate hypersensitivity reaction which peaked at 1 h PI and declined after 6 h. Subsequently, a second response was observed in the Nguni from 24 h peaking at 72 h PI (Figs. 1 and 2).

Table 3
Associations between tick count and dermatitis and delayed hypersensitivity in the Nguni and Bonsmara heifers.

Tick count	High	Low	χ^2	P
Dermatitis				
Present	7	1	4.2	0.043
Absent	2	10		
Delayed hypersensitivity				
Present	1	11	7.1	0.008
Absent	8	0		

Associations between tick infestation level, delayed skin hypersensitivity, and tick-induced dermatitis

There was an association ($P < 0.05$) between tick count and presence of a delayed hypersensitivity reaction and between tick count and presence of dermatitis in the heifers (Table 3). Heifers that had lower tick loads exhibited a delayed hypersensitivity response while those with high tick loads did not exhibit a delayed hypersensitivity response ($P < 0.05$). Similarly, heifers that had lower tick loads did not exhibit tick-induced dermatitis, whereas in the heifers with high tick loads tick bite wounds and dermatitis were observed ($P < 0.05$).

Discussion

Smooth coats and short hairs have been given as the cause of superior tick resistance in the Nguni cattle breed (Marufu et al., 2011b). However, low tick infestation in Nguni cattle with rough coats and long hairs suggested that tick resistance in the Nguni may be dependent on non-innate immunological mechanisms (Marufu et al., 2011a). To improve knowledge on the immunological mechanisms responsible for tick resistance in cattle, the current study investigated skin hypersensitivity reactions to unfed larval extracts of R. decoloratus and R. microplus in Nguni and Bonsmara heifers.

Nguni heifers were observed to have lower mean counts of engorged adult ticks than their Bonsmara counterparts, confirming

Table 2
Differences in ear thickness induced by the three inoculants in the study heifers.

Treatment	Nguni		Bonsmara	
	Mean ± standard error	Range	Mean ± standard error	Range
R. decoloratus	0.8 ± 0.01^c	0.49–0.99	0.9 ± 0.01^c	0.47–1.03
R. microplus	0.6 ± 0.01^b	0.52–0.79	0.7 ± 0.01^b	0.53–0.93
Control	0.5 ± 0.01^a	0.45–0.60	0.5 ± 0.01^a	0.47–0.61

a,b,c Means with different superscripts differ ($P < 0.05$).

the assertions of Spicket et al. (1989), Muchenje et al. (2008), and Marufu et al. (2010) that the Nguni breed has superior tick resistance. The evolutionarily long continuous contact between Nguni cattle and the blue ticks has most likely resulted in the development of greater resistance in this breed (Marufu et al., 2011b). Tick infestation was not associated with tick sore lesions and dermatitis in the Nguni heifers, suggesting that the Nguni breed has most likely developed superior immunological defences that limit tick damage on their skin. It should be noted that there were 2 Bonsmara heifers which had low mean tick counts similar to some Nguni heifers and if such animals were selected and used in breeding programmes, it could improve tick resistance in the Bonsmara breed. Resistance to natural tick infestation in cattle is a trait that has been reported to have a moderate heritability averaging 0.37 (Turner et al., 2010) which is sufficient to result in the successful selection for tick resistance. Given the high genetic variability among individuals and breeds (Morris, 2007), the identification of superior genes is thus important for the development of breeding programmes for tick resistance in cattle. It is, however, important to be mindful of the correlated responses in other economic traits such as growth, meat quality, or milk yield as a consequence of selection for tick resistance.

Bonsmara heifers showed only an immediate type hypersensitivity response to ULE of both blue tick species characterised by soft swelling at the inoculation site further strengthening the view that this is the only type of hypersensitivity response mounted in tick-susceptible cattle. The present findings agree with those of Bechara et al. (2000) and Prudencio et al. (2011) who reported only a non-specific immediate type hypersensitivity response to tick antigen in previously sensitized tick susceptible cattle. Immediate type hypersensitivity reactions, also called type I hypersensitivity reactions, are initiated by antigen binding to immunoglobulin E (IgE) pre-attached to mast cells or basophils leading to inflammatory mediator release (Szabo et al., 2004). The resultant increased vascular permeability causes oedema, and this could explain the subsequent swelling at the injection site in Bonsmara cattle. Nguni heifers, however, had a less pronounced immediate type hypersensitivity response to ULE of both ticks used in the present study which most likely led to less evident oedema.

In the Nguni heifers, a delayed type hypersensitivity response was observed over and above the immediate type response and was most likely the cause of tick resistance in this breed. Several authors have reported similar development of a delayed type hypersensitivity response and linked it to the expression of acquired resistance to ticks in a variety of tick-resistant hosts (Bechara et al., 2000; Szabo et al., 2004; Prudencio et al., 2011). *Rhipicephalus* tick larvae take at least 4 days after initial attachment to reach engorgement and hence host rejection of tick attachment within the first 24 h is critical for prevention of tick engorgement (Porto Neto et al., 2011). The larvae continuously change the repertoire of salivary molecules that they secrete to inhibit humoral and cellular defences of the host thus enabling early evasion of host defences resulting in engorgement. It would seem therefore that due to their long contact with ticks, Nguni cattle have developed the delayed hypersensitivity response as a key mechanism of protection against these immune evasive tactics by the ticks and thus limiting tick engorgement and fecundity. This view is supported by the low numbers of engorged adult ticks observed on the Nguni heifers in the present study. It should be noted that the results of the χ^2 test (Table 3) were important for showing the breed effect on the delayed hypersensitivity responses. The χ^2 test is not the most suitable test, however, especially when frequencies are below 5, it tends to inflate the values of the test.

The presence of only an immediate type response and lack of a delayed type hypersensitivity reaction to tick antigens in the Bonsmara heifers could point to a deficit response in this breed which

most likely led to increased tick susceptibility. Immunomodulatory molecules in tick saliva have been described (Brossard and Wikel, 2004; Steen et al., 2006; Mans et al., 2008), which reduce host ability to respond to tick antigens that could stimulate a protective immune response. Kovář et al. (2001) reported an inhibitory effect of these immunomodulatory molecules on T helper 1 and a stimulatory effect on T helper 2 cytokine elaboration. Helper 2 T cells assist B cells to develop into antibody-producing cells thus promoting humoral immunity. In addition, T helper 2 cytokines recruit eosinophils which are the most important source of indoleamine 2,3 deoxygenase (IDO), an enzyme that inhibits the T helper 1 lymphocytes (Odemuyiwa et al., 2004), thus inhibiting cellular responses to ticks. The T helper 2-type response which is associated with immediate hypersensitivity reactions is thus linked to the lack of development of cellular immunity and hence tick resistance in susceptible hosts (Ferreira et al., 2003). Though cellular responses were not the focus of the present study, it can be surmised from the present results of cutaneous hypersensitivity test that *Rhipicephalus* ticks induce, a predominantly T helper 2 response which inhibits local cellular immunity and increases tick susceptibility in the less resistant Bonsmara breed.

The observed delayed hypersensitivity in heifers with low tick loads and its absence in those with high tick loads could be indicative of differences in cellular responses between the tick-resistant and -susceptible cattle. Delayed type hypersensitivity reactions are thought to occur due to a cellular infiltration and triggering of cellular immunity in resistant hosts (Hlatshwayo et al., 2004). The T helper 1 cells are implicated in the mediation of delayed hypersensitivity leading to cutaneous basophil infiltrations (Ferreira et al., 2003). Basophils have long been recognized as important effectors in tick rejection in cattle, and 2 possible ways how they accomplish this have been proposed. Wada et al. (2010) observed that basophils cluster close to tick mouthparts in the skin and so assumed that they function as direct effectors of the anti-tick reaction mounted in response to tick antigens. The same authors also suggested that antigen/antibody-stimulated basophils function as activators of mast cells that in turn produce effector molecules against ticks. The cutaneous basophil hypersensitivity that is associated with delayed type hypersensitivity reaction may likely have caused the reduction of tick infestation in the tick-resistant Nguni heifers. Further studies are, however, required on the characterisation of the cellular infiltrations associated with delayed hypersensitivity responses to ticks in Nguni cattle to confirm the present findings.

Skin response to *R. decoloratus* ULE was observed to be more intense than that to *R. microplus* ULE suggesting higher antigenicity in the former tick species. In the current study area, *R. decoloratus* is the more dominant species though it co-exists with *R. microplus* (Jongejan and Uilenberg, 2004; Marufu et al., 2011a). It should be noted that development of immunity to *R. microplus* does not necessarily confer cross protection against *R. decoloratus* (De Vos et al., 2001). When investigating the antigenicity and control of *Rhipicephalus* ticks, it is thus not sufficient to extrapolate results from studies on one tick species. Considerations should be made of the distribution and population dynamics of both tick species in a particular area. The present study's findings thus highlight the need for identifying mechanisms of host resistance to specified tick species of economic importance. This could also have significant ramification in the search for tick-protective antigens which can be used as vaccine candidates in the control of ticks.

A crude larval extract was used to induce cutaneous hypersensitivity responses in cattle in the present study because it was difficult to extract larval saliva. The present results should be interpreted with caution as crude larval extract contains many bio-active components including metabolites and larval body proteins, which the host is not normally exposed to during tick feeding. Tick saliva, which is primarily injected into the host during tick feeding (Steen

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et al., 2006), would thus have been preferable and is recommended for similar future studies. The inclusion of protease inhibitors in the ULE could have also affected the observed inflammatory response in the study heifers by inhibiting the host enzymes involved in combating tick salivary antigens. Alternatively, the protease inhibitors could have affected the proteases in the tick saliva hence assisting the host's defence mechanisms. Given that the inhibitors were used in both control and test sites and that Nguni heifers exhibited greater immune responses than the Bonsmaras, it may thus be concluded that the inhibitory effect of protease inhibitors could have been minor.

Intradermal inoculation of tick salivary antigens to elicit cutaneous hypersensitivity reactions has been used to broadly evaluate immune responses to ticks in hosts and is useful in classifying them according to level of susceptibility. Use of the skin test to classify cutaneous hypersensitivity responses is documented for dogs (Ferreira et al., 2003), bovines (Bechara et al., 2000; Prudencio et al., 2011), and horses (Szabo et al., 2004). The present results show that the intradermal inoculation of 50 μg of ULE of *R. microplus* and *R. decoloratus* elicited immediate local inflammatory reaction in the ears of pre-sensitized Bonsmara heifers and delayed hypersensitivity responses in Nguni heifers. In view of these observations, investigations are needed to characterize the immune reactions in greater detail to measure the immune response in skin or lymph node biopsies. The intradermal test could be used in conjunction with the assessment of coat characteristics (Marufu et al., 2011b) and evaluation of skin or lymph node biopsies to aid in accurate characterisation of the level of tick resistance in cattle.

Conclusions

Unfed larval extracts of *R. decoloratus* and *R. microplus* induced an immediate type hypersensitivity reaction, which was associated with tick susceptibility in previously sensitized Bonsmara cattle. Immediate followed by delayed hypersensitivity reaction to ULE of *R. decoloratus* and *R. microplus* were observed and associated with tick resistance in previously sensitized Nguni cattle. The *R. decoloratus* ULE had higher antigenicity and elicited a more intense skin hypersensitivity response in both breeds than the *R. microplus* ULE. Intradermal testing of tick immune status in cattle can be used in selective breeding programmes for tick resistance. Further investigations into the associated cellular responses to tick infestation in Nguni and Bonsmara cattle were recommended.

Acknowledgements

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Appendix 3: Cellular responses to Rhipicephalus microplus infestations in pre-sensitised cattle with differing phenotypes of infestation

Cellular responses to *Rhipicephalus microplus* infestations in pre-sensitised cattle with differing phenotypes of infestation

Munyaradzi C. Marufu · Kennedy Dzama · Michael Chimonyo

Abstract The blue tick, *Rhipicephalus microplus*, threatens cattle production in most tropical and subtropical areas of the world. Delayed skin hypersensitivity reactions are thought to cause Nguni cattle to be more resistant to *R. microplus* than Bonsmara cattle yet the cellular mechanisms responsible for these differences have not been classified. Tick counts and inflammatory cell infiltrates in skin biopsies from feeding sites of adult *R. microplus* ticks were determined in 9-month-old Nguni and Bonsmara heifers to determine the cellular mechanisms responsible for tick immunity. Nguni heifers (1.7 ± 0.03) had lower ($P < 0.05$) tick counts than the Bonsmaras (2.0 ± 0.03). Parasitized sites in Nguni heifers had higher counts of basophils, mast and mononuclear cells than those in the Bonsmara heifers. Conversely, parasitized sites in Nguni heifers had lower neutrophil and eosinophil counts than those in the Bonsmara heifers. Tick count was negatively correlated with basophil and mast cell counts and positively correlated with eosinophil counts in both breeds. In the Bonsmara breed, tick count was positively correlated with mononuclear cell counts. Cellular responses to adult *R. microplus* infestations were different and correlated with differences in tick resistance in Nguni and Bonsmara cattle breeds. It is essential to further characterise the molecular composition of the inflammatory infiltrate elicited by adult *R. microplus* infestation to fully comprehend immunity to ticks in cattle.

Keywords Bonsmara · Cutaneous basophil hypersensitivity · Mast cells · Nguni cattle · *Rhipicephalus microplus*

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Introduction

Rangeland-based cattle production in most tropical and subtropical areas is greatly hampered by the blue tick, *Rhipicephalus microplus*, which affects growth and fertility, damages hides and transmits the haemoparasites *Babesia bigemina*, *Babesia bovis* and *Anaplasma marginale* in cattle (Jonsson 2006). Alternative strategies are required to control this one-host tick (*R. microplus*), due to its evolving resistance to commercially available acaricides (Brake and Perez de Leon 2012). The selection and rearing of breeds and individual cattle with high resistance to ticks is a promising tick control method (Kongsuwan et al. 2010) which could be useful in combating the blue tick. A large number of *Bos indicus* and indigenous Sanga breeds (*Bos taurus africanus*) have been reported to exhibit high levels of resistance to ticks and tick-borne diseases making them suitable for rearing in tropical and subtropical conditions (FAO 2007; Marufu et al. 2011a). The Nguni cattle breed (*B. t. africanus*) of Southern Africa is one such example.

Mechanisms responsible for the high levels of tick resistance in Nguni cattle are still unclear. Marufu et al. (2011a) identified the smoothness and shortness of the coats as important attributes that cause innate tick resistance in Nguni cattle but not in the Bonsmara breed. The Bonsmara is a composite breed which originated from a 5/8:3/8 combination of the Afrikaner (*B. t. africanus*) and Shorthorn/Hereford (*Bos taurus taurus*) (Strydom et al. 2001). Low tick infestation in Nguni cattle with rough coats and long hairs, however, suggested that tick resistance in the Nguni may be dependent on acquired immunological mechanisms (Marufu et al. 2011a). Acquired resistance to tick infestation involves humoral and cellular immune-regulatory and effector pathways (Camargo Mathias et al. 2011). Evidence from cutaneous hypersensitivity responses to *R. microplus* larval antigen strongly suggests that delayed hypersensitivity reactions could be associated with superior tick resistance in the Nguni cattle breed (Marufu et al. 2013). The same study has linked absence of a delayed hypersensitivity response coupled with the presence of an intense immediate hypersensitivity reaction to increased tick susceptibility in the less resistant Bonsmara breed. The differences in hypersensitivity reactions to *R. microplus* ticks in Nguni and Bonsmara cattle could be better understood if cellular responses at tick feeding sites were characterised in these breeds.

Several studies have been conducted on the histology of tick attachment sites on artificially infested cattle with the aim of elucidating the cellular responses of cattle to different tick species (Verrisimo et al. 2008; Constantinoiu et al. 2010; Carvalho et al. 2010). Findings from these studies have revealed that mast cells, eosinophils, basophils and lymphocytes play some part in resistance to artificial tick infestations in cattle, with varying importance and roles. Artificially induced animal–tick associations are generally characterised by more intense expression of acquired resistance than naturally occurring tick–host relationships (Ribeiro 1989; Boppana et al. 2005). Few studies have focused on comparing the immunological mechanisms of resistance to natural infestation with *R. microplus* in cattle of differing phenotypes of infestation. In addition, little attempts have been made to describe the relationships between the various cellular immune components to tick counts and therefore resistance to *R. microplus* in naturally infested cattle. Elucidating the cellular mechanisms by which tick resistant cattle prevent heavy infestation can be important for the comprehension of tick-borne disease transmission and can also aid the development of alternative immune-based tick control methods.

Differences in cellular responses to *R. microplus* infestation and their relationship with tick immunity in Nguni and Bonsmara cattle have not been studied, despite long standing evidence of differences in tick resistance between the two breeds. The correlation between breed and cellular responses to tick infestation and its relationship to tick immunity in

Nguni and Bonsmara cattle also requires investigation. The objective of the current study was, therefore, to determine the cellular responses at the attachment sites of *R. microplus* ticks in Nguni and Bonsmara heifers reared on a tick infested rangeland.

Materials and methodology

Study site

Sampling for the histopathology of tick attachment sites was conducted at Fort Cox College Farm (27°01′E and 32°46′S) in January 2012 when the highest adult tick loads occur on cattle. The farm lies in the Eastern Province Thornveld in which *Acacia karroo*, *Themeda triandra*, *Panicum maximum*, *Digitaria eriantha*, *Eragrostis* species, *Cynodon dactylon*, and *Pennisetum clandestinum* are dominant (Acocks 1988). It receives an average annual rainfall of about 480 mm, most of which occurs in the hot wet season, making it a typical semiarid area. The average daily temperature ranges from 7 to 35 °C. Blue ticks (*R. decoloratus* and *R. microplus*) are the most commonly occurring and numerous tick species at the farm, with *Hyaloma marginatum* and *Amblyomma hebraeum* also occurring in lower numbers.

Study animals

Nine-months-old Nguni (n = 12) and Bonsmara (n = 12) heifers were randomly selected and used for this study. The heifers were ear tagged for easy identification and grazed on the same natural tick-infested rangeland for 6 months prior to sampling to enable natural *R. microplus* infestation. All experimental procedures on the heifers were compliant with internationally accepted standards for welfare and ethics in animals (Austin et al. 2004) and were approved by the University of KwaZulu-Natal Animal Ethics Research Committee (Reference number: 097/11/Animal).

Tick load evaluation

Whole body tick counts were conducted on the experimental animals exposed naturally to ticks once just before skin biopsy sampling in January 2012 as described by Marufu et al. (2011a). Briefly, each heifer was restrained in a crush pen while two trained enumerators, one on either side of the heifer, counted and recorded all visible engorged adult *R. microplus* ticks on the whole body of the heifer. Since the study heifers were subjected to natural tick infestations, adult female *R. microplus* ticks were the easiest tick stage to identify and enumerate, and as these cause the most severe losses in cattle, hence their attachment sites were selected for biopsy sampling.

Skin biopsy sampling

The heifers were heavily sedated with 0.2 mg/kg body weight xylazine (Rompun®, Bayer, South Africa) administered intramuscularly in the rump. Four punch biopsies were taken from each animal, two normal skin biopsies from non-parasitized sites, and two parasitized skin biopsies from the feeding sites (parasitized) of fully engorged (4–6 mm diameter) adult *R. microplus* ticks respectively, using a 5 mm punch biopsy needle (Kyron

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Technologies, South Africa). Care was taken to ensure uniform sample collection method and depth. The biopsies were of full skin thickness, 5 mm diameter and 10 mm deep. Skin samples were immediately immersed in buffered formalin (pH 7.0) pending processing.

Histological processing

The skin samples were kept for 24 h in the fixative, embedded in paraffin and processed according to routine histological techniques. Each biopsy was serially sectioned at a thickness of 4 μm and stained with Haematoxylin–Eosin and May Grünwald Giemsa, to enable general histological evaluation and differential cell counting respectively (Van Der Heijden et al. 2005).

Section analysis

Sections were analysed under light microscopy. General features were evaluated on Haematoxylin–Eosin stained sections. Total cell counts were made on sections stained by May-Grünwald Giemsa. For this purpose, cells from two areas of 0.0052 mm^2 of the dermis, immediately below the epidermis and the cement cone, were counted. Means of each area were used for further analysis. The counting was limited by a Reichart integrating graticle (PK6 3X mn, Austria) on oil immersion fields (objective 100×). Differential cell counts (neutrophils, eosinophils, basophils, mononuclear cells and mast cells) were performed on the same sections and areas used for total cell counts. In the presence of a feeding cavity (area of liquefative necrosis in the dermis of the host which sometimes occurs under the tick attachment site) cells surrounding this cavity were counted.

Statistical analyses

All analyses were conducted in Statistical Analysis System version 9.2 (SAS 2009). The count data were checked for normality using PROC UNIVARIATE (SAS 2009) and observed to be not normally distributed. Log(x + 1) transformation was then performed and conferred normality to the count data. The Generalised Linear Models Procedure (SAS 2009) was used to determine the effects of breed and tick feeding on the cell counts and tick counts. The following statistical model was used:

$$Y_{ijk} = \mu + A_i + B_j + AB_{ij} + \varepsilon_{ijk};$$

where, Y_{ijk} = cell count; tick count, A_i = effect of breed (i = Nguni and Bonsmara), B_j = effect of tick feeding (normal skin site and tick bite site), AB_{ij} = the effect of the interaction of breed and tick feeding, ε_{ijk} = residual error.

Least square means were compared using the PDIFF option (SAS 2009). Correlations between cell and tick counts were determined using the PROC CORR (SAS 2009). The χ^2 test was used to determine associations between breed and histological characteristics of the skin biopsies.

Results

Tick counts and differential cell counts

Breed had a significant effect on the log(x + 1) tick counts of the study animals, with Nguni heifers (1.7 ± 0.03) having lower counts than their Bonsmara counterparts

(2.0 ± 0.03). Breed, parasitisation and the interaction between breed and parasitisation had a significant effect on the differential and total cell counts of the study heifers (Table 1). The non-parasitized sites in both Bonsmara and Nguni heifers had the least number of neutrophil and eosinophil counts followed by the parasitized sites in Nguni heifers while parasitized sites in the Bonsmara heifers had the highest counts. Conversely the parasitized sites in Nguni heifers had the highest basophil, mononuclear and mast cell counts followed by parasitized sites in Bonsmara heifers, non-parasitized sites in Nguni heifers and non-parasitized sites in Bonsmara heifers in descending order. Total cell counts were highest in parasitized sites of the Bonsmara heifers followed by parasitized sites in the Nguni heifers, non-parasitized sites in the Bonsmara heifers and non-parasitised sites in the Nguni heifers in descending order.

Correlations between tick and cell counts

Tick count was correlated to the differential counts of eosinophils, basophils, mononuclear and mast cells on parasitized sites in the study heifers (Table 2). There were significant positive correlations between tick count and eosinophil counts in both breeds and between tick count and mononuclear cell counts in the Bonsmara breed. Tick count was, however, negatively correlated to basophil and mast cell counts of parasitized sites in both breeds. In the Nguni breed, tick count had a significant negative correlation with mononuclear cell counts of parasitized sites. Generally, tick count had significant positive correlations with eosinophil counts, significant negative relationships with basophil, mononuclear and mast cell counts and was not significantly correlated to neutrophil and total cell counts. No significant correlations were observed between tick count and differential cell counts in non-parasitised sites in both breeds.

General features of parasitized skin biopsies

A summary of the categorisation of the histopathological changes on the parasitized skin biopsies in the study heifers is given in Table 3. Figure 1 shows that histopathological changes in the epidermis and dermis were more pronounced in parasitized skin samples from Bonsmara than Nguni heifers. In most of the parasitized skin samples from both Bonsmara and Nguni heifers, there was epidermal fracture associated with the penetration of tick mouth parts as far down as the upper dermis. Parasitized skin samples obtained from

Table 1 Differential log(x + 1) cell counts at normal and infested skin sites of Nguni and Bonsmara heifers

Cell type	Bonsmara		Nguni	
	Normal	Infested	Normal	Infested
Neutrophils	0.3 ± 0.03^a	2.3 ± 0.03^c	0.3 ± 0.03^a	1.4 ± 0.03^b
Eosinophils	1.0 ± 0.02^a	1.6 ± 0.02^c	1.0 ± 0.02^a	1.4 ± 0.02^b
Basophils	0.3 ± 0.02^a	0.8 ± 0.02^c	0.6 ± 0.02^b	1.3 ± 0.02^d
Mononuclear cells	1.3 ± 0.03^a	1.6 ± 0.03^b	1.3 ± 0.03^a	1.8 ± 0.03^c
Mast cells	0.4 ± 0.02^a	1.3 ± 0.02^c	0.8 ± 0.02^b	1.9 ± 0.02^d
Total cell count	2.3 ± 0.08^b	3.1 ± 0.08^d	2.0 ± 0.08^a	2.9 ± 0.08^c

[a, b, c, d] Means with different superscripts within a row differ at $P < 0.05$

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Table 2 Correlations between log(x + 1) tick count and differential log(x + 1) cell counts in Bonsmara and Nguni heifers

Parameter	Bonsmara	Nguni	Overall
Neutrophils	0.14	0.12	0.14
Eosinophils	0.61*	0.89**	0.39*
Basophils	−0.78**	−0.96**	−0.48
Mononuclear cells	0.68*	−0.79*	−0.52**
Mast cells	−0.45*	−0.94**	−0.28*
Total cell count	0.27	0.35	0.39

* Indicates significant correlations at $P < 0.05$

** Indicates significant correlations at $P < 0.001$

Bonsmara heifers had severe basal cell hyperplasia, epidermal necrosis accompanied by acantholysis and oedema while fewer parasitized samples from Nguni heifers exhibited these changes (Fig. 1a–d). More Bonsmara heifers exhibited severe pustule-like lesions in the epidermis and moderate to severe inflammatory infiltrates into the dermis. The infiltrates consisted predominantly of neutrophils with few eosinophils, basophils and mast cells. Most Nguni heifers exhibited few epidermal pustule-like lesions and the dermal inflammatory infiltrates were dominated by basophils, eosinophils and some mast cells.

Discussion

The observed lower tick counts in the Nguni heifers, may likely confirm assertions by Muchenje et al. (2008) and Marufu et al. (2010, 2011b) that the Nguni has superior tick resistance than the Bonsmara breed. Marufu et al. (2011a) suggested that the development of greater resistance in the Nguni breed has most likely resulted from its long continuous contact with the blue ticks. High tick infestation in the tropics and subtropics (Porto Neto et al. 2011) could have placed strong selection pressure on Nguni cattle enabling them to develop heightened immune responsiveness to ticks, a trait which may have allowed them to survive in this environment. Resistance to *R. microplus* could also be related to the portion of *B. t. africanus* genes in the cattle, however this requires further investigation. Tick counts are a reliable proxy for tick resistance (Verrisimo et al. 2008); however, tick biological parameters such as engorgement weight, feeding period, moulting period and tick yield (Wikel 1999; Camargo Mathias et al. 2011) may provide more accurate information on tick resistance. Further investigations into these tick biological parameters and their correlations with immunological parameters associated with tick resistance could be essential in providing comprehensive information on the Nguni breed's tick resistance status and immune responsiveness to ticks.

Epidermal hyperplasia, acantholysis, oedema and necrosis observed in all the parasitized biopsies in the current study were expected as these are common non-specific changes induced by noxious stimuli in the host's skin (Szabó and Bechara 1999; Monteiro and Bechara 2008). The more severe oedema and necrosis in the Bonsmara heifers could likely be a result of a chronic allergic-type (type I hypersensitivity) response to ticks which may have led to tick susceptibility. This chronic type I hypersensitivity reaction was reported to be advantageous to the tick (Tatchell and Moorhouse 1968; Piper et al. 2010) and could

Table 3 Histological characteristics of parasitized skin sites in Nguni and Bonsmara heifers

Parameter	Severity	Nguni (n = 12)	Bonsmara (n = 12)	χ^2	Significance
Epidermal hyperplasia	Moderate	8	0	4.8	$P < 0.05$
	Severe	4	12		
Epidermal necrosis	Absent	10	0	9.3	$P < 0.05$
	Mild	2	3		
	Moderate	0	3		
	Severe	0	6		
Dermal oedema	Mild	7	0	8.0	$P < 0.05$
	Moderate	5	4		
	Severe	0	8		
Vascular reaction	Mild	8	0	6.9	$P < 0.05$
	Moderate	3	6		
	Severe	1	6		
Hyperemia	Mild	6	2	5.7	$P > 0.05$
	Moderate	5	4		
	Severe	1	6		
Pustules	Absent	9	0	15.0	$P < 0.05$
	Mild	2	1		
	Moderate	1	6		
	Severe	0	5		

likely be linked to the lack of development of cellular immunity and hence decreased tick resistance in susceptible hosts. Reduced inflammatory response in the Nguni cattle, on the other hand, may likely be due to an evolutionary acquired ability to respond less vigorously to bioactive molecules in the *R. microplus* tick saliva as postulated by Piper et al. (2010) for tick resistant Brahman cattle.

Higher neutrophil and eosinophil counts on parasitized sites in the Bonsmara heifers could likely be associated with their lower tick resistance. The present findings are in accord with those of Wada et al. (2010) who observed increased neutrophil and eosinophil infiltrations in parasitized sites of less resistant hosts and surmised that recruitment of these cells was insufficient or dispensable for the manifestation of tick resistance. Neutrophils are highly motile phagocytic cells that constitute the first line of defence of the innate immune system (Francischetti et al. 2009). Increased neutrophilic infiltration may be due to molecules present in tick saliva or to chemokines secreted by degenerating epidermal cells in the inflammatory focus. Constantinoiu et al. (2010) suggested that neutrophils do not play a major role in resistance to *R. microplus* ticks in cattle. In the present study, neutrophils were associated with increased breakdown of extracellular matrix and necrosis around tick mouth parts in parasitized sites of tick susceptible Bonsmara cattle. The present findings support the reports of Tatchell and Moorhouse (1970) that neutrophils might be responsible for paving the way for tick feeding by destroying the extracellular matrix around the tick attachment lesion allowing ticks access to tissue fluids and blood.

Eosinophils are predominant in body surfaces that interact with the external environment, such as the skin, and are generally associated with parasitic infestation or allergic reactions (Francischetti et al. 2009). Earlier researchers proposed that eosinophils are involved in the translocation of mast cell histamine to the tick attachment site resulting in
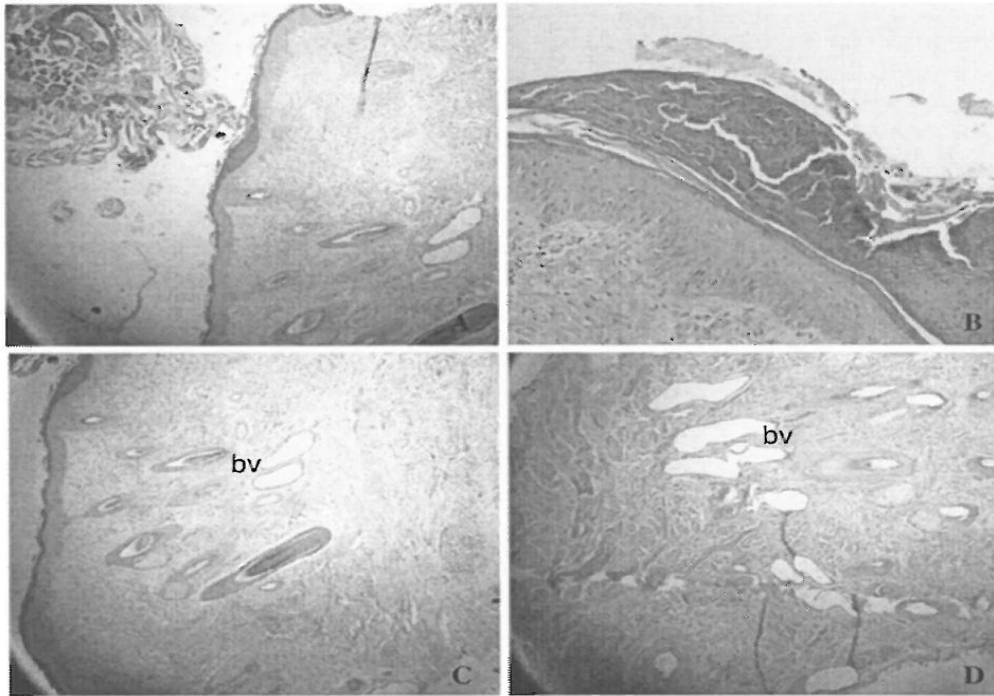
Fig. 1 Pictures of skin sections (stained with Haematoxylin and Eosin) taken from representative tick-infested Nguni (**a** and **c**) and representative tick-infested Bonsmara heifers (**b** and **d**). Bonsmara heifers (**b**) had epidermal fracture, severe basal cell hyperplasia, epidermal necrosis accompanied by acantholysis and oedema while fewer Nguni heifers (**a**) did not exhibit these changes. Nguni heifers (**c**) had fewer blood vessels (**bv**) in the dermis than the Bonsmara heifers (**d**)

increased grooming and tick rejection in cattle (Schleger et al. 1981). In the present study, high eosinophil presence in tick attachment sites of tick susceptible Bonsmara cattle seems to suggest that eosinophils are associated with reduced *R. microplus* resistance in cattle. Eosinophils have been linked to allergic-type reactions under the influence of immuno-globulin E and this chronic allergic type reaction may have led to increased tick suscep-tibility in Bonsmara cattle. The positive correlation between tick count and eosinophil count in the study heifers suggests that infiltration of the bite site by high numbers of eosinophils leads to reduced resistance to *R. microplus* ticks. This finding contrasts that of Carvalho et al. (2010) who reported that increased resistance to adult *R. microplus* ticks was associated with high eosinophil presence in the tick bite site of infested cattle. These authors concluded from their study that resistant bovines have a greater capacity than susceptible hosts to retain eosinophils in the lesion of adult tick-infested skin. The present study therefore highlights the need to further investigate the role of eosinophils in resis-tance to ticks in cattle.

In the indigenous Nguni heifers, the observed higher basophil and mast cell counts suggests superior tick resistance in this breed. The present findings are in agreement with Monteiro and Bechara (2008) and Carvalho et al. (2010) who reported that basophil accumulations in tick attachment sites are significantly associated with tick resistance in caprine and bovine hosts respectively. Basophils have long been reported to be responsible

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for the development of acquired tick resistance in cattle (Brown et al. 1984). Direct anti-tick reaction and antigen/antibody-mediated activation of mast cells to effect tick rejection, are the two ways in which basophils are thought to accomplish tick rejection (Wada et al. 2010). In the former instance, basophils are thought to migrate and cluster close to tick mouth parts in the skin, de-granulate and release local mediators which cause immune skin rejection of blood-feeding ticks, the so called cutaneous basophil hypersensitivity (CBH) reaction (Francischetti et al. 2009; Wada et al. 2010). The CBH is a form of delayed type hypersensitivity which is thought to be mediated by T helper 1 lymphocytes (Brossard and Wikel 2004). The local mediators released by basophils that are involved in the manifestation of tick resistance, however, remain to be fully identified.

Mast cells, and the histamine they contain inside cytoplasmic granules, are of fundamental importance to the self-grooming mechanism, which is thought to be critical to resistance of cattle to the R. microplus tick (Koudstaal et al. 1978; Kemp and Bourne 1980; Schleger et al. 1981). Self-grooming caused by histamine from degranulated mast cells is an important factor in reducing tick burdens (Maharana et al. 2011) and could have led to the lower tick loads in Nguni cattle in the present study. Mast cells also contribute to the expression of acquired immunity to ticks through the release of other bioactive molecules such as leukotrienes, prostaglandins and enzymes at the bite site (Wikel 1999). The observed higher mast cell counts in the Nguni cattle may thus have led to the breed's superior tick resistance compared with the Bonsmara breed. The present findings agree with those of Moraes et al. (1992) and Verrisimo et al. (2008) who also reported higher dermal mast cell counts in highly tick resistant indicine cattle breeds than in the tick susceptible taurine breeds.

The negative correlations observed between tick count and basophil and mast cell counts in the Nguni and Bonsmara heifers suggest that these cells have an important role in conferring tick immunity in cattle. The present findings support the reports of Wada et al. (2010) that both basophils and mast cells synergistically contribute to the manifestation of tick resistance in animals. Tick infestation has been thought to cause a modification in the skin of parasitized hosts leading to massive migration of basophils and mast cells to tick attachment sites thus effecting tick rejection in the highly resistant bovine hosts (Engracia Filho et al. 2006; Monteiro and Bechara 2008). The chemical mediators released by degranulated mast cells and basophils are thought to play an important role in the resistance mechanism of cattle to ticks (Verrisimo et al. 2008). Further elucidation of the roles of each inflammatory mediator released by basophils and mast cells may lead to better understanding of their roles in tick immunity in cattle. From the present findings however, it can be concluded that the higher the infiltration of basophils and mast cells in the tick attachment site of cattle the more resistant they are likely to be to R. microplus infestation.

The negative correlation between mononuclear cell counts and tick count in the Nguni breed and positive correlation between the two parameters in the Bonsmara breed could signify specific differences in cellular response to R. microplus infestation in tick resistant and susceptible cattle. Mononuclear cells include macrophages which process and present tick antigens to T-cells which in turn stimulate cellular and humoral (antibody production) specific immune responses (Francischetti et al. 2009). Tick saliva is known to contain molecules that inhibit lymphocyte and macrophage function thus affecting cellular immune responses in susceptible hosts (Castagnolli et al. 2008). The higher R. microplus tick loads in Bonsmara heifers in the present study could likely have led to tick-induced suppression of lymphocyte and macrophage function, despite their infiltration of tick attachment sites. It is possible that Nguni cattle produced humoral antibodies to neutralise immunosuppressive molecules secreted by the ticks and hence lymphocyte and macrophage function

was not affected in this breed. Studies on the characterisation of molecular responses at the tick–host interface in Nguni and Bonsmara cattle could give clarity on this postulation.

The present study shows the important role that mast cells, basophils and mononuclear cells play in the resistance of cattle to the *R. microplus* tick. It should be noted, however, that the present data represent a small number of biological replicates and thus caution should be taken when interpreting these results. Cellular responses to adult *R. microplus* tick bites were shown to differ in hosts of differing tick resistance and were correlated with variations in acquired resistance to tick infestation. The acquisition of tick resistance is associated with reduced pathogen transmission from infected ticks (Wikel 1999; Marufu et al. 2010). The current findings may, therefore, provide avenues toward the development of novel control strategies such as the development of anti-tick vaccines which can also be used to control tick-borne diseases. Considering the wide geographical distribution of *R. microplus* and its increasing range, its effect on the molecular immune responses in cattle of differing resistance need further detailing. Genetic polymorphisms originating in the host may have several effects on tick resistance and these need to be profiled in Nguni and Bonsmara cattle to elucidate the genetic mechanisms involved in tick resistance.

Conclusions

Cutaneous reactions to bites of adult *R. microplus* ticks in the Nguni breed differed significantly with those of the Bonsmara breed. The tick resistant Nguni heifers had more basophils, mononuclear cells and mast cells in their inflammatory infiltrates, while infiltrates in the less resistant Bonsmara heifers had more neutrophils and eosinophils. Tick resistance in the Nguni breed was correlated with CBH while tick susceptibility in the Bonsmara breed was associated with chronic allergic-type reaction on the tick bite sites. Further characterisation of the molecular composition of the inflammatory infiltrate elicited by adult *R. microplus* infestations remains essential for the comprehension of immunity to ticks in cattle.

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Appendix 4: University of KwaZulu-Natal Animal Ethics Research Committee Clearance (Reference number: 097/11/Animal)

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22 September 2011

Reference: 097/11/Animal

Dr. MC Marufu   —13327
Animal Science
c/o Prof. Michael Chimonyo
SASA
PMB CAMPUS

Dear Dr. Marufu

Ethical Approval of Research Projects on Animals

I have pleasure in informing you that the Animal Ethics Sub-committee of the University Ethics Committee has granted ethical approval for 2011/2012 on the following project:

"Resistance to ticks and diseases in Nguni cattle"

Yours sincerely

Professor Theresa HT Coetzer
Chairperson: Animal Ethics Sub-committee

Cc   Registrar   —> 133290
      Research Office —7 133294
      Head of School, Prof. G. Ortmann —> 133298

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