The comparison of immunological responses of Brahman, Nguni and Angus cattle infested with *Rhipicephalus microplus* and *Rhipicephalus decoloratus*

By

Cornelius Loftus Nel

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Supervisor: Prof K. Dzama

Co-supervisors: Dr MC Marufu

Prof NN Jonsson

Dr B Dube

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DECLARATION

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

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Summary

Ticks and tick borne diseases pose major threats to modern South African cattle production. *Rhipicephalus microplus* and *R. decoloratus* are important tick species currently spread throughout most of Southern Africa. Current control methods are not considered sustainable because of various economic, social and environmental concerns. Host resistance to ticks is a characteristic of cattle and is dependent on breed type. An understanding of these resistance mechanisms is necessary if host resistance is to be exploited as an alternative control method. Host reaction to infestation is specific to the tick species. It was hypothesized that tick resistance is a product of co-evolution between host and parasite and a breed will thus show superior resistance to tick species that it has a historical relationship with. The parasite-host pair with African origin, *R. decoloratus* and the *Bos taurus africanus* Nguni, was thus chosen along with the pair of Asiatic origin, *R. microplus* and the *Bos indicus* Brahman. A European breed, the *Bos taurus* Angus was also included due to their known susceptibility to *Rhipicephalus* ticks. Following the collection of control samples on all animals (n=36), one half (n=6) of breed group (n=12) was artificially infested with roughly 100 unfed larvae of *R. microplus* while the other half was similarly infested with *R. decoloratus*. Approximately 12 hours' post infestation, multiple blood samples were drawn and skin biopsy samples were collected from visible parasitized sites of all animals. The remaining ticks were allowed to mature and tick counts were performed on day 18 post infestation. The blood samples were used for comprehensive haematology and serum biochemistry profiles while the skin biopsy sites were sectioned for cell counts and histopathological scoring of tissue using hematoxylin and eosin staining. There was no significant interaction between breed and tick species for counts, haematology, biochemistry or cutaneous cell counts and breed and tick species was used as fixed effects for assessment. Regarding day 18 tick counts, the Brahman breed displayed lower ($p<0.01$) tick counts compared to both the Nguni and Angus breeds. *Rhipicephalus microplus* displayed a higher success rate ($p<0.05$) compared to *R. decoloratus* across all breeds. At the 12-hour time point, the Brahman breed displayed a lower ($p<0.05$) level of mean cell volume (40.94 fl). The Nguni breed displayed a lower ($p<0.05$) level of platelets (311.59 x 10^9/dl). No haematological differences were observed for tick species. The Angus breed displayed a lower ($p<0.05$) absolute level of circulating neutrophils (3.65 x 10^9/l) and a higher ($p<0.05$) level of lymphocytes (9.69 x 10^9/l) compared to the Nguni, but not Brahman breed. The Nguni displayed a higher ($p<0.05$) absolute level of eosinophils (0.43 x 10^9/l) compared to the Brahman, but not Angus breed. Regarding serum biochemistry, the Brahman breed displayed higher ($p<0.05$) albumin levels (28.85 g/l) compared to both breeds and higher ($p<0.05$) alanine transferase (59.70 U/l) levels compared to the Angus breed. The Angus breed displayed higher ($p<0.05$) levels of blood urea nitrogen (5.21 mmol/l) compared to the Brahman breed. The Brahman breed displayed lower levels of fibrinogen (1.77 g/l) than the Nguni and Angus breeds. Animals infested with *R. microplus* displayed a higher ($p<0.05$) serum globulin level (43.37 g/l) than those infested with *R. decoloratus*. Overall, alanine transferase (-0.36, $p<0.05$), alkaline phosphatase (-0.36, $p<0.05$) and fibrinogen (0.39, $p<0.05$) showed weak, but significant correlations to day 18 tick counts. No differences within breed and tick species groups were observed within the number of cellular infiltrates or histopathology scores.

Within all treatment groups, the level recorded for cutaneous infiltrates or histopathology scores post infestation was higher ($p<0.05$) than the control values. It was concluded that a specific evolutionary relationship is not necessarily the primary contributor to the manifestation of the resistant phenotype and a high level of cross resistance is possible. *R. microplus* has a superior parasitic aggression which will have an influence on its displacement of *R. decoloratus*. Immunological parameters are important when assessing tick-host relationships, but the influence on the host includes a wider range of factors. The 12-hour interval is promising for further investigations, but higher intensities of infestation are recommended to increase the reliability of assessments.

Opsomming

Bosluise en bosluis-oordraagbare siektes hou groot bedreigings vir moderne Suid-Afrikaanse vee produksie in. *Rhipicephalus microplus* en *Rhipicephalus decoloratus* is belangrike bosluisspesies tans versprei deur die grootste gedeeltes van Suider-Afrika. Huidige beheermaatreëls word nie as volhoubaar beskou nie as gevolg van verskeie ekonomiese, maatskaplike en omgewingskwessies. Weerstand teen bosluise is 'n kenmerk van beeste en is afhanklik van ras. 'n Begrip van die meganismes van weerstandigheid is nodig as gasheer weerstand uitgebuit gaan word as 'n alternatiewe metode van beheer. Die gasheer reaksies op infestasie is spesifiek teen opsigte van die bosluis spesie. Dit is gevolglik vermoed dat bosluis weerstandigheid 'n produk is van ko-evolusie tussen gasheer en parasiete. Rasse sal dus moontlik ‘n beter weerstand fenotiepe wys tot bosluis spesie met wie dit 'n historiese verhouding deel. Die parasiet-gasheer paar met Afrikaanse herkoms, *R. decoloratus* en die *Bos taurus africanus* Nguni, was dus gekies saam met die paar van Asiatiese oorsprong, *R. microplus* en die *Bos indicus* Brahman. 'n Europese ras, die *Bos taurus taurus* Angus, is ook ingesluit as gevolg van hul bekende vatbaarheid vir bosluise. Na die versameling van kontrole monsters op alle diere (N = 36), was een helfte (N = 6) van elke ras groep (N = 12) kunsmatig geïnfesteer met sowat 100 ongevoerde larwes van *R. microplus* terwyl die ander helfte op ‘n eenerse wyse geïnfesteer is met *R. decoloratus*. Ongeveer 12 ure na besmetting is verskeie bloedmonsters getrek en vel biopsie monsters is versamel van sigbare areas van infestasie. Die oorblywende bosluise was toegelaat om tot volwassenheid te ontwikkel en bosluis tellings is uitgevoer op dag 18 (na infestasie). Die bloedmonsters is gebruik vir omvattende hematologie en serum biochemie profiele terwyl die vel biopsies gesny is vir seltellings en histopatologiese evaluasie van weefsel met behulp van hematoxylin en eosin toepassing. Interaksie tussen ras en bosluisspesie was nie betekenisvol vir bolsuis tellings, hematologie, biochemie of kutane seltellings nie. Hoof effekte ras en bosluisspesie is dus oorweeg vir assessering. Met betrekking tot dag 18 bosluis tellings, het die Brahman ras laer ($p < 0,01$) tellings in vergelyking met beide die Nguni en Angus rasse vertoon. *R. microplus* het 'n hoër suksessyfer ($p<0.05$) in vergelyking met *R. decoloratus* oor alle rasse vertoon. Op die 12-uur-tyd punt, het die Brahman ras 'n laer ($p<0.05$) vlak van die gemiddelde sel volume (40,94 fl) vertoon. Die Nguni-ras het 'n laer ($p<0.05$) vlak van plaatjies (311,59 x 10^9/dl) vertoon. Geen hematologie verskille is waargeneem vir tussen bosluis spesies nie. Die Angus ras het ‘n laer ($p < 0.05$) absolute vlak van sirkulerende neutrofiele (3,65 x 10^9/l) en 'n hoër ($P < 0,05$) vlak van limfosiete (9,69 x 10^9/l) vertoon in vergelyking met die Nguni, maar nie Brahman ras. Die Nguni het 'n hoër ($p<0,05$) absolute vlak van eosinofiele (0,43 x 10^9/l) in vergelyking met die Brahman, maar nie die Angus ras, vertoon. Met betrekking tot serum biochemie, het die Brahman ras hoër ($p<0,05$) albumien vlakke (28,85 g/l) in vergelyking met beide rasse vertoon en hoër ($p<0,05$) alanien transferase (59,70 U/l) vlakke in vergelyking met die Angus ras vertoon. Die Angus ras het hoër ($P < 0.05$) vlakke van bloed ureum stikstof (5.21 mmol/l) in vergelyking met die Brahman ras vertoon. Die Brahman ras vertoon laer vlakke van fibrinogeen (1.77 g/l) as die Nguni en Angus rasse. Diere wat geïnfesteer is met *R. microplus* het hoër ($P < 0,05$) serum globulien vlakke (43,37 g/l) geïnfesteer as dié besmet is met *R. decoloratus*. Algeheel, het alanien transferase (-0,36, $p<0.05$), alkaliese fosfatase (-0,36, $p<0.05$) en fibrinogeen

(0,39, p<0.05) swak, maar betekenisvolle korrelasies getoon teenoor dag 18 bosluis tellings. Geen verskille tussen ras of bosluisspesies groepe is waargeneem in die aantal inflammatoriese seltellings of histopatologie tellings nie. Binne alle behandeling groepe, het die vlak vir kutane seltellings en histopatologiese tellings hoër (p<0,05) as die kontrole waardes. Daar is tot die gevolgtrekking gekom dat 'n spesifieke evolusionêre verhouding is nie noodwendig die primêre bydraer was tot die manifestasie van die weerstandige fenotipe nie en 'n hoë vlak van kruis weerstand is moontlik. *R. microplus* het 'n verhoogde parasitiese aggressie wat 'n invloed op sy verplasing van *R. decoloratus* sal hê. Immunologiese eienskappe is belangrik vir die ondersoek van bosluis-gasheer verhoudings, maar die invloed van infestasie op die gasheer sluit 'n wyer verskeidenheid van faktore in. Die 12 uur interval is belowend vir verdere ondersoeke, maar hoër intensiteite word aanbeveel om die betroubaarheid van assessering te verhoog.

CHAPTER 1 - GENERAL INTRODUCTION

1.1.1 Introduction

Tick infestation is a major constraint on cattle production in South African beef production systems. Problems associated with ticks include tick worry, immunosuppression, secondary infections and transmission of tick-borne diseases (TBD), all of which translate to decreased performance (Ghosh, Azhahianambi & De La Fuente, 2006). Ticks and TBD are thus associated with large economic losses in cattle production (de Castro, 1997; Dold & Cocks, 2001; Manjunathachar *et al.*, 2014). Acaricides and vaccines are currently the primary methods of tick control globally; however, they are not considered sustainable because of various economic, environmental and social concerns (Jonsson, 2006; Regitano *et al.*, 2008; Machado *et al.*, 2010). There is therefore the need for alternative tick control strategies that are sustainable and cost-effective. Host resistance to ticks is a characteristic of cattle, which when exploited may be vital in controlling ticks (Marufu *et al*., 2014).

Tick resistance among beef breeds is variable, with the Nguni having been shown to be more resistant to ticks than Bonsmara and Angus cattle (Jonsson, 2006; Muchenje *et al.*, 2008; Marufu, *et al.*, 2011a). Similarly, the Brahman breed has displayed superior resistance to ticks compared to its *Bos taurus* counterparts (Seifert, 1971; Utecha *et al.*, 1978; Piper *et al.*, 2009). The invasive *Rhipicephalus microplus* and the indigenous *Rhipicephalus decoloratus* are two economically important tick species included into the current parasitic threat to cattle production in South Africa (Vos, 1979; Nyangiwe *et al.,* 2013). No differences were observed when comparing Nguni and Brahman purebreds after exposure to *R. decoloratus* (Rechav & Kostrzewski, 1991) or when Brahman x British and Africander x British crosses were exposed to *R. microplus* (Seifert, 1971). In all instances, the *Bos indicus* and *Bos taurus africanus* cattle proved more resistant to ticks than type *Bos taurus.* Understanding the mechanisms of this resistance to ticks can form the basis of tick control programs. Coat characteristics, such as hair length and coat thickness, colour and smoothness affect the ability of ticks to attach upon an animal (Marufu *et al.*, 2011b; Ibelli *et al.*, 2012). Kemp & Bourne (1980) suggested that cutaneous hypersensitivity reactions are a key factor in the immune response of tick resistant hosts. High tick resistance has been associated with a strong delayed and less intense immediate type hypersensitivity response (Marufu *et al.*, 2013). Piper *et al.* (2010) observed that type I hypersensitivity reaction enables tick engorgement and could be associated with the host's poor development of a cellular immunity. Studies on tick infestation site histology have also found that eosinophils, basophils, mast cells and lymphocytes are associated with the degree of tick resistance (Marufu *et al.*, 2014a). Changes in parameters like hematocrit, white cell counts, plasma proteins, cholesterol and lactate dehydrogenase have been directly associated with the effects of tick infestation in cattle (O'kelly, 1968; O'Kelly *et al.*, 1970; O'Kelly & Kennedy, 1981; Piper *et al.*, 2009b). Observations of these parameters, amongst others, might help clarify differences in parasite-host relationships as well as provide insights into the physiological state of the animals during specific parasitic infestations.

Immune responses also vary depending on the degree to which an animal's immune system has evolved in its ability to generate vigorous responses in defense against a biting tick species (Marufu et al., 2014). This may be attributed to the variations that exist in the characteristics of the tick species, such as, mouthparts, saliva bioactive molecules and other physiological properties (Francischetti *et al.,* 2009). Since the immune response is tick species specific, long term association between a breed and a particular tick species may result in a more advanced targeted immune response against the tick species. Hence, resistant breeds might have experienced a long period of evolution in the presence of the tick species they are resistant to and accumulated resistance to that tick species (Frisch, 1999; Marufu et al., 2011). *Rhipicephalus decoloratus* is indigenous to Africa, while *R. microplus* is of Asiatic origin (Horak, Nyangiwe, De Matos & Neves L, 2009). It may thus be suggested that the Nguni breed, *Bos taurus africanus*, may be more resistant to tick species *R. decoloratus* than to *R. microplus*. The Brahman (*B. indicus*) can also be expected to be more resistant to *R. microplus* than to *R. decoloratus*. Likewise, the observed susceptibility of the European *B. taurus* types to the *Rhipicephalus* ticks (Wambura *et al.*, 1998) may be attributed to lack of long term association between them.

Tick-host associations can thus be classified as modern or ancient depending on the history of association between the breed and tick species. Comparison between the ancient and modern tick-host relationships will enable an understanding of the molecular basis of the superior levels of resistance displayed by certain breeds. It will also aid in the process of determining if the Nguni, for example, displays superior resistance due to a unique uncharacterized genetic makeup or if it is due to the long term evolutionary relationship between the breed and the ticks.

The inclusion of the tick species *R. microplus* and *R. decoloratus* thus allows for the simultaneous investigation of the parasitic relationships that are currently common in South African cattle production, as well as insights into the underlying genotypic mechanisms that constitute tick resistance. The two selected tick species are in the unique position of being economically important within South African cattle production, but also being in a position of possible historical association with selected cattle breeds. Side by side artificial infestations thus allow for the investigation of each individual relationship without the influence of the other tick type. The host reaction to artificial infestation is more intense than natural infestation (Boppana *et al.*, 2005), which is advantageous in comparative studies. Furthermore, the use of artificial infestation allows for a simulation of the field interactions while minimizing the environmental effects not under investigation. Predation, temperature and humidity affect the success rates of ticks during natural infestation (Regitano & Prayaga, 2010). A parallel artificial infestation thus allows equal opportunity for both tick species to infest and feed on all three breeds for the most accurate assessment of 'ancient' and 'modern' comparisons.

1.2 Aim

To investigate cellular and immunological mechanisms of resistance to *R. decoloratus* and *R. microplus* in Nguni, Brahman and Angus cattle.

1.2.1 Specific objectives

1. To compare systemic responses of historic (Nguni-*R. decoloratus* and Brahman-*R. microplus*) and modern tick-host associations (Nguni-*R. microplus*; Brahman-*R. decoloratus*; Angus- *R. microplus* and Angus- *R. decoloratus*).
2. To compare cutaneous histological reactions of historic (Nguni-*R. decoloratus* and Brahman-*R. microplus*) and modern tick-host associations (Nguni-*R. microplus*; Brahman-*R. decoloratus*; Angus-*R. microplus* and Angus- *R. decoloratus*).

1.2.2 Research questions

1. Has the *Bos indicus* type cattle built up a natural immunity more effective against tick species from the same region historically i.e. *R. microplus*?
2. Similarly, what is the level of resistance that the indigenous *Bos taurus africanus* type when exposed to ticks indigenous to the same area i.e. *R. decoloratus*?
3. How immunologically effective will each breed type be when exposed to a tick species it is not historically associated with?
4. What is the difference in immunological responses between resistant and susceptible comparisons?

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CHAPTER 2 - LITERATURE REVIEW

2.1 Introduction

Ticks and tick borne diseases pose a major threat to modern cattle production (Ghosh, Azhahianambi & de la Fuente, 2006; Jonsson *et al.*, 2008) which has detrimental implications for the industry's economic viability (de Castro, 1997; Manjunathachar *et al.*, 2014b). South African cattle are subject to infestation by the indigenous tick *Rhipicephalus decoloratus* as well as the Asiatic intruder, *R. microplus* (Nyangiwe *et al.*, 2013). The control of these tick species is heavily dependent on the use of chemical acaricides, but the control method faces severe challenges that include cost (Jonsson, 2006), environmental influence (Machado *et al.*, 2010) as well as the ability of tick species to build up resistance to chemical control (Mekonnen *et al.*, 2002). The use of resistant breed types has been suggested as an alternative method of control for South African cattle production (Marufu *et al.*, 2011). In order to determine the feasibility of such an approach, a clear understanding of the factors that constitute the current limitations to South African cattle production is necessary, followed by an investigation into host resistance as alternative control method. This review will thus briefly discuss the tick species involved, the breeds that have shown promising phenotypes and the current knowledge of the underlying mechanisms of resistance. Increased focus will be on reactions from cattle breeds exposed to *Rhipicephalus* ticks, with special attention given to cutaneous cellular and systemic responses.

2.2 Tick Classification and distribution

The world's tick population consists of 879 species which can be divided into 3 families: Argasidae (186), Ixodidae (692) and Nuttaliellidae (1) (Nava *et al.*, 2009). More than 650 different species can be found in Africa. These can also be divided into 7 genera, of which 3 are considered to be of economic importance: *Rhipicephalus, Amblyomma, and Hyalomma* (Jongejan & Uilenberg, 2004). South African cattle are subjected to infestations by the indigenous *R. decoloratus* as well as the Asiatic intruder *R. microplus*. Studies show that *R. decoloratus* might be under threat of displacement by *R. microplus*, possibly due to an increasing environmental temperature (Nyangiwe *et al.*, 2013b). *Rhipicephalus microplus* is presently found in almost all sufficiently warm and humid areas of South Africa and it is only the extremely cold and dry areas that has not been prone to a *R. microplus* invasion (Mapholi *et al.*, 2014).

The invasion of *R. microplus* that precedes the change in composition of the tick population livestock animals are exposed to could have serious implications for tick-borne diseases (TBD). *Rhipicephalus decoloratus*, the indigenous species, is responsible for the transmission of an organism known to be the cause of African redwater. *R. microplus*, in turn, not only transmits *Babesia bigemina*, but also *Babesia bovis*, known to cause Asiatic redwater (Nyangiwe *et al.*, 2013). Both tick species are also capable of the transmission of *Anaplasma marginale*, an intra-erythrocytic rickettsia known to cause anaplasmosis (Fyumagwa *et al.*, 2009). The disease has severe economic impacts (Jonsson, 2006) and is considered

one of the most prevalent causes of cattle losses in low input South African production systems (Mapiye *et al.*, 2009).

2.3 Economic losses due to ticks

Global economic losses from ticks and tick-borne diseases (TBD) have been estimated at around 13 to 18 Billion US Dollars annually (de Castro, 1997). Losses in Africa are estimated at US$ 160 Million (Dold & Cocks, 2001) and US$ 92 Million in South Africa (Mapholi *et al.*, 2014). The accuracy of these figures could benefit from a re-evaluation, but they aid in emphasizing the impact of ticks and TBD. The direct effect of tick infestations can also have a significant impact on cattle production systems. It has been reported that an animal that is infested with roughly 40 ticks per day can lose up to 20 kg of body weight per year (Frisch, 1999). Infestation has shown clear effects on cow productivity. Scholtz *et al.*, 1991 has calculated losses of calve weaning weights per individual tick, of which a very large portion *R. decoloratus*, to be 8.9g, 8.0g, and 8.6g for Hereford, Bonsmara and Nguni breeds, respectively. Although the Nguni cattle in the study carried significantly less ticks, the losses per tick were similar to what was seen in the remaining breeds. Sutherst *et al.*, 1983 reported weight loss estimates in *Bos indicus X Bos taurus* steers of 0.6 to 1.5g per *R. microplus* infestation. It has been shown that heavy tick infestations can lead to anorexia in Hereford *(B. taurus)* cattle (Seebeckt *et al.*, 1971). The authors suggested that the loss of appetite due to the pathological effects of tick infestation contribute 65 % of the cause of weight loss seen in the cattle. The weight loss could not be directly correlated to the number of engorged female ticks, but was considered to be determined by the variation in the extent to which infestation suppresses appetite (Seebeckt *et al.*, 1971). Furthermore, infestations have a detrimental effect on the hide value of cattle. Purposed for the leather industry, damage to hides can decrease their value decrease by 20 to 30%. (Frisch, 1999).

Ticks are also responsible for degrees of immunosuppression. *Rhipicephalus microplus* infestations have caused decreases in peripheral blood leukocytes in *Bos taurus* cattle (Inokuma *et al.*, 1993), while *R. sanguineus* inhibited T-cell proliferation in mice (Ferreira & Silva, 1998) as well as neutrophil function (Inokuma *et al.*, 1997) and antibody production (Inokuma *et al.*, 1997) in dogs. The suppression of these immunological functions increases the animal's susceptibility to tick-borne diseases. It is thus not surprising that cattle displaying a higher level of resistance to ticks are at a lesser risk of being subjected to pathogen transmission from tick infestations (Wikel, 1999). There has been evidence of cattle displaying some resistance to TBD (Axford *et al.*, 2000), but results can be conflicting and should be interpreted carefully, as it can be difficult to distinguish results between resistance to disease per se or resistance to the ticks that act as vectors for the disease.

2.4 Variation in resistance by breeds

The type of cattle breed has been found to be an important factor in determining a host's level of resistance to ticks (Utecha *et al.*, 1978; Wambura *et al.*, 1998a). Comparisons within this review are largely based on tick counts, which are considered to be a reliable form of assessing the level of resistance displayed by an animal (Veríssimo *et al.*, 2008). Variation for the level of resistance to ticks occurs both between and within

breeds (Seifert, 1971). The indigenous Nguni cattle breed (type *Bos taurus africanus*) is more resistant to tick infestations than the European meat breeds (type *Bos taurus*). It has displayed significantly lower tick counts when compared to exotic (Angus and Hereford) cattle as well as synthetic Bonsmara cattle on natural pasture and in feedlots (Scholtz *et al.*, 1991; Muchenje *et al.*, 2008; Marufu *et al.*, 2011). Both Scholtz *et al.*, (1991) and Marufu *et al.*, (2011) reported high levels of exposure to *R. decoloratus* within their studies, suggesting the Nguni has developed a strong resistance phenotype for the tick type. Similar results have been found when comparing *Bos indicus* type breeds to *Bos taurus* cattle. The *Bos indicus* cattle are significantly less susceptible to infestation and display a more effective level of resistance to a wide range of ticks when compared to the exotic type purebreds as well as *Bos indicus* x *Bos taurus* crosses (Wambura *et al.*, 1998a). Following artificial infestations with *R. microplus*, *Bos indicus* Brahman cattle also displayed superior resistance after they were compared to a wide range of *Bos taurus* cattle (Utecha *et al.*, 1978). There are few studies that include the side by side comparison of the *B. indicus* and *B. taurus africanus* breeds exposed to *R. microplus* or *R. decoloratus*. Rechav & Kostrzewski, (1991) assessed *R. decoloratus* tick burdens of various cattle including the Brahman and Nguni breeds under natural infestation. The Nguni breed had less infested ticks than the Brahman at all sampling time points, but the differences were not significant. They were, however, significantly less than the Simmentaler and Santa Gertrudus breeds also exposed and the authors suggested that resistance is strongly related to the portion of Indicine or Sanga genes within a breed. Brahman-British and Africander-British crosses have also been compared following exposure to *R. microplus*, which in turn, is likely historically related to B. indicus cattle (Seifert, 1971). The authors did not report significant differences between the Brahman or Africander (type *Bos taurus africanus*) crosses, but mentioned that unpublished data has indicated Brahman cattle to be slightly more resistant to *R. microplus* than *B. taurus africanus* breeds. *Bos taurus* cattle types are much more prone to exhibit intolerance to heavy infestation levels than *Bos indicus* breeds, displaying 'tick sore' lesions from infestation levels of 10 000 larvae (Constantinoiu *et al*., 2010).

The greater resistance displayed by Nguni and Brahman cattle when subjected to *Rhipicephalus* ticks can possibly be attributed to a long and continuous evolutionary relationship with the ticks. High levels of continuous infestation could thus have created an environment which placed strong selection pressure on the cattle breed to increase its ability to resist and tolerate the tick species (Marufu *et al.*, 2014). The question remains, however, if the breed type has an increased level of resistance to the specific *Rhipicephalus* tick species it is historically associated with. The Nguni can be expected to have evolved under the influence of continuous selection pressure applied by the well-known detrimental effects accompanying infestation, assumed to be by *R. decoloratus*. The resistant phenotype is thus likely the product of co-evolution as also suggested by Frisch, 1999 and Marufu *et al.,* 2011b. A similar relationship for Brahman cattle has been suggested in an attempt to explain its increased resistance to *R. microplus* ticks (Piper *et al*., 2010). There are tendencies toward differences observed when *Indicine* or *B. t. africanus* cattle are exposed to the same Rhipicephalus species, but significant differences are yet to be observed. It has to be considered that, as both the Brahman and Nguni breeds were subjected to *Rhipicephalus* ticks

during their historical evolution, the mechanisms of natural selection might be relatively similar between *R. decoloratus* and *R. microplus* ticks, as suggested by Seifert, (1971). Host resistance trait has shown a fairly high level of heritability (82 %) for *Bos indicus* cattle and lower levels (39 to 49 %) for *Bos taurus* types (Piper *et al.*, 2009b).

2.5 Current Methods of Control

If exploitation of the resistance phenotype is to be considered as a possible method of tick control, it is important that some of the current methods of control are evaluated. Key methods in modern cattle production include acaricides and vaccines.

2.5.1 Acaricides

Acaricide use is currently the primary method of tick control globally. It is, however, not considered a sustainable method of tick control due to the ability of ticks to exhibit resistance to acaricides. Both *R. microplus* as well as *R. decoloratus* have displayed resistance to a variety of acaricides (Rajput *et al.*, 2006; Robbertse *et al.*, 2016) including the widely used amitraz (Mekonnen *et al.*, 2002). Some authors have stated that the process of acquiring of resistance by the ticks is effective to the point where the introduction of almost any new acaricide will only have an effective lifetime of about 5 to 10 years (Wharton, 1976). As the use of these drugs are in effect exerting a very intense selection pressure on the parasitic population, it has the consequence that the 'resistance gene' becomes very highly concentrated in the subsequent populations. This evolutionary pressure enables the emergence of new chemical-resistant strains of ticks, faster than new chemicals can be produced (Li *et al.*, 2007). In addition, acaricide use can lead to residues of chemicals in meat, milk and the environment (Machado *et al.*, 2010). This does not comply with an environmentally friendly method of control as well as a growing demand for chemical residual-free products in the consumer market (Regitano *et al.*, 2008). Continuous acaricide use will thus affect profit margins in cattle production systems due to the high cost associated with frequent dipping procedures (Jonsson, 2006; Manjunathachar *et al.*, 2014b).

2.5.2 Vaccines

There has been work done on the development of a vaccine for the cattle tick, *B. microplus*, using the tick antigen Bm86 (Willadsen *et al.*, 1996). Vaccines have provided degrees of effective control, but only to the specific species of tick that is vaccinated for. They are also not effective for short term use, as they often affect the tick's reproductive capabilities and cause a gradual decrease in ticks over time and will not be effective for control if an immediate effect is required (Frisch, 1999; de la Fuente *et al.*, 2007). Vaccines are not economically feasible to small-scale farmers and ticks mutate the targeted epitopes into unfamiliar forms; hence nullifying the effect of a particular vaccine. Furthermore, the identification of candidate antigens for vaccines remains challenging, which is amongst the primary limiting factors regarding the development of vaccines that meet the requirement of effective tick control (de la Fuente *et al.*, 2007).

It thus becomes clear that the primary methods of tick control in modern cattle production face various limitations and challenges. The exploitation of host resistance might become a sustainable alternative for these control methods, which may be more sustainable and cost effective.

2.6 Mechanisms involved in resistance

Although there has been intensive research into the mechanisms that underlie the resistance displayed by certain breeds, they are still to be fully understood. Constantinoiu *et al.* (2010) argued that progress has been slow due to the widespread generalizations when comparing infestations between different tick species as well as hosts, where host-parasite relationships should be seen as relatively unique. If such a suggestion is valid, differential comparisons of responses to *R. microplus* and *R. decoloratus* become essential. The necessary segregation between relationships is in a large part due to reports that the complex salivary secretions differ significantly between different tick species (Mans *et al.*, 2008).

2.6.1 Tick Avoidance

Amongst the primary, but not necessarily most effective, methods with which cattle control tick populations is by avoiding dense tick populations in natural pasture. Sutherst *et al.*, (1986) reported avoidance behavior of cattle for *R. microplus* in Australian pastures. They also reported that host seeking larvae were less likely to successfully attach when densities of the larval populations were high. Avoidance behavior in cattle was also witnessed in Zimbabwe to the tick *Rhipicephalus appendiculatus* (Norval *et al.*, 1988).

2.6.2 Coat characteristics

The extent of resistance by a breed type is related to several coat characteristics of that breed. Breed types that display a high level of resistance will often have coats that prevent the attachment of ticks to a certain extent. Coat characteristics such as hair length as well as coat thickness, color and smoothness will significantly influence the ability of ticks to attach upon the animal. Those that a display a short haired, smooth and light colored coat will tend to have less ticks attached in comparison to long haired, rough and dark colored coats (Marufu *et al.*, 2011; Ibelli *et al.*, 2012)

2.6.3 Grooming

Tick infestation causes the release of histamine by granulocytes in the skin of cattle. This leads to skin irritation and self-grooming, the physical removal of ticks from the skin by cattle (Koudstaal *et al.*, 1978). Several authors have shown grooming to be a key part of the control of ticks by cattle (Riek, 1956; Bennett, 1969; Koudstaal *et al.*, 1978). Furthermore, histamine release as an inflammatory mediator plays an important in the grooming mechanism (Veríssimo *et al.*, 2008); it is also known to act on tick infestation directly (see section 2.6.4.1).

2.6.4 Immunological responses

Coat characteristics and grooming play an important role in the cattle breed's ability to display resistance to infestation. However, strong evidence suggests that they also rely on innate and acquired immunological

mechanisms (Marufu *et al.*, 2011). The animal's immunological response to infestation includes a range of components amongst which leukocytes, complement, cytokines, antigen presenting cells (APC). The intensity and mechanism of expression depends on the type of host and tick species involved. The effects of successful host responses on the tick vary from tick rejection, reduced engorgement weight, reduced viability and quantity of the female eggs up to death of the parasite (Willadsen, 1980a).

2.6.4.1 ***Histamine***

Histamine plays an important role in an animal's response to tick infestation. In vivo and in vitro studies show that histamine has a resisting effect on infesting larvae, causing detachment of the parasite from the host (Kemp & Bourne, 1980). They suggested that the pharmacological mediator has a direct effect on the parasite, while Wikel & Bergman, (1997) observed that this is achieved by preventing salivation and engorgement on to the host. This is supported by observations of histamine-rich basophils accumulating at infestation sites when guinea pigs were infested with *Dermacentor andersoni*, the Rocky mountain wood tick (Wikel, 1982). Resistance displayed by these animals was less successful after they were treated with histamine antagonists. The skin of resistant bovine hosts also contains higher amounts of histamine than that of susceptible cattle (Willadsen, 1980).

2.6.4.2 ***Hypersensitivity***

In inflammatory reactions, the intensity at which the host responds can play a key role in the outcome of the response (Kemp & Bourne, 1980; Reuben Kaufman, 1989). It has led to the development of an intradermal skin test which allows the measurement and comparison of cutaneous hypersensitivity responses between certain breeds as well as the association of these responses with levels of tick resistance (Marufu *et al.*, 2013). Marufu *et al.* (2013) compared hypersensitivity responses between tick susceptible (Bonsmara) and tick resistant (Nguni) breeds. When injected with unfed larvae extracts (ULE) of both *R. microplus* and *R. decoloratus* the Bonsmara cattle exhibited only an immediate type hypersensitivity reaction, also known as a type I reaction. The more tick resistant Nguni cattle displayed a less intense immediate type hypersensitivity response but rather a strong delayed type response to both tick species. The study is supported by several authors who have similarly associated the delayed type response with types that have shown a high level of resistance to ticks (Bechara *et al.*, 2000; Pablo Juan Szabó *et al.*, 2004; Prudencio *et al.*, 2011). Bechara *et al.*, (2000) and Prudencio *et al.*, (2011) reported the positive associations between delayed type hypersensitivity using extracts from *R. microplus* on *B. taurus* and *B. indicus* cattle. The use of both *R. decoloratus* and *R. microplus* ULE on *B. indicus* and *B. taurus. africanus* cattle could thus benefit the understanding of these inflammatory reactions and their specificity towards tick types. Studies have reported that the type I hypersensitivity reaction enables tick engorgement (Piper *et al.*, 2010a) and could be associated with the host's poor development of a cellular immunity. The less pronounced inflammatory response from Nguni cattle might likely be due to an ability to prevent an extensive reaction to the biomolecules that is found in the tick saliva. This might have been obtained due to the breed's evolutionary development in the presence of the parasite (Marufu *et al.*, 2014).

2.6.5 Cutaneous cellular responses

The histology of the tick attachment binding sites has been studied by several authors in order to improve the understanding of the cellular responses when cattle are exposed to different tick species. Mast cells, basophils, eosinophils and lymphocytes play a role in the animal's cellular response and degree of resistance when artificially infested with ticks (Veríssimo *et al.*, 2008; Carvalho *et al.*, 2010; Constantinoiu *et al.*, 2010; Marufu *et al.*, 2014). It should be noted that reactions to artificial infestations have been shown as more pronounced than those following natural infestation (Ribeiro, 1989; Boppana *et al.*, 2005), although the intensity of artificial infestation will likely have a strong influence. Piper *et al.*, 2010 confirmed the findings of Tatchell & Moorhouse, (1968) that infestation site histology analysis has shown the *B. taurus* breeds to display a more intense cellular response than its *indicine* counterparts.

2.6.5.1 ***Leukocytes***

Neutrophils form part of the first line of defense of the innate immune system as very motile phagocytes (Francischetti *et al.*, 2009). Tick susceptible hosts have displayed a higher number of neutrophil and eosinophil counts at infestation sites suggesting that these cells could be associated with an increased susceptibility (Wada *et al.*, 2010; Marufu *et al.*, 2014). Tatchell & Moorhouse, 1970 and Marufu *et al.*, 2014 found neutrophils to be associated with the breakdown of the extracellular matrix and necrosis of parasitized tissue and consequently suggested that they might enable tick feeding by facilitating the tick's access to host fluids. The observations are consistent with early suggestions that specific vascular damage is due to components found in tick saliva, but that general tissue damage can in a large part be attributed to the host's own response. The suggestion was based on observations that collagen damage below the mouthparts of infesting ticks was preceded by a substantial influx of neutrophils following *R. microplus* infestation (Tatchell & Moorhouse, 1968). Constantinoiu *et al.*, 2010 also found granulocytes (stated to very likely be neutrophils) clustered in parasitized sites close to tick mouthparts after *R. microplus* infestation. The authors studied infestation histology at several time points and found the granulocyte presence to peak earlier and maintain for longer in *B. taurus* cattle than in *B. indicus* breed types. Granulocyte specific antibodies was repeatedly seen inside the ticks, suggesting that the parasite ingests neutrophils within very early stages of infestation. It is possible that neutrophils are a source of nutrition for the larvae (Constantinoiu *et al.*, 2010). *B. taurus* cattle have also exhibited increased levels of expression for chemokine CXCL-8, which functions to attract and activate neutrophils (Piper *et al.*, 2010a). Bovine neutrophils are known to produce bovine alpha-1 acid glycoprotein (α-1AGP) (Rahman *et al.*, 2008). This acute phase protein (ACP) is responsible for reducing the chemotaxis of bovine monocytes (Lecchi *et al.*, 2008) as well as inhibiting the aggregation of platelets (Costello *et al.*, 1979). The levels of α-1AGP have been reported as consistently higher in susceptible hosts both in the presence or absence of infestation (Carvalho *et al.*, 2010).

Eosinophils constitute roughly 1 to 6% of the leukocytes circulating in the blood and will usually emigrate to tissue, where their majority is found, after about 8 to 12 hours of circulation. The cells play a key role in the modulation of inflammatory responses and can function as pro-inflammatory leukocytes (Young *et al.*, 2006). Eosinophils are commonly found in the body surfaces that interact with external surfaces and thus often play a role in allergic reactions or parasitic infestations (Francischetti *et al.,* 2009). There has been contrasting findings when investigating the role of eosinophils in tick resistance. Marufu *et al*., (2014) found positive correlations between eosinophils and tick counts, concluding that they are likely associated with susceptibility to *Rhipicephalus* ticks. Carvalho *et al.,* (2010) reported a higher number of eosinophils the more resistant hosts, suggesting that the resistant cattle had a greater capacity to retain eosinophils in the lesion of parasitized skin.

Amongst mononuclear cells are macrophages which stimulate specific immune responses by presenting tick antigens to T-cells (Francischetti *et al.,* 2009). A role within a T-cell mediated response would suggest that monocytes would play a positive role in resistance. This is supported by Marufu *et al.*, 2014, who reported negative correlation of mononuclear cell counts to tick count in the Nguni breed while mononuclear cell and tick counts had a positive correlation in the more susceptible Bonsmara breed. Carvalho *et al.,* (2010) found mononuclear cells to be significantly less in parasitized skin, but did not report a significant difference between the susceptible (Holstein) and resistant (Nelore) breeds.

Taking to account the aforementioned studies regarding cutaneous cellular responses, it becomes clear that a lot focus has been placed on *R. microplus* as well as the differences between *indicine* and *taurine* cattle. If the questions regarding the specificity of reactions, especially regarding ancient and modern associations, are to become more clear, further investigation is necessary. The roles of the various cellular components could also benefit from further investigation and may aid in elucidating certain immunological components of resistance.

2.6.5.2 ***T-cells***

In animals considered to be naïve to tick infestation, the skin of B. indicus type cattle were shown to have significantly higher numbers of T-cell sub populations and $CD25^{+}$ than the *B. taurus* counterparts, which might represent a superior capacity to elicit responses to infestation (Constantinoiu *et al.*, 2010). Within the T-cell sub-populations, γδT-cells appeared to be the most dominant. Within the same study, T-cell sub-populations increased significantly in both breed types after infestation, but the γδ T-cell sub type appeared in significantly larger numbers in the *B. indicus* cattle than in *B. taurus*. Carvalho *et al.*, (2010) also reported significantly higher numbers of CD3+ and γδ T-cells in the infestation sites of resistant cattle than susceptible phenotypes. Constantinoiu *et al.*, (2010) has consequently suggested that the γδ T-cells might likely play a key role in the manifestation of resistance. The multiple functions of the γδ T-cell sub-type is still to be fully articulated, but they are thought to be amongst the primary components to respond to disease or tissue damage by integrating the innate and adaptive systems of the immune system (Born *et al.*, 2006).

2.6.5.3 ***B-cells***

B cells form a key part of the lymphatic immune system and specialize in the synthesis and secretion of antibodies as immunoglobulins (Ig) (see following section).

In an attempt to assess the effect of *R. microplus* infestation on B-cell populations of B. taurus and B. indicus cattle, very few B-cells were present in the parasitized skin sections of both breeds (Constantinoiu *et al.*, 2010a). Establishing the role of B-cells has been difficult as reports do not show great consistency. Ovine animals displayed the presence of B-cells after infestation with *Hyalomma anatolicum* (Boppana *et al.*, 2005), but B-cells were not detected in mice after infestation with *Ixodesricinus* nymphs (Mbow *et al.*, 1994).

2.6.5.4 ***Immunoglobulins***

Humoral factors are thought to play a role in the acquisition of resistance in animals. Evidence was displayed by passively transferring immune serum to tick-naïve laboratory rabbits, enabling partial immunity to *Ixodes ricinus* (Brossard & Girardin, 1979). A similar immunity to *R. microplus,* expressed to a lesser extent than the original resistant cattle, has been observed in naïve calves after passive transfer (Roberts *et al.*, 1976). A positive correlation between antibody titres and resistance to *R. microplus* was originally reported (Mattioli *et al.*, 2000). Conversely, antibody levels were negatively correlated to resistance after repeated manifestations of sheep to *Amblyomma americanum* ticks (Barriga *et al.*, 1991). Noticeable differences have been reported in the ability of a tick to induce antibody responses at different stages of its lifecycle (Hernandez *et al.*, 1994). They observed that antigen extractions from adult ticks had a much greater reaction than those of larvae and nymph after infesting rabbits with *Rhipicephalus sanguineas*. Various reports, however, question the role of antibodies in resistant animals. High levels of antibodies are often seen in animals susceptible to infestation (Willadsen, 1980b; Schorderet & Brossard, 1993; Piper *et al.*, 2016). The level of antibodies tends to decline after repeated infestations (Barriga *et al.*, 1991), while the animal's level of resistance increases (Fivaz *et al.*, 1991). Reports on antibody responses in mice also suggest that high titers of antibodies do not lead to high levels of resistance to infection (Biozzi *et al.*, 1986). Passive transfer of resistance is more effective when using lymph node cells than serum from tick resistant animals, suggesting that the T-cell responses may play a larger role in the manifestation of resistance (Wikel & Allen, 1976).

2.6.5.5 ***General tissue evaluation and the extracellular matrix***

Szabó & Bechara, (1999) suggested that cutaneous changes caused by skin infestation are non-specific and the presence of hyperplasia, oedema, dermal infiltration, haemorrhage and necrosis can be expected in most cases of any noxious stimuli. Marufu *et al.*, (2014) compared the general tissue evaluations of parasitized sites on Bonsmara and Nguni heifers after *R. microplus* infestations. The Bonsmara heifers tended to have more pronounced changes in the dermis and epidermis than the Nguni's. The parasitized samples from the Bonsmara heifers also exhibited severe basal cell hyperplasia, epidermal necrosis along with acantholysis and oedema. The increased severity of necrosis and oedema in the Bonsmara heifers

could likely be associated with a type I hypersensitivity response to ticks leading to an increased susceptibility to infestation (Marufu *et al.*, 2014). The Bonsmara heifers were also more prone to exhibit severe pustule-like lesions in the epidermis as well as moderate to severe inflammatory infiltrates into the dermis of parasitized skin samples. While these cutaneous changes are likely to appear during most infestations, the extent to which they are pronounced might likely depend on the susceptibility of the animal towards the tick species. Similar observations were made by Piper *et al.*, 2010, who reported more severe cutaneous changes in both the epidermis and dermis of Holstein-Friesian cattle compared to Brahman cattle. While the epidermis of the Brahman cattle were entirely free of any changes, the Holstein Friesian cattle displayed at least one or more cases of necrosis, acantholysis, sub epidermal clefting, basal cell hyperplasia and hyperkeratosis. Piper *et al.*, (2010) studied the expression of several collagen transcripts between Brahman and Holstein-Friesian animals. Expression was observed to be higher in the parasitized skin of the *B. indicus* Brahmans compared to the Holstein-Friesians. These findings supported similar observations in the skin of B. taurus cattle that displayed a phenotype of increased resistance (Wang *et al.*, 2007). Collagen fibril diameter will increase in skin that is under repetitive mechanical stress (Wang & Sanders, 2003). Fibrils of a larger diameters allow for skin with an increased physical strength in comparison to small diameters (Ottani *et al.*, 2001). It is well known that collagen expression is involved in the wound healing process of damaged and inflamed tissue. It has been suggested, however, that resistant cattle may respond by remodeling the extracellular matrix for an environment less vulnerable to infestation (Piper *et al.*, 2009b). The previous study strongly suggests a possible mechanical defense by the *B. indicus* breed. It would be beneficial to compare the ability of ticks to form an advantageous tick feeding lesion between susceptible and resistant breeds. Differential cellular infiltrates have been included in numerous studies (see section 2.6.5.1), while general tissue evaluation at infestation sites is not often included. Furthermore, as reactions to tick species are considered specific (Constantinoiu *et al.*, 2010), possible differences in cutaneous changes between tick types could improve the understanding regarding the specificity of responses, and the previous studies are lacking in including a wider assessment of breeds and often only focus on a single tick species.

2.7 Systemic responses to infestation

Early studies on tick infestations by *R. microplus* showed depression of the host animal's growth rate and blood hematology (Francis, 1960; Little, 1963). It is important to note that tick infestation influences the animal's appetite when interpreting data related to metabolism (Seebeckt *et al.*, 1971). In this regards, it has been suggested that ticks have a direct suppressing effect on certain metabolic processes through the release of a toxin (O'Kelly *et al.*, 1970).

2.7.1 Hematology

2.7.1.1 *Leukocytes*

Leukocytes are thought to generally perform their biological functions in tissues. Their presence in circulation is usually part of transportation between the areas of formation, storage or activity. (Schalm,

1961; O'Kelly *et al.*, 1970). It is important to note the time period and level of infestation in O'Kelly *et al.*, (1970) which reported an average of 4240 mature ticks on the 33rd day and leukocyte values tend to show a wide range of variation between breeds and locations. Neutrophils are not known to exit circulation and enter tissues in large amounts unless initiated by acute inflammation. Basophils, monocytes and eosinophils are more prone to enter tissues under normal circumstances and will appear in circulation in relatively low numbers (Young *et al.*, 2006).

Platelets, also known as thrombocytes, are small and non-nucleated cells with various essential functions to any vascular damage. Platelets adhere to collagenous tissue at sites of vascular trauma to form plugs which would later be reinforced by fibrin. They also provide a surface for the assembly of proteins complexes responsible for coagulation which enables clotting as well as secreting factors responsible for the modulation of vascular repair and modulation (Young *et al.*, 2006). The function of platelets suggests they could play a primary role in parasite-host interaction, but platelets have not been cited as a primary method for the defense of a resistant host. The effectivity of platelets within the manifestation of resistance can be questioned, however, as the ability of a large majority of ticks to inhibit platelet aggregation has been reported (Francischetti *et al.*, 2009).

2.7.1.2 ***Lymphocytes***

Lymphocytes make up for about 20 to 50% of the white cells in circulation. These white blood cells are in constant circulation within an animal's fluids, including the blood and lymph, and pauses within the organized lymph tissues (Young *et al.*, 2006). Piper *et al.*, (2010) studied the differential expression of genes between tick-naïve and tick infested Holstein-Friesian cattle. The authors found a higher expression of chemokines as well as an up regulation of CCL-8 and CCL-2, genes which are responsible for targeting T-cells, natural killer cells as well as monocytes. It was concluded that the animals recruit non-resident cells toward infestation sites as part of an inflammatory response, which was supported histological evaluation. Normal ranges for bovine cattle can be seen in Table 2.2.1.

Table 2.2.1 Estimates for circulating levels of erythrocytes (red blood cells) and leukocytes for adult bovine animals

Erythrocytes		
	Extreme Range	Average Range
Erythrocytes	5.0-10.0	6.5-8.0
Hemoglobin	8.0-14.0	10.5-12.0
PCV	24-48	34-38
MCV	40-60	45-55
MCH	11-17	13-15
MCHC	26-34	28-32

Leukocytes		
	Range	Average
Leukocytes	4-12000	7-9500
Band Neutrophils	0-2	0.5
Neutrophil	15-45	28
Lymphocyte	45-75	58
Monocyte	2-7	4
Eosinophil	2-20	9
Basophil	0-2	0.5

Source: (Schalm 1975) (PCV: packed cell Volume; MCV: mean cell volume; MCH: mean cell hemoglobin; MCHC: mean cell hemoglobin concentration)

2.7.1.3 ***Red Blood Cell Count, Hematocrit (PCV) and Blood Composition***

In animals under normal circumstances, it should be noted that Grade Brahmans showed higher levels of PCV, hemoglobin and red cell count compared to Brahman- and Africander-crosses as well as British breeds in a tropical environment. The breeds could be ranked in the order mentioned, ending with the British breeds displaying about 75% of the values of the Grade Brahman. The Grade Brahmans did, however, display a lower mean corpuscular volume (MCV) than the remaining breeds Evans & Turner, (1965).

Tick susceptible breeds like the Holstein-Friesian display a significantly lower Red Blood Cell Count (RBCC) than the more resistant Brahman breed after artificial *R. microplus* infestations. The Holstein Friesians also exhibited low levels of hemoglobin and packed cell volume, thought to be a common occurrence in heavily infested animals (Piper *et al.*, 2009). The values displayed by the animals in the study by Piper *et al.*, 2009 were very close to what would be classified as blood anemia (Cole *et al.*, 1997). Within the same study, the Holstein-Friesian animals displayed a significantly higher white blood cell count. They suggested that the slightly higher than normal values are a consequence of sustained inflammation and stress due to the excessive levels of infestation. Their results are consistent with those found by Rechav *et al.*, 1990, where significantly higher WBC counts in the *B. taurus* Simmentaler cattle were noted when compared to Brahmans after infestation with African tick species.

Similarly, studies have reported decreased hematocrits as consequences of tick infestations (Riek, 1957; Little, 1963). There seems, however, to be a difference in responses regarding the intensity of infestation. While O'Kelly & Seifert, 1969 reported observations that light infestation caused an increase in hematocrit and hemoglobin in Shorthorn x Hereford steers, O'Kelly & Seifert, (1970) observed depression in these parameters following heavy infestations. Similar observations were made by Kennedy & O 'kelly, (1981) who reported decreased PCV levels for British crosses, but not Africander (*B. t. africanus*) after intense *R.*

microplus infestation. The most likely causes of blood anemia include the loss of blood due to tissue hemorrhage or blood sucking parasites; an increased rate of erythrocyte destruction and a decrease in the rate of erythrocyte production (Schalm, 1961). Also, cattle on a low level of nutrition are prone to display reduced levels of hemoglobin and hematocrit (Springell, 1968) and tick infestations have a depressing effect on the animal's appetite (Seebeckt *et al.*, 1971). In a study designed to separate the direct effects of infestation from the anorectic effects due to decreased intake, O'Kelly *et al.*, (1971) was able to associate depressed levels of hematocrit and hemoglobin to the direct effect of ticks.

2.7.2 Metabolic function through serum biochemistry

Tick infestations have shown to have both direct and indirect effects on the metabolism of an animal (O'Kelly *et al.*, 1970; O'Kelly & Kennedy, 1981). Plasma proteins, cholesterol and lactate dehydrogenase have been shown to under the influence of the effects of infestations (O'kelly, 1968; O'Kelly *et al.*, 1970; O'Kelly & Kennedy, 1981). It is been suggested that ticks might secrete an hepatoxic compound (Jonsson, 2006) that, if present in sufficient amounts, should alter the efficiency of liver metabolism. A blood biochemistry profile of animals could thus be beneficial to improve the understanding of the physiological and metabolic state of resistant/susceptible breeds during infestation. Furthermore, as a majority of studies up to date have been based on indicine and taurine breeds, there is a need for information regarding the *Bos taurus indicus* Nguni breed regarding these parameters if differential relationships between parasites and hosts are to be more understood.

2.7.2.1 Intake, immunity and lipid metabolism: plasma proteins and cholesterol.

Plasma proteins are known to display changes in their profiles during infections and the subsequent immunological responses. After the animal's exposure to an exogenous antigen, the levels of albumin are prone to decreasing, while the globulin levels will likely increase. The increase in globulins is most likely due to the strong relationship between globulins and antibodies, while the decrease in albumin is thought to be a secondary response to the change in globulin concentration in order to maintain colloid osmotic pressure at acceptable levels. The extent to which the plasma protein profile changes is also thought to be related to the severity of the infection and the magnitude of the host response (Dimopoullus, 1963).

Assessing segregated levels of albumin and globulin may provide a better response as the protein sub-classes can respond in different directions. According to O'Kelly *et al.*, (1971), tick infestations cause increased levels of serum globulin and lead to decreased albumin concentrations. Although it is thought that infestation leads to the removal of blood by ticks, albumin decreases were due to changes in catabolism or synthesis of the protein, which could result from liver damage or decreased protein intake. Further observations by Kennedy & O 'kelly, (1981) confirmed the increase of λ-globulin animals in heavily tick infested animals. They observed a λ-globulin decrease over the treatment period, where the British breeds displayed a significantly higher level than the Africander X British crosses. The study also showed hypoalbuminia in the tick infested animals; suggesting a possible alteration in metabolism due to decreased absorption or liver damage. Similar responses regarding albumin and globulin changes could be observed

in other assessments of parasitic infestation (Herlich & Merkal, 1963). It can possibly be considered as a non-specific reaction to situations regarding parasites and their consequent infections.

Fibrinogen is one of the plasma proteins that plays a vital role in the blood clotting process and, because of its solubility characteristics, is classified as a globulin (Dimopoullus, 1963). Fibrinogen levels are also known to rise during inflammatory reactions, with levels of above 10g/l indicative of a severe reaction. The extent of an inflammatory response might be represented more accurately in the changes in fibrinogen levels rather than the total leukocyte counts (Schalm *et al.*, 1975). Moderate increases have also been associated with sub-clinical conditions and responses are visible within 24 hours of the onset of the disease or foreign antigen responsible for the inflammatory process (McSherry *et al.*, 1970). Interpretation of fibrinogen levels is thus not limited to very severe inflammatory cases.

Blood cholesterol levels have been reported to differ significantly between *B. taurus* and *B. indicus* breeds as by O'kelly, 1968, who reported Brahman (*B. indicus*) to have display higher levels of plasma cholesterol than British (Hereford and Shorthorn) after assessment in the same tropical environment. The author also negatively correlated plasma cholesterol levels to the animals' degree of displayed resistance to *R. microplus*. Due to the strong statistical correlations seen, the author suggested that plasma cholesterol might be a viable index for resistance to the cattle tick. Higher cholesterol levels have also been reported for Africander x British crosses than pure British breed crosses, although the effect of intense artificial infestation by *R. microplus* lowered the values in both breeds over a 28 day period (O'Kelly & Kennedy, 1981).

2.1.1.1 Muscle damage: Alanine Amino Transferase, Creatine Kinase and Lactate Dehydrogenase

Alanine amino transferase (ALT) (also known as serum alanine transaminase) is found in the cytoplasm of liver cells (hepatocytes) of certain animals. Significant increases in serum ALT levels are associated with pathological liver cell damage in some species, but ALT concentrations are considered not to be of value for clinical diagnosis of liver diseases in the horse, cattle or sheep (Stogdale, 1981). This is due to the very low levels of ALT found in the livers of domestic farm animals. It does provide a useful indication of muscle damage within these domestic species, as it has been shown to increase during traumatic myopathy in cattle (Boyd, 1988). Creatine Kinase (CK) is an enzyme found in the skeletal muscle, heart muscle as well as significantly lower amounts in neurological tissues of various species, including cattle (Boyd, 1988). Increases in the serum concentration are associated with damage within the muscular tissue. As the levels will normally return to basal levels within two to three days, it is most efficient as an indicator of acute muscle damage as opposed to the chronic type (DiBartola & Tasker, 1977). Along with ALT and lactate dehydrgenase, rises in CK are often associated with nutritional myopathy, also known as white muscle disease, in cattle. Inherited myopathies are considered uncommon in farm animals (Boyd, 1988).

Increases in LDH levels are used as an indicator of various diseases including muscular dystrophy and hemolytic anemia in humans, O'Kelly *et al.*, (1971) reported depressed LDH values due to the specific

effects of tick infestations and was able to negatively correlate serum LDH levels to tick counts. The high intensity and extended period of infestation in this experiment has to be noted.

2.1.1.2 Liver function: Alkaline Phosphatase, Gamma-Glutamyl Transferase, Uric acid and Bilirubin

Alkaline phosphatase (Alkp) is an enzyme which functions to hydrolyze organic esters such as glucose or glycerol in order to yield the inorganic phosphate intracellularly in the spleen, liver, kidneys and intestines. In the bone, Alkp functions extracellularly after production by osteoblasts, and most of the Alkp observed in the blood entered circulation from bone tissue (Stogdale, 1981). The level of Alkp in the serum will increase significantly in any disease or malfunction of the liver that inhibits the normal flow of bile. Examples include hepatocellular damage, swelling and obstruction of the hepatic bile canaliculi as well as or obstructive jaundice (Stogdale, 1981). Cattle subject to heavy levels of tick infestation have shown depressed levels of serum Alkp. Both pure British crosses as well as Africander X British crosses had significantly lower levels of Alkp activity after infestation with 40 000 *R. microplus* larvae. The decreased levels, along with lowered levels of albumin, could be associated with possible liver damage (O'Kelly & Kennedy, 1981).

Gamma Glutamyl Tranferase (GGT) (also known as Gamma Glutamyl Transpeptidase) is an enzyme found in the epithelial cells of the bile canaliculi within the liver and in the proximal convoluted tubule cells of the kidney. The profile of GGT in blood serum is associated with cholestasis (decrease in bile flow) or damage to the bile ducts in horses and cattle (Mullen, 1976). GGT increases are sensitive to obstructive liver diseases and are used to determine whether increases in alkaline phosphatase are due to liver or bone disease (Lum & Gambino, 1972). This is because GGT levels are not affected during bone tissue disorders as is Alkp. In cattle, GGT is considered an accurate estimate of the extent of liver damage caused by the hepatobiliary parasite *Fasciola Hepatica* (Simesen *et al.* , 1973).

Uric acid is derived from the degradation of the components of nucleic acids, purines and pyrimidines (Stogdale, 1981). Elevated levels of uric acid are thus considered to be an indication of liver disease or malfunction (Cornelius, 1963). The levels in blood serum are influenced by the amount of nucleic material ingested, the rate of cellular breakdown within the body and the livers ability to convert uric acid to allantoin through oxidation. Increases in the serum concentration of uric acid can be indicative of diffuse liver diseases and in hepatocellular jaundice (during liver cell necrosis), but not of obstructive or hemolytic jaundice (Stogdale, 1981).

Increased concentrations of bilirubin in the blood is known as bilirubinemia, a possible symptom of hemolytic icterus (also known as jaundice). Bilirubin is a product of the catabolism of hemoglobin. Excess levels could also be the consequence of liver disease or malfunction, known as hepatic icterus. (Cornelius, 1963). It has been stated, however, that even advanced levels of liver disease will only lead to marginal gains in serum bilirubin levels, and that more extensive increases in levels is more likely due to hemolysis of erythrocytes (Wellde *et al.*, 1974).

2.1.1.3 Kidney function: Blood Urea Nitrogen and Creatinine

The presence of these metabolic products in the blood can be used as a measure for determining possible renal malfunctions. Severe rises in blood urea nitrogen BUN occur during insufficient glomerular filtration rates, a consequence of decreased renal blood flow, a decrease in the number of functioning nephrons or due to a decrease in urine outflow (Finco & Duncan, 1976). It is important to note, however, that ruminants recycle circulating blood urea back into the rumen where it is degraded by the presiding microbial populations. A significant rise in BUN levels in ruminants is thus not observed until the kidney malfunction has progressed until a very severe stage. Decreases in BUN levels is associated with a very low level of protein intake, likely indicative of starvation, as well as during insufficient liver function (Stogdale, 1981). In a study evaluating selected serum biochemical levels in cattle infected with *Trypanosoma congolense,* elevated levels of BUN were observed during the same periods as decreased levels of serum protein. Consequently, the authors suggested that the increased levels of blood urea nitrogen might be due to the catabolism of serum protein (Wellde *et al.*, 1974). Creatinine is an end product of the process of muscle metabolism as it is derived from creatine found in muscle tissue. It is released into circulating blood and the amount depends only on the amount of muscle mass of the animal. As it is filtered but not reabsorbed or secreted by the tubule within the kidneys, a rise in blood creatinine is a marker for extensive kidney damage with an approximation of less than 25% of total function (Bentick-Smith, 1963; Stogdale, 1981).

2.8 Conclusion

It is clear from this review that the current approaches to tick control is not sustainable and alternative methods of control needs to be explored. Host resistance to ticks seems to be a promising option, but the mechanisms of resistance needs investigation. There is evidence that parasite-host relationships might be specific to breed and tick type and that the resistant phenotype displayed by some cattle are dependent on various factors. Local cutaneous responses play a key role in the manifestation of resistance, but the effect of ticks is not limited to cutaneous reactions. The RBCC and PCV are essential assessments in animals subjected to blood sucking parasites, while the total and differential WCC are important immunological indicators. There is a lack of knowledge surrounding the effects of salivary excretions on host metabolism, and a thorough biochemistry profile of proteins, lipids and enzymes could likely provide insights into the effects accompanying infestation. All of these parameters will necessarily play a key role in resistance per se, but their observed changes aid in emphasizing the extent of parasitic influence on the host and aid in the understanding of the wider scope of each parasite-host relationship. Further investigation into these relationships as well as the respective mechanisms of resistance is necessary.

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CHAPTER 3 - TICK COUNTS AND DIFFERENCES IN HAEMATOLOGY AND SERUM BIOCHEMISTRY FOLLOWING ARTIFICIAL INFESTATION

3.1 Introduction

It has been well documented that cattle breed type has a valuable influence on the animal's ability to resist infestation (Utecha *et al.*, 1978; Wambura *et al.*, 1998b). While the mechanisms underlying the animals' differential ability to resist infestation needs clarification, valuable insights can also be gained by the comparison of the dynamics of specific tick-host associations. *Rhipicephalus microplus* and *R. decoloratus* infestations are important current contributors to the parasitic threat to cattle production in South Africa (Horak *et al.,* 2009; Nyangiwe *et al.,* 2013). The volume of research that focuses on *R. decoloratus* is less compared to that devoted to *R. microplus.* The progressive displacement of *R. decoloratus* by the Asiatic intruder, *R. microplus*, also raises concerns (Tønnesen *et al.*, 2004; Nyangiwe *et al.*, 2013). Several considerations are taken into account surrounding the design of the infestation protocol. Firstly, it is an artificial representation of the circumstances that will likely determine modern parasite-host relationships in South-Africa. Secondly, it allows for side by side comparison of the susceptibility or resistance of divergent evolutionary relationships of host genotype and parasite. An understanding of the historical context of acquired resistance will be useful if selection on the trait is to be applied as a possible control method in present day production systems. A 12-hour post-infestation sampling time-point is considered early for the measurement of responses on a systemic level. It could, however, be insightful to obtain thorough hematological and biochemical profiles at this interval, as it is possibly a key stage in the manifestation of resistance. Furthermore, there is a lack of information available on parasite host interactions in the earliest stages of the larvae phase. Due to ethical and practical constraints, a 24 and 48-hour sampling points could not be additionally included to the protocol, despite the increased likelihood of differential responses on a systematic level.

3.2 Materials and Methods

3.2.1 Study site

Animals were housed at the feedlot of the Agricultural Research Council campus in Irene, Pretoria. The location of the feedlot is 25˚54'25.1''S 28˚12'51.8''E. Pretoria is located in the Gauteng Province of South Africa, about 1340m (4396 ft.) above sea level. According to the Köppen-Geiger classification system (Peel *et al.*, 2006), the city is classified as Cwa which relates to a humid subtropical climate. The annual average temperature is roughly 18° C with 700mm of rain. The area is characterized by long and wet summers with short and dry winters.

3.2.2 Cattle breeds

The study was conducted using three different cattle breeds. Twelve Nguni (*Bos Taurus Africanus*), 12 Brahman (*Bos indicus*) and 12 Angus cattle (*Bos Taurus*) were sourced from nearby areas. Within breeds, all animals have been sourced from the same region. The Nguni cattle were obtained from Loskop farm in Limpopo, while the Brahman and Angus groups were obtained from Mpumalanga and the Free State provinces, respectively. A recent survey confirmed the significant presence of *R. microplus* and *R. decoloratus* at or nearby these locations (Mapholi, 2015). The animals are uniformly considered as sensitized to *Rhipicephalus* ticks through natural exposure. The possibility exists that the breed groups might have been subjected to different levels of tick densities with a contributing effect to the animal's ability to respond to the same species. This is seen as a shortcoming in using non-naïve animals.

3.2.3 Sex and age

All the Brahman and Nguni cattle were uncastrated males between 16 and 18 months of age, whereas the Angus cattle comprised of six female and six (uncastrated) males, between 12 and 14 months old.

3.2.4 Tick larvae and intensity of infestation

The tick species used were *R. microplus* and *R. decoloratus.* Unfed larvae for both tick species were obtained from ClinVet Laboratories, Bloemfontein, South Africa. Females were incubated at 27 °C and 80 to 90 % relative humidity until hatching of the eggs. The larvae were kept unfed and allowed to mature for 8 weeks prior to infestation. The preparation of the larvae was done in a sterile environment and they were considered aseptic. This method minimizes the risks involved surrounding any tick-borne diseases. The intensity of infestation used was roughly 100 larvae per animal. This was the maximum intensity allowed within the ethical constraints imposed on this study protocol.

3.2.5 Animal management

For the duration of the trial, the animals were kept in a feedlot. This allowed for housing in individual pens that provided sufficient space for the animal to turn, lie down and stand up. Water was provided *ad libitum*. The animals were maintained on a high-quality complete ration (Herbivore All in One®, EPOL, South Africa) that was fed twice a day at 8am and 5pm. There were exceptions at two intervals (see biopsy sampling). The feedlots were cleaned with water daily to provide a sanitary environment. All animals were ear tagged

with codes for identification. Codes were based on breed (A, B or N), the type of tick to be applied (D or M) and the number ID within that treatment group (1 to 6). The first Angus infested with *R. decoloratus* was thus AD1. Within breeds, animals were randomly assigned to treatment groups, with the exception of the females in the Angus group, which were divided into each tick species treatment group accordingly so each group contains equally n=3 females. All the animals were subject to the administration of a short acting acaricide before arrival at the study site. Acaricides used were either Deltamethrin- or Amitraz-based. The animals were allowed a resting period of at least 14 days to acclimatize to the environment and to ensure no residual effects of the acaricide remained.

3.2.5.1 ***Timeline:***

Trial protocol proceeded as follows:

Day-0: Blood samples and skin biopsies were collected from all animals and then the animals were prepared for infestation by the adhesion of the calico bags.

Day-1: 6 Nguni, 6 Brahman and 6 Angus cattle were artificially infested with *R. microplus* larvae and 6 Nguni, 6 Brahman and 6 Angus cattle were infested with *R. decoloratus* larvae.

Day-2: Approximately 12-hours post infestation, blood samples were collected and a skin biopsy was collected from any identifiable tick attachment site.

Day-3 to Day-17: Animals were inspected twice daily: at 9am and 5:30pm. Any abnormalities were reported and any damage to the calico bags was mended.

Day-18: The calico bags were carefully removed from the animals and tick counts were performed on all animals. The animals were subsequently treated with an amitraz-based acaricide (Decatix, Cooper Veterinary Products (Pty) Ltd, South Africa) to kill all ticks.

3.2.6 Artificial Infestation

Infestation proceeded as follows:

1. Animals were restrained in a crush pen for processing.
2. A circular part of the upper back was shaved using an industrial cattle clipper. Shaved areas were adjacent to the cervico-thoracic humps of Brahman and Nguni cattle while maintaining a similar position on the Angus Breed.
3. A calico bag (See Figure 3.1) was attached to the clean-shaven area using a contact adhesive (Contact Adhesive, Alcolin®, South Africa) applied to the outer ring followed by a 24-hour drying period.
4. Roughly 100 larvae were placed into plastic vials (see Figure 3-4) and inserted into the calico bags (see Figure 3-1 and Figure 3-2)
5. The caps were opened to allow the ticks to exit and the bag was twisted shut and secured with a rubber castration ring (see Figure 3-3)

6. At the 12-hour sampling point, the plastic vials were removed and re-sealed by closing the caps.
7. The bags were carefully monitored for the duration of the trial.
8. On day 18, the animals were restrained to allow for careful removal of the bag.
9. The area of the skin where the outer ring was attached was disinfected with chlorfenvinphos 0.48% (Supona Aerosol Spray®, Zoetis, South Africa) to prevent infection and wound myasis from any possible skin trauma.
10. A short acting acaricide (Decatix, Cooper Veterinary Products (Pty) Ltd, South Africa) was applied to kill of all the ticks.

3.2.7 Blood Collection

Blood was collected from the tail vain by making a coccygeal venipuncture and draining blood into 9ml vacuum tubes (Vacuette®, Lasec, South Africa). The animal was restrained in a crush pen during blood sampling. The tail was lifted with the left hand and the groove lying in the ventral midline of the tail was located. A venipuncture was made midway along the coccygeal vertebrae by inserting an 18-gauge needle perpendicularly to the skin, approximately 150mm from the base of the tail. Blood was then drained from the site and pressure was applied immediately after the removal of the needle to stop the bleeding. Two blood samples were taken per animal for a single sampling point. One sample was collected into a 9ml Vacuum tube containing EDTA (Vacuette® K2EDTA, Lasec, South Africa), while the other was collected into a plain 9ml vacuum tube (Vacuette® Z Serum Clot Activator, Lasec, South Africa). The blood samples were inverted and stored at room temperature for 1 to 2 hours followed by refrigeration at 4°C until sampling was complete and all samples were collected for processing.

3.2.8 Biosecurity measures

All personnel on the experimental site were equipped with gumboots and overalls - which were washed and disinfected daily. All personnel that contributed to sampling procedures wore gloves which were discarded immediately after processing was completed. The housing pens were cleaned daily and disinfected with an acaricide (deltamethrin 0.05%) to prevent the spread and contamination of ticks. All materials and biological waste (Calico bags, syringes, needles etc.) were sealed in plastic and sent for incineration at the ARC furnace.

3.2.9 Blood Biochemistry

Blood to be used for biochemical analysis was collected into plain 9ml vacuum tubes (Vacuette® Z Serum Clot Activator, Lasec, South Africa). All blood biochemistry analysis was performed by IDEXX® Laboratories, Johannesburg, shortly after collection. Blood serum was analyzed for total serum protein, albumin, globulin, alanine transferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), uric acid, bilirubin, cholesterol, creatine kinase (CK), blood urea nitrogen (BUN), creatinine, lactate dehydrogenase (LDH) and fibrinogen.

All serum biochemistry analysis was performed using a VITROS® 350 Dry Slide Chemistry Analyzer (The Scientific Group®, South Africa). Analysis proceeded with the use of the parameter-specific VITROS® sides, the appropriate VITROS® products calibrator kit and the VITROS® 5600 Integrated System. The VITROS slide is an analytical element that consists of multiple layers. In general, a drop of the sample is deposited on the slide and evenly distributed to the underlying layer. The reaction of the biochemical component with its specific reagent is measured by reflectance spectrophotometry and is proportional to the concentration of the component in the sample. A short summary of the reactions and agents involved can be seen in addendum A.

3.2.10 **Haematology**

Blood for haematology was collected into 9ml Vacuum tubes containing EDTA (Vacuette® K2EDTA, Lasec, South Africa). All haematology analysis were performed by IDEXX® Laboratories, Johannesburg within 4 hours of collection of samples. Whole blood was analyzed for red blood cell count (RBCC), hemoglobin, hematocrit (packed cell volume), mean cell volume (MCV), red cell distribution width (RCDW), mean cell hemoglobin concentration (MCHC), white cell counts (WCC) and platelets. Differential analysis was also performed for segmented neutrophils (% and abs), band neutrophils (% and abs), lymphocytes (% and abs), monocytes (% and abs), eosinophils (% and abs), basophils (% and abs).

All haematological analysis were performed using a SYSMEX® XT2000i Automated Hematology Analyzer (SYSMEX®, South Africa). A brief description of the analysis procedures and the active ingredients of all reagents are shown in addendum A.

3.2.11 **Statistical analysis**

The data were analyzed using Statistical Analysis System (SAS) Enterprise guide software (Version 7.1, 2014; SAS Institute Inc, Cary, NC, USA). All tick count data were square root-transformed to confer normality. The linear models procedure was used to perform ANOCOVA (Type III) analysis of the effects of treatments breed and tick species on the respective biochemical, hematological parameters and ANOVA (type III) analysis of the square root transformed tick counts. Mean effects of treatments were determined using LSMEANS option and compared using Bonferroni t-tests. Correlations among variables were determined using the PROC CORR function. The pre-infestation values for each parameter were used as the covariate in all ANOCOVA models. The statistical model for all biochemical and haematological parameters can be summarized as follows:

$Y_{ijkl} = \mu + \beta X_i + A_j + B_k + A_jB_k + \varepsilon_{ijkl}$

Where, Yijkl = All biochemical and haematological parameters

βX_i = effect of covariate for the i'th animal

A_j = Effect of breed (j = Brahman, Nguni, Angus)

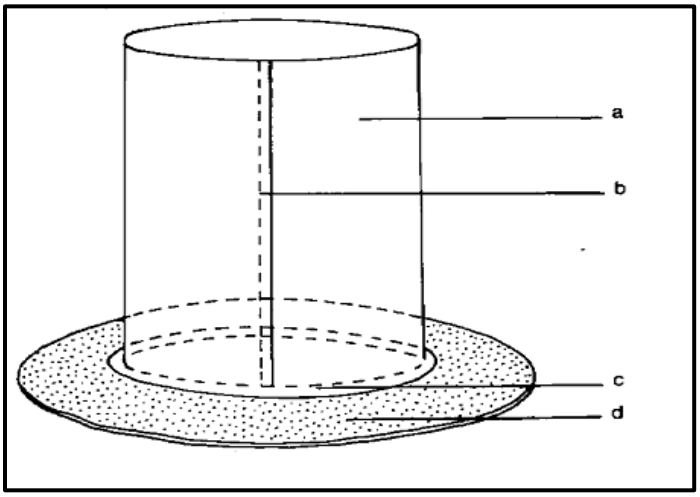
B_k = Effect of tick species (k = *R. microplus*, *R. decoloratus*)

B_jC_k = The effect of breed and tick species interaction (k= Brahman**R. microplus*, Brahman**R. decoloratus*, Nguni**R. microplus*, Nguni**R. decoloratus*, Angus**R microplus*, Angus**R. decoloratus*)

ε_{ijk} = residual error

3.2.12 **Ethical Clearance**

All procedures involved in the research protocol were submitted for review and approved by the University of Stellenbosch Research Ethics committee: Animal Care and Use (Protocol#SU-ACUD15-00084). All animal procedures performed were in compliance with internationally accepted standards for animal welfare (Austin et al., 2004). The study also obtained permission (Ref: SU-ACUDIS-00084) to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO 35 of 1984) from the Department of Agriculture, Forestry and Fisheries (DAFF).



a) 200mm x 280mm calico cloth stitched to form a tube
b) Stitching
c) Stitching on to a suede ring
d) 35mm diameter suede ring

Figure 3-1 Calico bag used in artificial infestation.

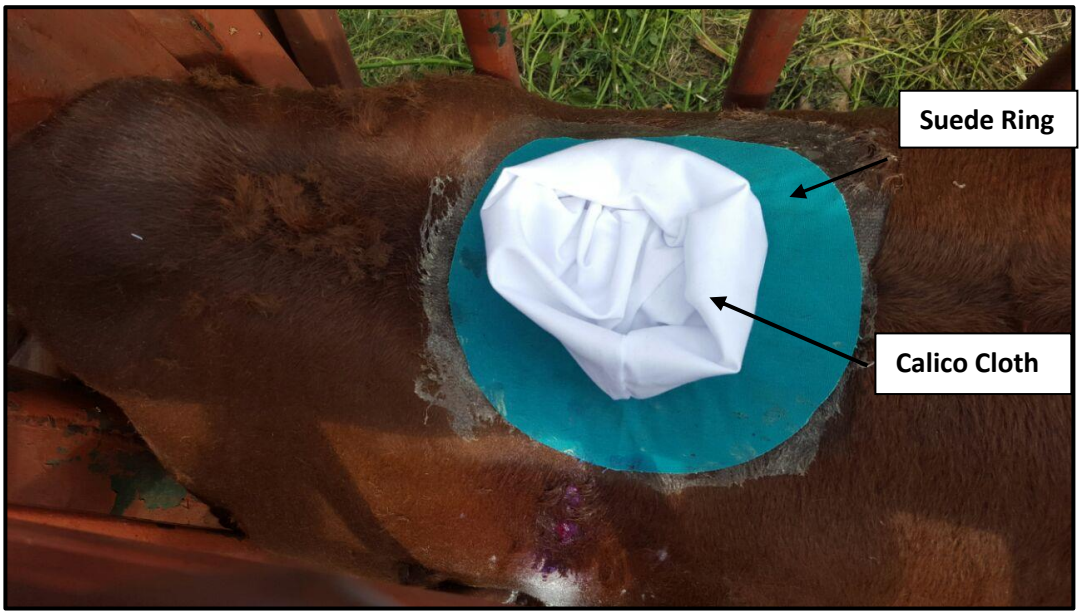


Figure 3-2 Calico bag attachment prior to infestation

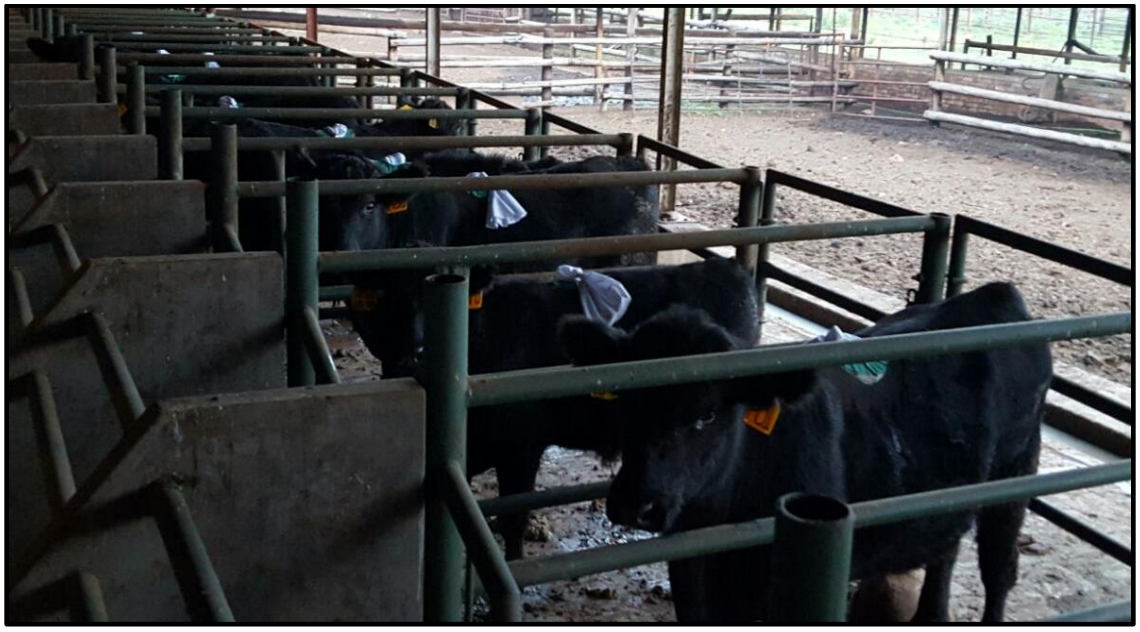


Figure 3-3 The Angus cattle in the housing pens. Here the elastic ring is attached, sealing off infestation area to the outside environment.

Figure 3-4 Plastic vials containing larvae prior to artificial infestation.

3.3 Results

3.3.1 Tick counts

Raw tick counts have been presented for illustrative purposes and only transformed data will be discussed. The Angus cattle displayed the highest square root-transformed tick counts on Day 18 at 5.99 (± 3.73), but did not differ significantly ($p<0.05$) from the Nguni cattle (5.67 ± 3.77). The Brahman cattle had a square root-transformed tick count of 3.60 (± 2.94) which was significantly ($P<0.01$) lower than both Nguni and Angus group. Table 3.1 presents the least square means as well as standard deviations for all groups.

Table 3.1 Tick counts (LS means and SD) recorded on day 18.

Parameter	Brahman	Nguni	Angus
Tick Counts (Raw)	20.95 ± 23.301^{b}	45.17 ± 41.64^{a}	48.75 ± 44.16^{a}
Tick Counts (SQRT)	3.60 ± 2.94^{b}	5.67 ± 3.77^{a}	5.99 ± 3.73^{a}

^{a,b} Means with different superscripts in the same row differ significantly ($p< 0.05$)

Cattle infested by *R. microplus* had higher ($p<0.01$) tick counts than the *R. decoloratus*-infested cattle. Transformed means of 8.27 (± 1.80) *R. microplus* ticks were counted on day 18 in comparison to 1.91 (± 1.22) *R. decoloratus* ticks. The transformed least square means for each tick species can be observed in Table 3.2.

Table 3.2 Tick counts (LS means and SD) recorded on Day 18

Parameter	*R. microplus*	*R. decoloratus*
Tick Counts (Raw)	71.52 ± 26.57[a]	5.06 ± 5.69[b]
Tick Counts (SQRT)	8.27 ± 1.80[a]	1.91 ± 1.22[b]

[a,b] Means with different superscripts in the same row differ significantly (p< 0.05)

The p-value for overall breed*tick species interactions was not significant (p>0.05). It is worth noting, however, that within individual treatment group comparisons the Brahman**R. microplus* group mean differed (p<0.05) from the Nguni**R. microplus* and Angus**R. microplus* groups. The Brahman**R. microplus* group displayed a transformed tick count of 6.20 (± 1.56), while the Nguni**R. microplus* and the Angus**R. microplus* groups had higher (p-value) count values of 9.13 (± 0.84) and 9.48 (± 0.66), respectively. On the other hand, there were no significant differences between breeds within the *R. decoloratus* groups, although the Brahman**R. decoloratus* group maintained the lowest tick count (1.00 (± 0.78)). Table 3.3 presents the least square means as well as standard deviations for the breed × tick species interactions.

Table 3.3 Tick counts (LS means and SD) recorded on Day 18.

	Brahman		**Nguni**		**Angus**	
Parameter	*R. microplus*	*R. decoloratus*	*R. microplus*	*R. decoloratus*	*R. microplus*	*R. decoloratus*
Tick Count (Raw)	40.4 ± 17.99[b]	1.5 ± 1.23[c]	84 ± 15.07[a]	6.33 ± 7.20[c]	90.17 ± 11.84[a]	7.33 ± 5.79[c]
Tick Count (SQRT)	6.20 ± 1.56[b]	1.00 ± 0.78[c]	9.13 ± 0.84[a]	2.21 ± 0.84[c]	9.48 ± 0.66[a]	2.52 ± 1.08[c]

[a,b] Means with different superscripts in the same row differ significantly (p< 0.05)

3.3.2 Haematology

3.3.2.1 *Haematology for individual (breed*tick species) treatment groups post infestation*

All breed*tick species interactions for all haematological parameters were not significant (p>0.05); The discussion could thus be focused on the main effects breed and tick species. For illustrative purposes, Table 3.4 shows the least square means as well as standard deviations for each breed*tick species treatment group. The Brahman**R. microplus* group (502.9 (± 133.86) x10^9/dl) displayed a higher (p<0.05) level of platelets than the Nguni**R. microplus* (268.85 (± 201.66) x10^9/dl) group.

Table 3.4 Haematology parameters (LS means and SD) of all individual (breed*tick) treatment groups measured at the second timepoint (post-infestation)

	Brahman		**Angus**		**Nguni**	
Parameter	***R. microplus***	***R. decoloratus***	***R. microplus***	***R. decoloratus***	***R. microplus***	***R. decoloratus***
Red blood cell count (x 10^{12}/l)	8.6 ± 1.22	8.24 ± 1.04	9.11 ± 2.63	9.24 ± 1.43	9.35 ± 0.95	8.62 ± 0.82
Hemoglobin (g/dl)	11.46 ± 1.04	10.97 ± 0.9	11.93 ± 2.51	12.18 ± 1.23	12.01 ± 0.86	11.15 ± 1.35
Packed cell volume (%)	34.77 ± 2.97	33.33 ± 2.82	36.25 ± 6.53	37.05 ± 3.07	37.14 ± 3.34	34.27 ± 4.33
Mean cell volume (fl)	40.96 ± 4.04	40.94 ± 2.25	41.28 ± 14.17	41 ± 5.62	41.56 ± 3.64	41.49 ± 3.08
Red cell distribution width (%)	29.89 ± 2.74	29.61 ± 1.57	29.75 ± 1.89	29.61 ± 4.35	28.75 ± 1.93	28.86 ± 1.46
Mean cell haemoglobin cons. (g/dl)	33.14 ± 2.02	32.9 ± 0.74	32.81 ± 2.12	32.84 ± 0.93	32.23 ± 0.74	32.49 ± 0.77
White cell count (x 10^{9}/dl)	15.41 ± 3.2	14.44 ± 2.13	15 ± 3.07	16.9 ± 3.94	17.46 ± 3.65	13.53 ± 5.63
Platelets (x 10^{9}/dl)	502.9 ± 133.86a	501.6 ± 224.47ab	431.13 ± 109.49ab	540.28 ± 165.49ab	268.85 ± 201.66b	354.33 ± 132.93ab

[a,b] Means with different superscripts in the same row differ significantly ($p < 0.05$)

3.3.2.2 *Haematology for breeds post infestation*

No significant differences were observed for red blood cell count, haemoglobin, packed cell volume, red cell distribution width, mean cell haemoglobin concentration and white cell count between breeds. The Angus cattle displayed the highest mean cell volume at 41.14 (± 4.83) fl, which did not significantly differ from that of the Nguni (41.53 (± 3.24) fl).The Brahman breed had a mean of 40.94 (± 3.00) fl which was lower ($p<0.05$) than the other breeds. The level of platelets displayed by the Nguni cattle (311.59 (± 162.25) x 10^9/dl) were lower ($p<0.05$) compared to both the Brahman and Angus breeds, which displayed 502.25 (± 179.11) x 10^9/dl and 485.70 (± 263.25) x 10^9/dl, respectively. Table 3.5 presents the least square means as well as standard deviations for haematology parameters observed on each group.

Table 3.5 Haematological parameters (LS means and SD) measured during the second sampling time point (post-infestation).

Parameter	Brahman	Nguni	Angus
Red blood cell count (x 10^{12}/l)	8.41 ± 1.14	8.89 ± 0.84	9.17 ± 2.00
Hemoglobin (g/dl)	11.21 ± 1.10	11.22 ± 1.11	12.06 ± 1.89
Packed cell volume (%)	34.05 ± 2.83	35.70 ± 3.86	36.65 ± 5.00
Mean cell volume (fl)	40.94 ± 3.00[b]	41.53 ± 3.24[a]	41.14 ± 4.83[a]
Red cell distribution width (%)	29.75 ± 2.01	28.80 ± 1.67	29.67 ± 3.63
Mean cell haemoglobin cons. (g/dl)	33.02 ± 1.63	32.36 ± 0.86	32.83 ± 1.42
White cell count (x 10^9/dl)	14.92 ± 2.47	15.49 ± 4.90	15.95 ± 3.49
Platelets (x 10^9/dl)	502.25 ± 179.11[a]	311.59 ± 162.25[b]	485.70 ± 263.25[a]

[a,b] Means with different superscripts in the same row differ significantly ($p<0.05$)

3.3.2.3 *Haematology for tick species post infestation.*

No significant ($p<0.05$) differences were observed between tick species *R. microplus* and *R. decoloratus* after blood measurements taken at the second sampling point (12h post infestation). Table 3.6 presents the least square means and standard deviation for all haematology parameters observed in each group.

Table 3.6 Haematological parameters (LS Means and SD) measured during the second sampling time point (Post infestation)

Parameter	*R. microplus*	*R. decoloratus*
Red blood cell count (x 10^{12}/l)	9.02 ± 2.37	8.70 ± 1.59
Hemoglobin (g/dl)	11.80 ± 2.04	11.43 ± 1.52
Packed cell volume (%)	36.05 ± 5.07	34.88 ± 4.83
Mean cell volume (fl)	41.14 ± 4.82	41.26 ± 5.06
Red cell distribution width (%)	29.46 ± 3.58	29.36 ± 3.90
Mean cell hemoglobin cons. (g/dl)	32.72 ± 2.01	32.74 ± 0.96
White cell count (x 10^{9}/dl)	15.96 ± 3.63	14.95 ± 4.20
Platelets (x 10^{9}/dl)	400.96 ± 170.82	465.40 ± 182.77

[a,b] Means with different superscripts in the same row differ significantly (p< 0.05)

3.3.2.4 *Differential leukocyte counts and percentages for individual (breed*tick species) treatment groups post infestation*

The breed*tick species interactions for all leukocyte parameters were not significant (p>0.05). Both the Angus**R. microplus* (61.73 (± 9.18) %) group as well as the Angus**R. decoloratus* (60.68 (± 7.01) %) group displayed a higher (p<0.05) lymphocyte % than the Nguni**R. microplus* (37.92 (± 17.59) %) group. For illustrative purposes, Table 3.7 shows the least square means as well as standard deviations for each breed*tick species treatment group

Table 3.7 Leukocyte parameters in percentages and absolute amounts (LS means and SD) of all individual (breed*tick) treatment groups measured at the second timepoint (post-infestation)

	Brahman		Angus		Nguni	
Leukocyte parameter	***R. microplus***	***R. decoloratus***	***R. microplus***	***R. decoloratus***	***R. microplus***	***R. decoloratus***
Segmented neutrophils %	39.72 ± 7.62	38.88 ± 7.85	24.96 ± 7.51	25.04 ± 6.23	38.28 ± 8.2	32.88 ± 9.57
Lymphocytes %	53.29 ± 7.07^{ab}	54.62 ± 9.71^{ab}	61.73 ± 9.18^{a}	60.68 ± 7.01^{a}	37.92 ± 17.59^{b}	51.68 ± 11.87^{ab}
Monocytes %	7.83 ± 2.16	7.66 ± 2	12.25 ± 5.32	10.27 ± 3.78	8.96 ± 3.13	13 ± 4.52
Eosinophils %	0.9 ± 1.41	0.54 ± 0.55	1.65 ± 1.73	1.8 ± 1.03	2.82 ± 2.17	2.53 ± 1.52
Segmented neutrophils (abs x 10^9/l)	5.2 ± 1.67	5.5 ± 1.17	3.36 ± 1.03	3.94 ± 1.11	7.38 ± 2.92	5.35 ± 3.42
Lymphocytes (abs x 10^9/l)	7.45 ± 1.58	7.89 ± 2.25	9.29 ± 2.6	10.1 ± 2.92	6.81 ± 2.91	6.46 ± 2.24
Monocytes (abs x 10^9/l)	1.41 ± 0.31	1.24 ± 0.27	1.76 ± 0.79	1.71 ± 0.79	1.59 ± 0.89	1.85 ± 1.13
Eosinophils (abs x 10^9/l)	0.13 ± 0.25	0.1 ± 0.08	0.3 ± 0.36	0.31 ± 0.17	0.46 ± 0.33	0.39 ± 0.21

^{a,b} Means with different superscripts in the same row differ significantly ($p < 0.05$)

3.3.2.5 *Differential leukocyte counts and percentages for breeds post infestation.*

All values for band neutrophils and basophils were recorded as zero; hence these parameters were removed from the statistical analyses. No significant differences ($p>0.05$) were found among breeds for monocytes (% and absolute). The Nguni and Brahman cattle had similar neutrophil counts of 35.58 (± 8.93) % and 39.30 (± 7.34) % respectively. These values were higher ($p<0.01$) than those observed in the Angus group (25.00 (± 6.40) %). A lymphocytes percentage of 61.20 (± 7.49) was observed on the Angus, which differed ($p<0.01$) from the 44.80 (± 15.83) displayed by the Nguni group. On the Brahman group, a mean 53.96 (± 8.12) % was recorded, which did not differ ($p>0.05$) from either the Angus or Nguni animals. The highest percentage of eosinophils of 2.67 (± 1.78) was observed on the Nguni, which was different ($p<0.05$) from that of the Brahman (0.72 (± 1.00) %). The mean eosinophil recorded on the Angus cattle was 1.72 (± 1.26) % that did not differ ($p>0.05$) from either the Nguni or Brahman group. Assessing the levels in absolute amounts produces different relationships than those observed in percentages in some of the parameters. The neutrophil counts of 6.37 (±3.21) x 10^9/l in the Nguni cattle differed ($p<0.05$) from the 3.65 (± 1.06) x 10^9/l observed in the Angus group. The 5.35 (± 1.32) x 10^9/l observed in the Brahman cattle did not differ ($p>0.05$) from either breed group. Lymphocytes were highest in the Angus cattle count (9.69 (± 2.66) x 10^9/l) and differed ($p<0.05$) from the 6.64 (± 2.42 x 10^9/l) displayed by the Nguni group. The 7.67 (± 1.87) x10^9/l observed in the Brahmans did not significantly differ from either breed group. The Nguni cattle displayed the highest absolute level of eosinophils at 0.47 (± 0.26), which was higher ($p<0.05$) than the 0.11 (± 0.17) observed in the Brahman group. There was a mean of 0.30 (± 0.24) displayed by the Angus cattle, which did not differ ($p<0.05$) from either group. Table 3.8 shows the least square means as well as standard deviations for leucocyte percentages and absolute amounts observed in each breed.

Table 3.8 Differential leukocyte in percentages and absolute amounts (LS means and SD) measured at the second sampling time point (Post infestation)

Leukocyte	Brahman	Nguni	Angus
Segmented neutrophils %	39.30 ± 7.34[a]	35.58 ± 8.93[a]	25.00 ± 6.40[b]
Lymphocytes %	53.96 ± 8.12[ab]	44.80 ± 15.83[b]	61.20 ± 7.49[a]
Monocytes %	7.74 ± 2.01	10.98 ± 4.00	11.25 ± 4.17
Eosinophils %	0.72 ± 1.00[b]	2.67 ± 1.78[a]	1.72 ± 1.26[ab]
Segmented neutrophils (abs x 10^9/l)	5.35 ± 1.32[ab]	6.37 ± 3.21[a]	3.65 ± 1.06[b]
Lymphocytes (abs x 10^9/l)	7.67 ± 1.87[ab]	6.64 ± 2.42[b]	9.69 ± 2.66[a]
Monocytes (abs x 10^9/l)	1.32 ± 0.28	1.72 ± 0.98	1.73 ± 0.75
Eosinophils (abs x 10^9/l)	0.11 ± 0.17[b]	0.43 ± 0.26[a]	0.30 ± 0.24[ab]

[a,b] Means with different superscripts in the same row differ significantly (P< 0.05)

3.3.2.6 *Differential leukocyte values for tick species post infestation.*

All values for band neutrophils and basophils were recorded as zero; thus, both parameters were removed from statistical analyses. No significant differences (p>0.05) were observed between tick species for any of the parameters, including assessment in percentages, as well as in absolute amounts. Table 3.9 shows the least square means as well as standard deviations for leucocyte percentages and absolute amounts observed in each tick species.

Table 3.9 Differential leukocyte values in percentages and absolute amounts (LS means and SD) measured during the second sampling time point (post infestation).

Parameter	*R. microplus*	*R. decoloratus*
Segmented neutrophils %	34.32 ± 10.65	32.26 ± 10.16
Lymphocytes %	50.97 ± 16.49	55.66 ± 10.11
Monocytes %	9.68 ± 4.64	10.31 ± 5.01
Eosinophils %	1.79 ± 1.96	1.62 ± 1.37
Segmented neutrophils (abs x 10^9/l)	5.31 ± 2.58	4.93 ± 2.21
Lymphocytes (abs x 10^9/l)	7.85 ± 2.69	8.15 ± 2.87
Monocytes (abs x 10^9/l)	1.59 ± 0.87	1.60 ± 1.03
Eosinophils (abs x 10^9/l)	0.30 ± 0.34	0.26 ± 0.21

[a,b] Means with different superscripts in the same row differ significantly (P<0.05)

3.3.2.7 *Correlations*

There were no significant (p>0.05) correlations between any of the haematology or differential leukocyte parameters overall or within any of the breeds.

3.4 Serum Biochemistry

All breed*tick species interactions for serum biochemistry parameters were not significant (p>0.05) and therefore the discussion is focused on the main effects of breed and tick species. For illustrative purposes, Table 3.10 presents the least square means and standard deviations of all individual breed*tick species treatment groups. The Nguni**R. decoloratus* group displayed lower (p<0.05) albumin levels (25.84 (± 1.60) g/l) than the Brahman**R. microplus* (28.96 (± 2.88) g/l) as well as the Brahman**R. decoloratus* (28.74 (± 2.07) g/l) groups. The Nguni**R. decoloratus* group also displayed a cholesterol level of 2.48 (± 0.15) which is significantly lower than the 3.31 (± 0.98) mmol/l observed in the Brahman**R. microplus* group. The Angus**R. decoloratus* group displayed a blood urea nitrogen level of 5.39 (± 1.20) mmol/l which is higher (p<0.05) than the 3.80 ± 0.60 mmol/l and 3.67 ± 0.35 mmol/l observed in the Brahman**R. microplus* group and the Brahman**R. decoloratus* group, respectively. The Angus**R. microplus* group also displayed a fibrinogen level of 3.03 (± 2.28) g/l which is higher (p<0.05) than observed levels in the Brahman**R. microplus* (1.81 (± 1.34) g/l) as well as Brahman**R. decoloratus* (1.72 (± 1.21) g/l) groups.

Table 3.10 Serum biochemistry parameters (LS means and SD) measured during the second sampling time point (12 h post infestation) for individual treatment groups.

	Brahman		Angus		Nguni	
Parameter	***R. microplus***	***R. decoloratus***	***R. microplus***	***R. decoloratus***	***R. microplus***	***R. decoloratus***
Albumin (g/l)	28.96 ± 2.88[a]	28.74 ± 2.07[a]	27.60 ± 1.41[ab]	26.75 ± 1.10[ab]	26.26 ± 1.67[ab]	25.84 ± 1.60[b]
Globulin (g/l)	44.48 ± 2.05	42.93 ± 2.68	42.44 ± 2.11	41.61 ± 2.27	43.17 ± 2.71	41.41 ± 3.03
Alanine Transferase (U/l)	58.46 ± 12.38	60.95 ± 9.02	42.64 ± 6.05	43.52 ± 6.25	49.48 ± 8.33	53.36 ± 6.31
Alkaline Phosphatase (U/l)	191.32 ± 98.26	202.25 ± 57.69	168.99 ± 29.61	174.94 ± 18.07	152.69 ± 43.24	178.85 ± 72.62
Gamma Glutamyl-Transferase (U/l)	52.69 ± 46.41	41.29 ± 10.44	52.89 ± 18.06	40.48 ± 9.73	39.38 ± 19.67	37.72 ± 10.29
Uric Acid (µmol/l)	69.79 ± 30.52	65.51 ± 14.34	60.99 ± 4.68	65.86 ± 9.12	63.34 ± 12.35	55.47 ± 18.25
Bilirubin Total (µmol/l)	19.90 ± 4.66	19.89 ± 3.22	17.93 ± 6.51	19.20 ± 6.99	17.97 ± 6.92	16.62 ± 3.33
Cholesterol (mmol/l)	3.31 ± 0.98[a]	3.10 ± 0.44[ab]	3.00 ± 0.16[ab]	2.81 ± 0.57[ab]	2.72 ± 0.56[ab]	2.48 ± 0.15[b]
Creatine Kinase (U/l)	2381.26 ± 2144.08	1344.18 ± 858.85	167.02 ± 66.13	723.03 ± 1574.85	959.10 ± 1704.7	498.63 ± 180.23
Bun Urea (mmol/l)	3.80 ± 0.60b	3.67 ± 0.35b	5.02 ± 1.04ab	5.39 ± 1.20a	4.74 ± 0.60ab	4.15 ± 0.90ab
Creatinine (µmol/l)	91.52 ± 25.71	100.91 ± 15.37	98.52 ± 15.75	103.42 ± 15.12	105.58 ± 7.18	105.47 ± 7.39
Lactate Dehydrogenase (U/l)	6800.82 ± 2740.92	5672.64 ± 1175.96	5708.80 ± 1105.58	5650.56 ± 654.60	5485.12 ± 1295.28	5185.22 ± 511.43
Fibrinogen (g/l)	1.81 ± 1.34[b]	1.72 ± 1.21[b]	6.25 ± 2.56[a]	3.03 ± 2.28[ab]	5.06 ± 2.16[ab]	4.80 ± 1.44[ab]

[a,b] Means with different superscripts in the same row differ significantly (P< 0.05)

3.4.1.1 *Serum biochemistry parameters between breeds post infestation.*

No significant differences (P>0.05) between breeds were observed for the serum levels of globulin, alkaline phosphatase, gamma-glutamyl transferase, uric acid, bilirubin, creatine kinase, creatinine or lactate dehydrogenase. The Brahman cattle displayed serum albumin levels of 28.85 (± 2.34) g/l, which differed ($p<0.05$) from the 27.18 (± 1.21) g/l and the 26.05 (± 1.68) g/l displayed by the Angus and Nguni cattle, respectively. Alanine transferase was highest in the Brahman at 59.70 (± 10.20) U/l, which was different ($p<0.05$) from the 43.08 (± 5.89) U/l observed on the Angus group. The 51.42 (± 7.37) U/l observed in the Nguni did not differ significantly from either group. The Brahman animals had a mean cholesterol level of 3.21 (± 0.78) mmol/l that was higher ($p<0.05$) than the 2.59 (± 0.41) mmol/l recorded on the Ngunis, whereas the 2.90 (± 0.40) mmol/l recorded on the Angus group did not differ ($p<0.05$) from either group. Blood urea nitrogen of 5.21 (± 1.09) mmol/l was the highest for the Angus cattle, and this differed ($p<0.05$) from the 3.74 (± 0.74) mmol/l displayed by the Brahman cattle. The 4.45 (± 0.45) mmol/l observed in the Nguni cattle did not differ ($p>0.05$) from either group. The Angus and Nguni groups had fibrinogen levels of 4.64 (± 2.63) g/l and 4.94 (± 1.71) g/l, respectively. Both values were higher (p-value) than the 1.77 (± 1.19) g/l observed in the Brahman group. Table 3.11 presents the least square means as well as standard deviations for serum biochemistry parameters measured during the second sampling time point for all breeds.

Table 3.11 Serum biochemistry parameters (LS means and SD) measured during the second sampling time point (12 h post infestation).

Parameter	Brahman	Nguni	Angus
Albumin (g/l)	28.85 ± 2.34[a]	26.05 ± 1.68[b]	27.18 ± 1.21[b]
Globulin (g/l)	43.70 ± 2.38	42.29 ± 2.77	42.02 ± 2.09
Alanine Transferase (U/l)	59.70 ± 10.20[a]	51.42 ± 7.37[ab]	43.08 ± 5.89[b]
Alkaline Phosphatase (U/l)	196.78 ± 75.60	165.78 ± 61.09	171.97 ± 26.01
Gamma Glutamyl-Transferase (U/l)	46.99 ± 31.39	38.55 ± 15.11	46.68 ± 14.05
Uric Acid (µmol/l)	67.35 ± 22.00	59.41 ± 14.86	63.42 ± 7.59
Bilirubin Total (µmol/l)	18.38 ± 4.08	17.29 ± 5.20	18.56 ± 6.46
Cholesterol (mmol/l)	3.21 ± 0.78[a]	2.59 ± 0.41[b]	2.90 ± 0.40[ab]
Creatine Kinase (U/l)	1862.72 ± 1560.73	728.86 ± 1179.82	445.02 ± 1107.81
Bun Urea (mmol/l)	3.74 ± 0.45[b]	4.45 ± 0.74[ab]	5.21 ± 1.09[a]
Creatinine (µmol/l)	96.21 ± 20.95	105.53 ± 7.7	100.97 ± 15.16
Lactate Dehydrogenase (U/l)	6236.73 ± 2189.01	5335.162 ± 944.18	5679.68 ± 896.13
Fibrinogen (g/l)	1.77 ± 1.19[b]	4.94 ± 1.71[a]	4.64 ± 2.63[a]

[a,b] Means with different superscripts in the same row differ significantly (P< 0.05)

3.4.1.2 *Serum biochemistry parameters between tick species post infestation.*

The least square means for serum biochemistry parameters measured during the second sampling time point for the tick species are presented in Table 3.8. No significant (p>0.05) differences for any serum biochemistry parameters could be found between tick species, except for serum levels of globulin at the second sampling point (12h post infestation). *R. microplus*-infested cattle produced a mean 43.37 (± 2.23) g/l level of serum globulin, which was higher (p<0.05) than the 41.98 (± 2.54) observed in the *R. decoloratus*-infested cattle.

Table 3.12 Serum biochemistry parameters measured during the second sampling time point (Post infestation).

Parameter	*R. microplus*	*R. decoloratus*
Albumin (g/l)	27.61 ± 3.63	27.11 ± 2.91
Globulin (g/l)	43.37 ± 2.23[a]	41.98 ± 2.54[b]
Alanine Transferase (U/l)	50.19 ± 11.31	41.98 ± 10.82
Alkaline Phosphatase (U/l)	171.00 ± 87.04	185.35 ± 86.00
Gamma Glutamyl-Transferase (U/l)	48.32 ± 30.39	39.83 ± 12.27
Uric Acid (µmol/l)	64.50 ± 19.35	62.28 ± 14.55
Bilirubin Total (µmol/l)	18.60 ± 5.91	17.56 ± 4.67
Cholesterol (mmol/l)	3.01 ± 0.97	2.80 ± 1.24
Creatine Kinase (U/l)	1169.13 ± 1667.34	855.28 ± 1035.11
Bun Urea (mmol/l)	4.52 ± 0.92	4.41 ± 1.17
Creatinine (µmol/l)	98.54 ± 25.00	103.27 ± 16.31
Lactate Dehydrogenase (U/l)	5998.24 ± 2059.48	5502.81 ± 900.87
Fibrinogen (g/l)	4.38 ± 2.69	3.19 ± 2.06

[a,b] Means with different superscripts in the same row differ significantly (P< 0.05)

3.4.1.3 *Correlations*

All blood biochemistry parameters were tested for possible correlation to transformed tick counts both over all animals and within breeds. Considering the post-infestation data, both ALT (r=-0.36; p<0.05) and ALP (r=0.36; p<0.05) were negatively correlated to the square root-transformed tick counts across all animals. Fibrinogen levels displayed a positive significant correlation (r=0.39, 0.03) to transformed tick counts. Post infestation cholesterol levels could not be significantly correlated to count data, but showed a tendency (r=-0.32; p<0.1) towards a negative relationship. Correlations are presented in Table 3.13.

Table 3.13 Correlation values between biochemistry parameters and tick counts measured post infestation. The correlation coefficient (r) and the p-value for significant correlation is given for each parameter across all breeds (overall) as well is within breeds.

Parameter		Overall	Brahman	Nguni	Angus
Albumin	r-value	-0.15	-0.10	-0.25	0.02
Globulin	r-value	-0.09	-0.31	-0.12	-0.01
Alanine tranferase	r-value	**-0.36***	-0.37	-0.10	-0.19
Alkaline phosphatase	r-value	**-0.36***	-0.04	-0.36	-0.43
Gamma glutamyl-transferase	r-value	0.06	0.34	0.16	0.09
Uric acid	r-value	-0.10	0.17	-0.12	-0.33
Bilirubin total	r-value	0.06	0.11	0.22	-0.07
Cholesterol	r-value	-0.32	-0.48	0.51	-0.03
Creatine kinase	r-value	-0.14	0.07	0.17	-0.30
Urea	r-value	0.14	-0.08	0.18	-0.21
Creatinine	r-value	-0.05	0.23	-0.21	0.29
Lactate dehydrogenase	r-value	0.09	0.48	-0.05	0.29
Fibrinogen	r-value	**0.39***	0.10	0.24	0.44

Correlation coefficients (r-values) with asterisk (*) superscript are statistically significant ($p<0.05$).

3.5 Discussion

3.5.1 Tick Counts

When assessing breed effects individually, the Brahman cattle showed strong evidence for a higher ($p<0.01$) level of resistance in comparison to both the Nguni and Angus breeds. These results are consistent with previous results showing that Brahman cattle display a superior level of resistance to *R. microplus* ticks in comparison to its European counterparts (Seifert, 1971; Utecha *et al.*, 1978; Piper *et al.*, 2009). The means produced by the individual treatment groups show that there was a larger difference between the Nguni and Brahmans in the *R. microplus* infested group than the same two breeds in the *R. decoloratus* infested group, suggesting that the Brahman cattle might have an increased advantage regarding the *R. microplus* ticks. Although their superior resistance over the European breeds is evident, there are few studies that compare the indicine Brahman and African *taurine* Nguni regarding their susceptibility to the *Rhipicephalus* ticks. Rechav & Kostrzewski, 1991 compared the two breeds after exposure to the African *R. decoloratus* tick and found them to be similar. The superiority of the Nguni over the non-Brahman purebred and crossbred cattle in the study led to the suggestion that the level of resistance to *Rhipicephalus* ticks could be correlated to the proportion of Sanga or Zebu genes within a breed. Brahman X British and Africander x British crosses have also been compared in terms of resistance to *R. microplus* in a study by Seifert, 1971. The *indicine* and African *taurine* crosses did not produce significant differences, although the *indicine* Brahman did consistently produce the lowest level of tick counts. The results in the current study, however, displayed clear differences between the Brahman and Nguni cattle, especially regarding *R. microplus* infestation. As Brahman cattle and *R. microplus* have the same historical origin, the result supports the hypothesis that levels of resistance are acquired through continuous co-evolution of the Asiatic tick species and cattle breed. Similarly, the hypothesis would have expected the Nguni to display a superior level of resistance to *R. decoloratus*. The Nguni breed, however, did not display higher resistance to *R. decoloratus* than the *taurine and* disagrees with previous studies that have provided strong evidence of the breed's superior level of resistance (Rechav & Kostrzewski, 1991; Marufu *et al.*, 2011a). Various possibilities should be considered when assessing the Nguni breed's resistance phenotype regarding *R. decoloratus*. First of all, the trial protocol is possibly lacking in its reliability in the assessment of resistance. It has been shown that the reliability of tick counts increase along with the intensity of artificial infestations (Wharton *et al.*, 1970) and the intensity of the current trial is very low. Furthermore, the reliability of assessment would likely benefit from the use of an average of replications, while the protocol in this study allows for only a single observation and an average is thus not possible. It is also possible that the Nguni breed did not express its optimal resistant phenotype, as despite the animals being fed a high-quality feedlot diet leading up to the trial, the Nguni animals were noted as physiologically poor based on visual inspection. The visual observations are supported by the Nguni breed's low cholesterol levels. It is unknown if it is related to a poor genotype or a lack of optimal nutrition during the animal's growth into adulthood. The condition of an animal is well known to have an effect on the animal's ability to display its optimal level of resistance (Riek, 1957; O'Kelly & Seifert, 1969). Furthermore, for the Nguni to display superior levels would

depend on high levels of infestation by *R. decoloratus* on at least one of the remaining breeds, and the overall potency of the tick species was below the levels expected.

There were clear differences in the success rate between the tick species, *R. decoloratus* and *R. microplus*. The results show *R. microplus* to possess a much higher success rate across all breeds. The tick species higher success rate might be due to various factors, including more effective mechanisms of host evasion. It is possible that *R. decoloratus* is not as well suited for use in artificial infestations or requires a much higher intensity to display a more accurate success rate. It is also possible that the vigour of the *R. decoloratus* larvae that were used in this particular study was less than that of the *R. microplus* larvae. Nonetheless, the observations reported here contribute to the concerns regarding the displacement of *R. decoloratus* by *R. microplus* as discussed by Nyangiwe *et al.* (2013). If the *R. microplus* ticks have a higher survival rate after attachment to the host, it will very likely have advantageous consequences for its reproduction. A certain degree of cross resistance is expected regarding tick species that belong to the same genus (McTier *et al.*, 1981). Thus, while differences could be expected to a certain extent, the differences observed in this study are quite pronounced. The results reported by Nyangiwe *et al.*, (2013), also showed considerable differences when directly comparing the two species, but *R. microplus* was not the most prevalent tick species at all locations. The superior success rates from the *R. microplus* ticks indicates the tick species's parasitic potency beyond the concerns and differences commented on by Horak *et al.*, (2009). They attributed the displacement to life cycle differences and mating advantages of the *R. microplus* ticks. As this protocol allowed for separate infestations, many of these factors are not valid to explain the observed differences in success rates. Results from artificial infestations can contribute to the knowledge of cattle exposed to natural infestation, but the distribution of the two-tick species in natural South African pastures is under the influence of many factors. A thorough investigation of the mechanisms and effects of the displacement of the indigenous *R. decoloratus* by the intruding *R. microplus* would be beneficial, but is beyond the scope of this study.

There is consensus that the domestication of cattle began about 10,000 years ago (Chen *et al.*, 2010), but the date and manner of the introduction of taurine and *indicine* breeds into Africa remains area subject of debate (Hanotte *et al.*, 2002). Although artificial selection does interfere with or entirely displaces natural selection, modern systematic breeding in Africa has only been practiced within the last century (O'Neill *et al.*, 2010). Cattle would thus still have been subjected to considerable selection pressure by the environment, including tick infestation. Considering the well-known detrimental effect accompanying infestation, it is not surprising that *R. microplus* would apply strong and continuous selection pressure upon the Brahman breed, allowing for the evolution of an increasingly resistant genotype. Using mitochondrial DNA (mtDNA) surveys, Chen *et al.*, (2010) was able to confirm the *Indicine* cattle's origins from the northern part of the Indian subcontinent. As the South African *R. microplus* strain also finds its origins in India (Labruna *et al.*, 2009), it is very likely that the parasite-host relationship would have been allowed many generations to develop and the Brahman's superior resistance is not surprising with regard to *R. microplus*.

Despite the concerns surrounding the infestation of *R. decoloratus*, it is still evident that Brahman cattle displayed a high level of resistance toward this tick species. In addition, a level of cross resistance can be expected to ticks within the same genus, as suggested by McTier *et al.*, (1981), and should be taken into consideration. As *R. decoloratus* and *R. microplus* have a similar phylogeny (Barker & Murrell, 2004), the mechanisms of natural selection that these tick species suppress upon their hosts are possibly similar. This might help to explain why the Brahman, with its indicine origins, seems well adapted to *R. decoloratus* exposure as well.

3.5.2 **Haematology**

Before the interpretation of haematological parameters, notably red blood cell counts (RBCC), it should be taken into consideration that the parameters are under the influence of various factors. It is well known that erythropoiesis can be influenced by catecholamine concentrations (Penn *et al.*, 2010). All animals likely experienced a moderate level of stress during handing. Due to the Brahman breeds' larger size and more aggressive temperament, these animals might have experienced higher levels and stress. Furthermore, estrogen is known to suppress erythropoiesis in multiple ways (Schalm *et al.*, 1975) and half of the Angus cattle were female, while the Brahman group was entirely male. This might further contribute to the different RBCC observations. After taking these factors into consideration, the similar counts (RBCC) displayed by the Brahman cattle disagrees with observations that tick resistant cattle show higher levels of RBCCs than their susceptible counterparts (Evans & Turner, 1965; Piper *et al.*, 2009b). Riek, (1957) also reported significant decreases in RBBC's on artificially infested cattle and similar results could have been expected for the susceptible groups in this study. It is essential, however, to take into account the extremely high intensities of infestation (approx. 100,000 larvae) and RBCC decreases in the study were attributed to blood loss rather than any toxic effect from the parasites. As normal values for bovine animals range between 5 to 10 x 10^{12}/l (Schalm, 1961), the post-infestation values of the groups can be considered as moderately high and there is no indication of anemia as reported in Piper *et al.*, (2009). As the post-infestation sampling time point is very early, it is not surprising that there is no evidence of blood loss to the parasites, as fluid acquisition/loss will be very little at this interval. Similar comparisons were observed for the packed cell volume (PCV) and haemoglobin levels. A noticeable relationship between PCV, RBCC and hemoglobin levels is expected considering the strong biological relationship between these parameters.

The results observed in the current study also disagree with reports by Evans & Turner, 1965 that Africander cattle display PCV and hemoglobin values that are intermediate between Brahman and British cattle under normal circumstances. Similarly, Marufu *et al.*, (2009) reported higher PCV values for Nguni cattle compared to non-descript alternatives. It is important to note that the group means have been adjusted to correct for the pre-infestation co-variate during ANOCOVA analysis. The mean differences at the post infestation time point are strongly influenced by responses at the timepoints between infestations and values during normal circumstances would not be comparable.

Regarding the effects of infestation, depressed levels in packed cell volume and hemoglobin have been associated with intense artificial infestations (O'Kelly & Seifert, 1970), which can also be attributed to blood loss. Contrary to our findings, O'Kelly & Seifert, (1969) observed increases in PCV and haemoglobin following light infestations. It is also important to note that they observed these measurements on British Hereford x Shorthorn crosses at days 34 and 62 during prolonged infestation periods. It is likely that the susceptible Angus group in the current trial could have displayed similar tendencies following extended periods of infestation.

The mean MCV values observed in the Nguni and Angus cattle fell in the lower end of the 40 to 60 fl range considered normal for cattle (Schalm *et al.*, 1975), while the Brahmans displayed a mean 40.94 fl, which was significantly lower than the levels observed in the other breeds. The observation in this study agrees with that of Piper *et al.*, (2009), where MCV values for Brahman cattle were lower compared to British breeds. Low MCV is often defined as *microcytic hypochromic* anemia and is often associated with an iron deficiency (Schalm *et al.*, 1975), but the explanation is likely invalid as the groups displayed similar levels of mean corpuscular hemoglobin concentration of which the Brahman cattle had the highest. The Brahman cattle were also a season older than the Angus group and MCV is known to increase with age (Mirzadeh *et al.*, 2010). Blood parameters are known to differ between breeds (Claxton & Ortiz, 1996); however, Evans & Turner, 1965 similarly reported lower MCV levels in Brahmans compared with British and Africander breeds.

The lower levels of platelets in the Nguni breed is unlikely directly related to the animals' response to infestation. Platelets are essential in blood clot formation in response to tissue trauma (Young *et al.*, 2006) and could thus have been expected as a primary defense against fluid loss to blood sucking parasites, but strong evidence exists for the ability of ticks to inhibit platelet aggregation, as explained by Francischetti *et al.*, (2009). The levels of the Nguni could likely be related to the groups physiological disadvantages, as previously discussed, during a period of stress.

It is important to clarify the interpretation of changes of leukocyte levels following infestation. According to Young *et al.*, (2006), the levels observed within circulation is directly proportional to its demand in tissues. According to Schalm *et al.*, (1975), however, the neutrophilic response is initially masked as both the currently circulating as well as the newly formed neutrophils are attracted to the formation of an acute inflammatory lesion. Only when the disease transitions toward the chronic state after 24 to 48 hours does neutrophilia set in. With a sampling time point as early as 12 hours, a lower level of circulating leukocyte values would most likely be regarded as reflecting an increased demand in tissue.

In the assessment of circulating leukocyte values, it should be noted that the sampling process might have certain levels of influence on results. The release of corticosterone occurs very soon after the onset of acute stress (Romero & Reed, 2005), which will likely be caused by animal handling and perhaps at higher levels for the Brahman group. Stress is well known to have an influence on white cell counts in animals

through the release of corticosterone, causing increases in neutrophil levels, while lymphocytes decrease (Dhabhar *et al.*, 1995).

Although no differences could be observed for total white cell counts (WBC) between the breeds, all mean WBC values displayed would be classified as leukocytosis (>11 x 10^9/L) (Schalm *et al.*, 1975). The absence of differences among breeds in WBC count disagrees with previous reports (Piper *et al.*, 2009; Rechav *et al.*, 1990); however, periods and intensity of infestation should be considered. The differences in WBC counts in the aforementioned studies were suggested to be a result of continuous stress and inflammation due to intense infestations. Obtaining a similar result within this study thus seems very unlikely due to protocol differences surrounding infestation. Differential leukocyte counts might provide a more insightful indication of possible inflammatory states within the animal than total WBC levels (Schalm *et al.*, 1975).

The Nguni and Angus groups could be clearly distinguished in terms of circulatory leukocytes. Both in percentage and absolute amounts, the Nguni cattle displayed higher levels of neutrophils and lower levels of lymphocytes. Results suggesting that susceptible British breeds undergo a larger exodus of neutrophils from circulation would agree with infestation site histology studies that have identified greater amounts of the leukocyte in susceptible cattle (Wada *et al.*, 2010a; Marufu *et al.*, 2014a). The lower levels of circulatory eosinophils in the Brahman breed are suggestive that the cells play a role in the infestation response within the breed. They are well known to be associated to parasitic infestations (Francischetti *et al.*, 2009b). It should be noted, however, that the cellular composition within the circulation might not be a direct reflection of the local parasitic response, especially at low intensities of infestation. The differences in neutrophil levels were observed in breeds that proved susceptible in this trial and the contribution of eosinophils to the manifestation of resistance is still uncertain. There were significant differences in the lymphocytes, although clear conclusions may be difficult as differentiation among the cell subsets was not possible. Various authors have found positive T-cell involvement regarding responses to infestation in *Bos indicus* cattle (Piper *et al.*, 2009b; Constantinoiu *et al.*, 2010b). Simultaneously, high antibody titres have been reported in tick-susceptible animals (Willadsen, 1980a; Fivaz *et al.*, 1991; Schorderet & Brossard, 1993; Piper *et al.*, 2016), which is a B-cell mediated response.

At this time point, the breeds could not be separated with regard to most of the hematological parameters. It is well known that ticks are able to remove sufficient quantities of blood to induce levels of anemia, but at a sampling time point as early as 12 hours it is an unlikely influence. Certain studies have, however, shown the ticks' influence on growth rate to be beyond the removal of blood (Rechav *et al.*, 1980). This suggests a level of toxicity that accompanies the parasitic excretions during infestation. At the level of intensity used in the current study, such an effect is not visible within the whole blood parameters; however, numerous tendencies ($p<0.10$) suggest that higher intensities might display different results. The observations within the differential leukocyte values allow for distinction between the breeds regarding parameters that relate to their immunological responses. Assessment of local tick-host interfaces may be needed to enlighten the role of individual leukocytes.

3.5.3 Serum Biochemistry

The lower levels of albumin in the susceptible breeds are supported by similar studies that have reported lower levels in infested animals (O'Kelly *et al.*, 1970; O'Kelly & Kennedy, 1981). Albumin decreases are well known in animals subjected to infection and thought to be associated with a compensatory mechanism for globulin increases to maintain osmotic pressure (Dimopoullus, 1963). The albumin-globulin relationship is unlikely a contributing factor as globulin levels are a poor reflection of albumin differences. O'Kelly *et al.,* (1971) reported albumin decreases due to *R. microplus* infestation on Hereford steers induced by the specific effect of ticks. They suggested that the ticks have a specific toxic effect on the liver's ability to produce albumin. Differences in the current study, however, could also likely be due to decreased levels of protein consumption in the susceptible animals as the anorectic effect and direct effect of infestation could not be separated. While the susceptible animals did show lower levels of albumin, the levels are not considered in the region of hypoalbuminaemia. The higher level of blood urea nitrogen (BUN) in the Angus group suggests some extent of altered protein metabolism compared to the Brahman group. Increased levels of BUN could be related to the increase catabolism of serum proteins, a suggestion which is supported by the lower albumin level within the Angus group. Similar results were reported for British Hereford cattle after experimental infection with *Trypanosoma congolense* (Wellde *et al.*, 1974). Severe kidney or liver malfunction is unlikely as such conditions would have very likely been reflected in the serum creatinine or alkaline phosphatase levels.

Although the globulin level is not an entirely accurate representation of immunoglobulins alone (Dimopoullus, 1963), considerable humoral responses would likely be reflected in total plasma globulin levels. High λ-globulin levels have been observed in tick-susceptible animals under much higher intensities of infestation (40 000 and 60 000 larvae) (O'Kelly & Kennedy, 1981). Globulin levels were similar between breeds, suggesting that their level of B-cell mediated response, at this time point, was similar. Higher intensities of infestation for prolonged periods might produce globulin differences as well as more severe influences on albumin levels. Considering suggestions that the change in globulin and albumin profiles is a non-specific reaction to parasitic infestation (Herlich & Merkal, 1963), it is interesting to note that *R. microplus*-infested cattle produced higher globulin levels than *R. decoloratus*-infested cattle. It should be considered however, that the success rate of the tick is much higher and would have had an influence despite the early time point. The difference might thus be because of a higher level of infesting ticks and their associated secretions (Francischetti *et al.*, 2009b) rather than a more severe reaction to an individual tick. If this were the case, however, it could have been expected that more of the parameters would differ significantly between tick species.

There were clear differences between the Brahman and Angus breeds in circulating alanine aminotransferase (ALT) levels. In cattle, however, ALT is not considered to be a good indication of liver damage due to the very small amounts found in bovine liver cells (Stogdale, 1981). It is a possible indication of muscle damage (Boyd, 1988). In this study, it might not be the case as the Brahman breed, which

displayed the highest levels of ALT, were the least infested and would not have been subjected to significant tissue trauma. The functional relationship between ALT and the animals' response to infestation is unclear. This might be a promising subject of further investigation, as serum ALT level also showed a weak, yet significant negative correlation (r-value: -0.36; p-value<0.04) to day 18 tick counts.

The total cholesterol levels in this study were not in complete agreement with O'kelly, 1968, where *Bos indicus* breeds displayed higher cholesterol levels than *Bos taurus* breeds. In the current study, the Brahman cattle displayed cholesterol levels significantly higher than those observed on the Nguni group. Similar results were, however, observed by O'Kelly *et al.*, 1988 who reported constant cholesterol levels in Brahman animals while, decreases were observed in both Afrikaner and British breeds due to ectoparasites *R. microplus* and the buffalo fly (*Haematobia irritans, exigua*). Parasitic infection is well known to influence lipid levels in humans (Bansal *et al.*, 2005). O'Kelly, 1968 suggested cholesterol levels as an index for resistance after reporting a significant inverse correlation with tick counts. While not in complete agreement with previous studies, the current results reinforce the suggestion of a functional relationship between cholesterol levels and tick resistance. The factors that determine the relationship between an animal's total cholesterol level and its susceptibility to ticks, however, remains unknown. Plasma cholesterol levels are difficult to assess as they are influenced by multiple factors. Hypothyroidism is often associated with increases in cholesterol levels and decreased levels of thyroid activity have been associated with parasitism (Ogwu *et al.*, 1992), misdiagnosis remains a risk as normal ranges tend to vary and is also under the influence of the diet, hepatic function and bile duct obstruction (Kaneko, 1970). Also, as the Brahman breed with the highest cholesterol levels were the least infested, the association with thyroid activity is unlikely within the current study. Different levels will likely be a consequence of infestation due to ticks' influence on diet and liver function, but the possibility exists that blood cholesterol could play a role in an animals' ability to respond to an external/foreign threat. One of the functional roles of cholesterol includes the maintenance of specific cell membrane "lipid-rafts" which are responsible for enabling cell transduction cascades (Simons & Toomre, 2000), allowing for the processing of certain stimuli and cellular function. Further investigation into the role of cholesterol during infestation is necessary.

The fibrinogen levels observed in the less susceptible Brahman displayed substantial differences post infestation with significantly lower values. The protein is known to increase during almost all inflammatory reactions, although responses related to more severe disorders in cattle will commonly produce levels in excess of 10g/l (Schalm *et al.*, 1975). Plasma fibrinogen levels have been considered more indicative of the extent of an inflammatory process than total leukocyte counts (Schalm *et al.*, 1975). Fibrinogen increases are also associated with sub-clinical conditions and are considered to be evident within 24hours of the onset of disease (McSherry *et al.*, 1970). The results provide considerable evidence that the Nguni and Angus animals suffered from light to intermediate inflammatory reactions not observed in the Brahman group. The suggestion is supported by observations by Piper *et al*., (2008) who reported a higher level of expression of inflammatory-associated genes in the more susceptible Holstein Friesian cattle compared to

Brahmans after *R. microplus* infestation. Marufu *et al*., 2013 also observed acute type I hypersensitivity responses in susceptible cattle following intradermal injection with unfed larval extracts from *R. microplus* and *R. decoloratus*, but the study has contrasting findings regarding the susceptibility of Nguni cattle.

The adsorption of certain leukocytes as well as the fibrinogen profiles were suggestive that the Brahman group displayed less of an inflammatory response. The individual involvement of leukocytes at infestation sites requires confirmation through histology site assessments. The biochemistry parameters provided some insights into the metabolic function of the animals. It is interesting to observe that the more susceptible animals did have different systemic responses despite a very early time point, when blood loss could effectively be ruled out. The functional differences can thus only be responses to the components of early infestation, such as the immunomodulatory excretions found in tick saliva. There was no indication of severe hepatic or renal malfunction, but analysis suggested altered protein metabolism, possibly decreased production or increased breakdown. Biochemical results also contributed to evidence of a functional relationship of tick infestation with blood cholesterol.

3.6 Conclusion

The relative success of *R. microplus* across all breeds makes comparisons surrounding the 'ancient' and 'modern' associations difficult. After taking these factors into account, it could not be concluded that 'ancient parasite-host relationships' provide the primary platform for the manifestation of effective resistance. The results had shown tendencies that support the hypothesis, but the Brahman group's performance regarding *R. decoloratus* indicates a reasonable level of heterospecificity by the breed. The level of infestation was too low and the sampling time point too early to have severe effects in the whole blood composition of susceptible animals, but prolonged intense infestation may produce different results. Differences in circulating leukocytes and fibrinogen levels are a strong indication that the immune responses between breeds are not similar, but investigation of local interactions is needed. It is likely that the Brahman breed was able to function more effectively despite the presence of infestation. It should also be considered that reactions similar to the Nguni and Angus were not induced because of a lower level of infesting ticks. Regardless, it is an important indication of the extent to which tick species can influence their host aside from the removal of blood. Irrespective of the performance concerns surrounding the Nguni breed, the Brahman breed has proven itself a strong candidate for a host that should maintain function well despite the widespread parasitic threat that face cattle production in South Africa. Further investigation into the responses of Nguni breed is necessary, which should include improved animals and *R. decoloratus* larvae with higher infestation capabilities, perhaps at a greater intensity. The Brahman group, in turn, should be compared to another African tick that is not as closely related to *R. microplus*. An African tick species that is from another genus, like *Amblyomma hebraeum,* might provide valuable insights into the level of cross resistance the breed is capable of. If successful, the breed could become an important target of molecular studies toward elucidating the resistant genotype.

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CHAPTER 4 - THE EFFECT OF TICK INFESTATION ON CUTANEOUS CELLULAR RESPONSES IN BRAHMAN, NGUNI AND ANGUS CATTLE.

4.1 Introduction

It has been well illustrated that local, cutaneous cellular responses play a key role in the bovine host's manifestation of resistance to infestation (Veríssimo *et al.*, 2008; Carvalho *et al.*, 2010; Constantinoiu *et al.*, 2010; Piper *et al.*, 2010; Marufu *et al.*, 2014). These previous studies have not only shown that the cellular responses between resistant and susceptible comparisons differ, but also that the animal's genotype, determined by breed, plays an important part in determining its response. Infiltrates of neutrophils, eosinophils, basophils, mast cells and lymphocytes contribute to the host's responses to tick attachement (Veríssimo *et al.*, 2008; Carvalho *et al.*, 2010; Constantinoiu *et al.*, 2010; Wada *et al.*, 2010; Marufu *et al.*, 2014). High densities of inflammatory cell infiltrations appear in certain instances to cause local tissue damage which might create an environment that is advantageous to the tick (Tatchell & Moorhouse, 1968; Constantinoiu *et al.*, 2010; Marufu *et al.*, 2014). It is possible that certain breeds have a superior ability to maintain an extracellular matrix that is less susceptible to tick feeding. Recent studies have indicated that susceptible breeds have more pronounced subcutaneous changes than resistant cattle (Piper *et al.*, 2010; Marufu *et al.*, 2014). Piper *et al.*, (2010) also reported increased expression of collagen transcripts in the skin of indicine Brahman cattle compared to the Holstein Frisian cattle, which supported suggestions by Piper *et al.*, (2009) that Brahman cattle might possess an ability to modify the extracellular matrix in a way that increases resistance to infestation. Marufu *et al.*, (2014) associated the more severe cutaneous changes to differences to the type of hypersensitivity response of host, suggesting that the more severe necrosis and oedema is due to an acute, type I hypersensitivity reaction. A comparison of ancient and modern parasite-host relationships could thus benefit from an assessment of the cutaneous environment and level of cellular infiltration during early infestation. Furthermore, previous studies included limited breed comparisons and focus was only on a single tick species, which could be a disadvantage in an attempt to elucidate host responses.

As the majority of previous studies has focused on cellular reactions to *R. microplus* by *B. taurus* and *B indicus* breeds, there is a need to investigate the cutaneous changes between a wider scope of breeds and tick species. Parallel artificial infestation of *B. taurus*, *B. indicus*, and *B. t. africanus* breeds with both tick types *R. microplus* and *R. decoloratus* thus allow for the comparison of 'ancient' and 'modern' relationships while allowing for tissue sampling during the same phase of infestation over all treatment groups. The initial reaction from the host is essential for the manifestation of resistance (Tatchell & Moorhouse, 1970) and because there is less value in the assessment of ticks that have already successfully fed to maturity, sampling is taken at 12 h post infestation in order to assess the cutaneous reaction before or during possible tick detachment within the resistant hosts. Sampling of attachment sites at 12 h, although providing a good

indication of the early host response to infestation, does not allow for determination as to whether the attachment would ultimately be a successful one. Hence interpretation is limited to relating the characteristics of an attachment site at 12 h with the average success rate of the breed, tick type or treatment group. The study's objective is to compare cutaneous responses between treatments in order to assess if certain breed and tick species interactions can be regarded as specific to that particular relationship.

4.2 Material and Methods

For all materials and methods regarding study site, cattle breeds, animal sex and age, tick larvae, management, protocol timeline and artificial infestation, see section 3.2.1 to 3.2.6 and 3.2.8 for biosecurity measures and 3.2.12 for ethical clearance.

4.2.1 Skin Biopsy Sampling

All animals were sedated prior to sampling with administration of xylazine (Sedaxylin®, Bayer, South Africa) which was administered 0.2mg/kg body weight in Angus and Nguni breeds. The dose was lowered to 0.05mg/kg bodyweight for all Brahman cattle due to the breed's known sensitivity towards xylazine. Punch biopsy sites were de-sensitized with the local administration of 2.0 ml of 2 % lignocaine hydrochloride (Lignocaine®, Bayer, South Africa) subcutaneously using a 25-gauge needle. After the application of the local anesthetic and sedation of the animal, two biopsies were taken from each animal using a 5mm punch biopsy needle (Stel+Medcc®, South Africa). At the time of the first sampling time point, samples were taken from the left side of the animal on the upper part of the rib-cage. At the second-time point (12-hours post infestation), biopsies were taken from the skin inside of the calico bags on the upper back of the animal. Where possible, sites of active infestation were visually identified as biopsy site and samples were taken to include the mouthparts of the parasite. Samples were taken with utmost care to ensure they were uniform and precisly full skin thickness, 5 mm diameter and 10 mm deep. The biopsy sites were treated with oxytetracycline (Terramycin Wound Powder®, Fivet, South Africa) and chlorfenvinphos 0.48% (Supona Aerosol Spray®, Zoetis, South Africa) to prevent bacterial infection and wound myiasis in the skin. After the completion of sampling, the animal was injected with 10 mg/kg yohimbine HCl (Reverzine®, AR, South Africa) to reverse the sedation. Skin samples were immersed in neutral buffered formalin (pH 7) immediately after collection.

4.2.2 Histological Processing

Biopsy samples were fixed in neutral buffered formalin (pH 7) for 48 h to ensure thorough fixation of the 5mm samples. At 48 h, the samples were processed according to routine histological techniques (Addendum C) and embedded in pre-heated paraffin wax (60˚C) (Paraplast, melting point 58°C) using a Leica EG 1160 embedder (SMM Instruments Ltd., South Africa) by IDEXX® laboratories, Pretoria. The 5 mm skin samples were sectioned with a microtome blade perpendicular to the outer surface of the skin, allowing for the visual inspection of all three skin layers.

Samples were individually sectioned at 2 µm using a Leica RM 2125RT microtome (SMM Instruments Ltd., South Africa) to allow for differential staining. Each sample was stained with Hematoxylin-Eosin (H&E) for histopathology score and total cell counts. The H&E stain should color the chromatin blue and the nuclei red while the cytoplasm will be stained shades of red and pink.

4.2.3 Section Analysis

Sample sections were individually analyzed under light microscopy using an Olympus IX70 inverted microscope fitted with a Colorview II camera (Wirsam®, South Africa), which allowed for analysis and visual documentation of sections (see Figure 4.1 to Figure 4.3). All histological evaluation was conducted by a technician blind to the breed and tick type treatment groups.

4.2.4 Histopathology score

Histopathology score was performed on H&E stained sections. Skin sections were evaluated and given an overall score for tissue damage. Changes were characterized on a 4-increment scale from 0 to 3 as follows: 0=None; 1=Mild; 2=Severe; 3=Very Severe. Each sample was evaluated twice and assigned a total score out of 6 based on the sum of the two individual scores. Cutaneous characteristics that were considered during the general tissue assessment included: epidermal hyperplasia, epidermal necrosis, dermal oedema, vascular reaction, hyperemia and pustules (Piper *et al.*, 2010; Marufu *et al.*, 2014)

According to Piper *et al.*, 2010, the characteristics can be defined as follows:

Cell Hyperplasia: An increase in the number of cells; Necrosis: The death of cells; Sub epidermal clefting: detachment of the epidermis; Hyperkeratosis: The thickening of the keratinised layer of the epidermis; Oedema: The accumulation of fluid; Vasculitis: inflammation of blood vessels.

4.2.5 Total cell counts:

Cell counts were enabled by performing H&E stains. Counting proceeded by performing two dermal cell counts from two images of a section and a mean value was calculated to be used for analysis. Each single count was performed using ImageJ version 1.51i software (ImageJ®, U.S National Institutes of Health, Bethesda, Maryland, USA). On a 4x objective, an area considered a representative of the dermis was selected and the result is produced as cells per μm^2. The software was programmed include only inflammatory cells.

4.2.6 Statistical Analysis

The data were analyzed using Statistical Analysis System (SAS) Enterprise guide software (Version 7.1, 2014; SAS Institute Inc, Cary, NC, USA). All cell count data were square root-transformed to confer normality. The Linear Models procedure was used to perform ANOCOVA (Type III) analysis of the effects of treatments, breed and tick species on the respective square root transformed tick counts. The pre-infestation values were used as the covariates in all ANOCOVA models. The adjusted mean effects of treatments were determined using LSMEANS option and compared using Bonferroni t-tests. Correlations

among variables were determined using the PROC CORR function. Associations between histopathology score and treatment groups were done using The FREQ Procedure. Due to low cell frequencies, P-values presented were calculated using Fisher's Exact Test.

4.3 Results

4.3.1 Total cell counts

4.3.1.1 *Total cell counts for individual breed*tick species treatment groups*

Interaction terms for transformed dermal cell counts between breed and tick species was not significant (p>0.05). Assessments could thus be focused on the main effects of breed and tick species. Table 4.1 presents the adjusted least square means as well as standard deviations for all individual breed*tick species treatment groups.

Table 4.1 Infestation site dermal cell counts (LS means and SD) for all breed*tick species treatment groups

	Brahman		**Nguni**		**Angus**	
Parameter	*R. microplus*	*R. decoloratus*	*R. microplus*	*R. decoloratus*	*R. microplus*	*R. decoloratus*
Dermal cell counts (SQRT)	48.78 ± 6.25	44.80 ± 6.43	45.52 ± 4.11	44.06 ± 3.16	50.57 ± 8.96	41.45 ± 3.86

[a,b] Means with different superscripts in the same row differ significantly (p< 0.05)

4.3.1.2 *Total cell counts between breed groups*

There were no significant (p>0.05) differences in square root-transformed infestation site dermal cell counts between any of the breeds. Table 4.2 presents the adjusted least square means as well as standard deviations for all breed groups.

Table 4.2 Infestation site dermal cell counts (LS means and SD) for all breed treatment groups

Parameter	**Brahman**	**Nguni**	**Angus**
Dermal cell counts(SQRT)	46.79 ± 7.54	44.79 ± 3.64	46.01 ± 7.92

a,b Means with different superscripts in the same row differ significantly (p< 0.05)

4.3.1.3 *Total cell counts for tick species treatment groups*

Differences in square root-transformed infestation site dermal counts between tick species treatment groups showed a tendency (p<0.10) to be higher in *R. microplus* attachment sites. Tendencies (p-values that are below 0.10) will not be discussed. Table 4.3 presents the adjusted least square means as well as standard deviations for all tick species treatment groups.

Table 4.3 Infestation site dermal cell counts (LS means and SD) for tick species treatment groups

Parameter	*R. microplus*	*R. decoloratus*
Dermal cell counts (SQRT)	48.29 ± 6.71	43.44 ± 5.58

[a,b] Means with different superscripts in the same row differ significantly (p< 0.05)

4.3.1.4 *Total cell counts for pre- and post-infestation time points*

There were no significant (p>0.05) differences in the transformed dermal cell counts among breeds. The parasitized skin displayed a higher level (p<0.05) of dermal cell counts than the non-parasitized skin in all three breeds. The parasitized tissue samples displayed square root-transformed dermal cell counts of 42.18 (± 7.54), 44.85 (± 3.63) and 43.10 (± 7.92) in the Brahman, Nguni and Angus groups, respectively. These were higher (p<0.05) than the 18.81 (± 3.62), 18.36 (± 13.47) and 20.31 (± 13.81) observed in the pre-infestation tissue of the same respective groups. Table 4.4 presents the least square means as well as standard deviations for skin samples before and after infestation within all breed groups.

Table 4.4 Dermal cell counts for pre-and post-infestation samples (LS means and SD) within all breed groups

	Brahman		Nguni		Angus	
Parameter	**Non-parasitized**	**Parasitized**	**Non-parasitized**	**Parasitized**	**Non-parasitized**	**Parasitized**
Dermal cell counts (SQRT)	18.81 ± 3.62[b]	42.18 ± 7.54[a]	18.35 ± 13.47[b]	44.86 ± 3.63[a]	20.31 ± 13.81[b]	43.10 ± 7.92[a]

[a,b] Means with different superscripts in the same row differ significantly (p< 0.05)

4.3.2 Histopathology scores

4.3.2.1 *Histopathology score for pre- and post-infestation time points*

General cutaneous tissue damage was more pronounced (p<0.01) in the parasitized skin of Brahman, Nguni as well as Angus cattle, compared to the non-infested skin samples of all three breeds. In the Brahman, Nguni and Angus breeds, parasitized tissue had a general evaluation score of 3 or higher with a frequency of 8 (Brahman) or 9 (Nguni and Angus), while a score above 2 only occurred in non-parasitized tissue once (within the Nguni group). Table 4.5 presents the frequencies of histopathology scores between before and after infestation within all breed groups.

Table 4.5 Frequency of histopathology scores for pre- and post-infested cutaneous samples within breed groups

Breed	**Brahman**		**Nguni**		**Angus**	
P-Value	**p<0.01**		**p<0.01**		**p<0.01**	
Histopathology score	**Non-Parasitized**	**Parasitized**	**Non-Parasitized**	**Parasitized**	**Non-Parasitized**	**Parasitized**
0	7	1	9	1	6	1
1	4	0	1	0	4	0
2	0	1	1	2	2	2
3	0	3	0	2	0	2
4	0	5	1	7	0	7
5	0	1	0	0	0	0
6	0	0	0	0	0	0

General cutaneous changes were more pronounced (P<0.05) in the parasitized skin samples of animals infested by both tick species groups. The non-parasitized sections had a single observation (within the *R. decoloratus* group) that displayed a histopathology score higher than 2, while the parasitized sections displayed 27 observations that scored 3 or higher. Table 4.6 presents the frequencies of histopathology scores between before and after infestation within all tick species groups.

Table 4.6 Frequency of histopathology scores for pre- and post-infested time points within tick species groups

Tick species	***R. microplus***		***R. decoloratus***	
P-Value	**p<0.01**		**p<0.01**	
Histopathology score	**Non-Parasitized**	**Parasitized**	**Non-Parasitized**	**Parasitized**
0	11	1	11	2
1	3	0	6	0
2	3	2	0	3
3	0	2	0	5
4	0	11	1	8
5	0	1	0	0
6	0	0	0	0

4.3.2.2 ***Histopathology score for breed and tick groups.***

No significant (p>0.05) associations between histopathology scores and breed groups were observed within any of the time points. Table 4.7 presents the frequencies of histopathology scores between before and after infestation within all tick species groups.

Table 4.7 Frequency of histopathology scores between breed groups within pre- and post-infestation time points.

Time point	**Non-parasitized**			**Parasitized**		
P-Value	**p<0.34**			**p<0.98**		
Histopathology score	**Brahman**	**Nguni**	**Angus**	**Brahman**	**Nguni**	**Angus**
0	7	9	6	1	1	1
1	4	1	4	1	2	2
2	0	1	2	3	2	2
3	0	1	0	5	7	7
4	0	0	0	1	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0

No significant (p>0.05) associations between histopathology scores and tick species groups were observed within any of the time points. Table 4.8 presents the frequencies of histopathology scores for all breed groups for both pre- and post-infestation time points.

Table 4.8 Frequency of histopathology scores between tick species groups within pre- and post-infestation time points.

Time point	**Non-parasitized**		**Parasitized**	
P-Value	**p<0.19**		**p<0.57**	
Histopathology score	***R. microplus***	***R. decoloratus***	***R. microplus***	***R. decoloratus***
0	11	11	2	1
1	6	3	3	2
2	0	3	5	2
3	1	0	8	11
4	0	0	0	1
5	0	0	0	0
6	0	0	0	0

4.3.2.3 ***Histopathology scores in different cell count categories***

Square root-transformed cell counts were assigned to categories as follows: values between 0 and 10 were assigned 1. Values between 10 and 20 assigned 2; 20 and 30 assigned 3… etc. Within samples taken post-infestation, cell count categories were then compared regarding possible associations with histopathology scores. General tissue damage was significantly (p<0.01) more pronounced in higher cell counts categories. Table 4.9 presents the frequency of histopathology scores for all cell count categories at the post infestation time point.

Table 4.9 Frequency of histopathology scores between cell count categories (Post-infestation)

	Cell Count Class						
Histopathology score	**1**	**2**	**3**	**4**	**5**	**6**	**7**
0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0
2	0	0	1	3	1	0	0
3	0	0	0	0	7	0	0
4	0	0	0	0	0	13	5
5	0	0	0	0	0	0	1
6	0	0	0	0	0	0	0

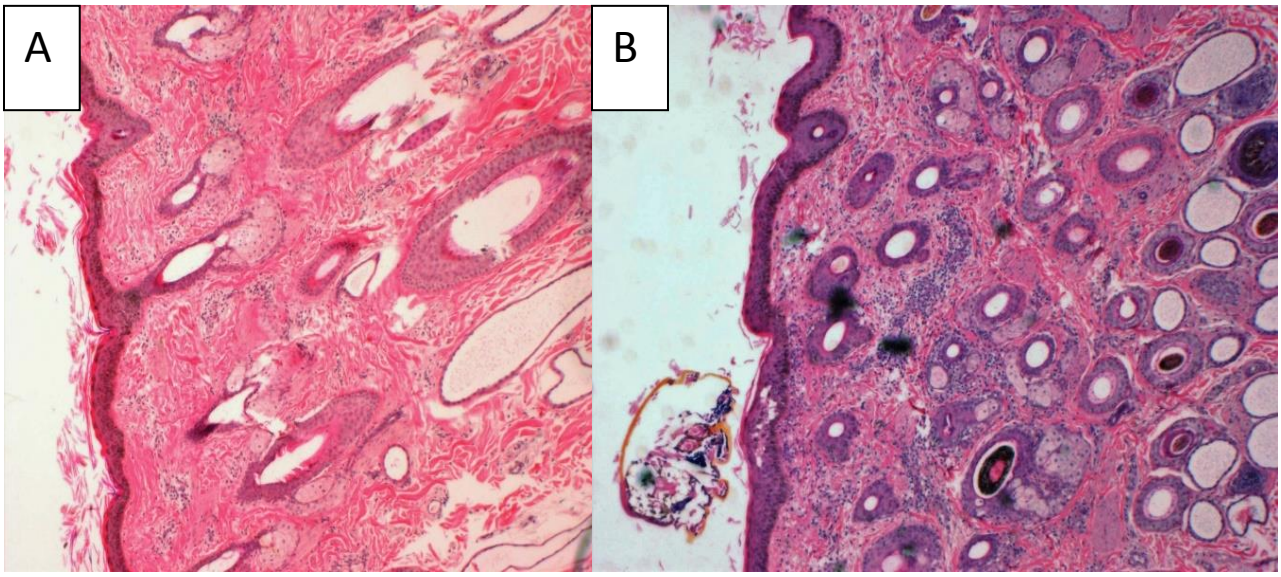

Figure 4-1 Images of skin sections (stained with H&E) taken from animals Brahman*R. decoloratus-2 (A) and Brahman*R. microplus-3 (B)

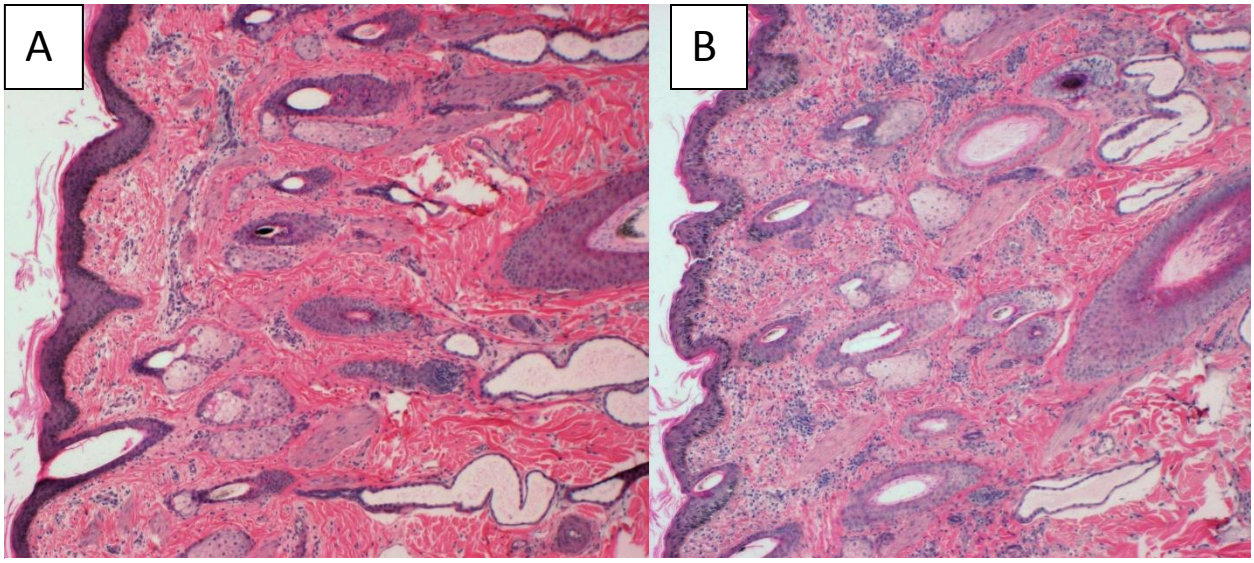

Figure 4-2 Images of skin sections (stained with H&E) taken from animals Angus*R. decoloratus-5 (A) and Angus*R. microplus-4 (B)

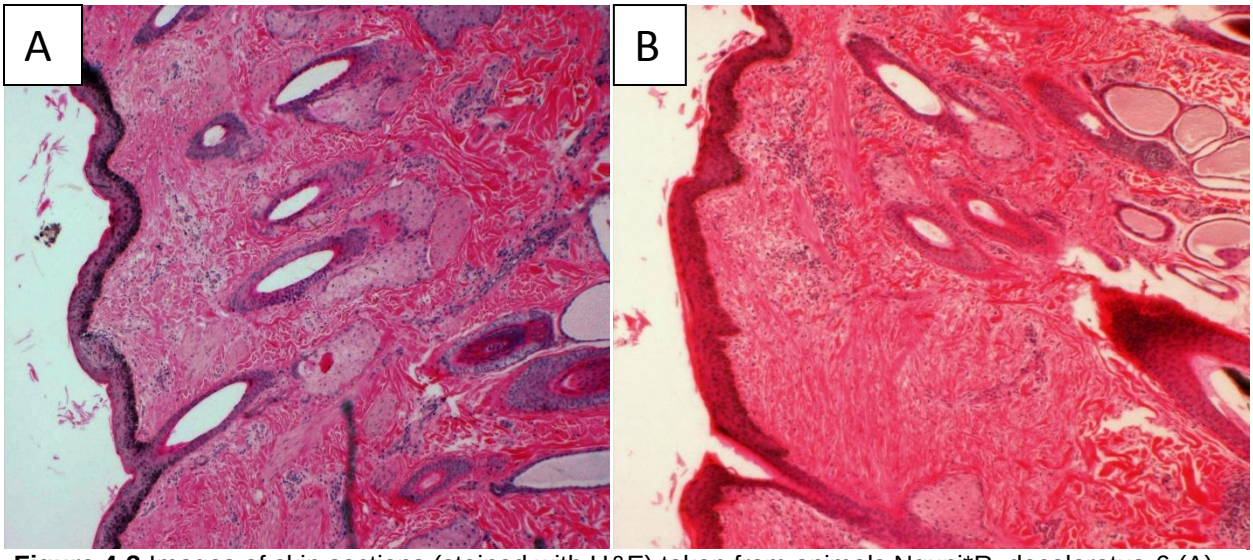

Figure 4-3 Images of skin sections (stained with H&E) taken from animals Nguni*R. decoloratus-6 (A) and Nguni*R. microplus-2 (B)

4.4 Discussion

The results observed in this study for total cell counts partially agree with observations by Marufu *et al.*, (2014), who reported increased total cell counts in parasitized skin compared to normal skin within breeds. It can be safely assumed that the presence of the cells within the post-infestation time point is due to their role as inflammatory infiltrates in response to larval infestation in the host's skin. The presence of infiltrates within parasitized tissue is not surprising considering the presence of leukocytes at infestation sites has been well documented (Veríssimo *et al.*, 2008; Carvalho *et al.*, 2010; Constantinoiu *et al.*, 2010; Wada *et al.*, 2010) and their presence will likely have an influence on the tick's ability to infest and feed. However, a unique aspect of the current study is the 12-hour post infestation sampling time point. The high density of infiltrates is thus an indication that the host response, and possibly the determining factor between successful or unsuccessful attachment, is already activated at very early stages of infestation. The results support early suggestions that the larval stage of the life cycle is the most critical for the manifestation of resistance (Roberts, 1968; Kemp *et al.*, 1976). It is not possible to distinguish between which of the sampled infestation sites would have developed into a successful tick feeding episode, but if the cellular differences between susceptible and resistant hosts are to be elucidated, it is possible that the 12-hour post infestation point could provide the optimal platform for comparisons of responses.

In breed comparisons of parasitized tissue, no differences in the total cell counts were observed, while tick count results from Chapter 3 clearly separated some of the breeds regarding their susceptibility to ticks. It is important to note that the cellular composition of infiltrates play a key role in the host's resistance or susceptibility to ticks, as neutrophils and eosinophils have been associated with increased susceptibility to ticks (Wada *et al.*, 2010; Marufu *et al.*, 2014), while mast cells and basophils have been shown to increase resistance (Veríssimo *et al.*, 2008; Carvalho *et al.*, 2010; Marufu *et al.*, 2014). It is thus important to note that differential assessment of infiltrates will provide an improved insight into the cellular influences at a cutaneous level compared to the total cell counts. The current results did, however, disagree with observations by Marufu *et al.*, (2014) who reported higher levels of total cell counts in the more susceptible Bonsmara breed than the more resistant Nguni counterpart. Furthermore, results in chapter 3 provided strong evidence that the breeds differed in their responses regarding acute inflammation, and a higher level of infiltrates could thus have been expected for the Nguni and Angus groups. The systemic and cutaneous results are thus not in complete agreement. However, it is necessary to evaluate sampling protocol before making conclusions surrounding cell count results. It is possible that skin sections were not always an accurate representation of a tick-host interaction site. An image of parasitized skin from each treatment group is presented in Figure 4-3 Images of skin sections (stained with H&E) taken from animals Nguni*R. decoloratus-6 (A) and Nguni*R. microplus-2 (B) The images support the observation that a considerable number of infiltrates were present in the parasitized skin of all breeds, but that there is also a large amount of variation within and between tissue sections, also observed in the large standard deviations of the group means. The model also has a very low R-square value (R^2=0.21), which is evidence of a large amount of

unexplained variation between and within treatment groups. Figure 4-1 (B) illustrates the infiltrate arrangement surrounding an infesting *R. microplus* larva in the skin of a Brahman host. It is evident that a large portion of the infiltrates tend to accumulate in very close proximity to the parasite. Similarly, Constantinoiu *et al.*, (2010) and Tatchell & Moorhouse, (1968) described granulocyte infiltration surrounding the mouthparts of infesting ticks. An accurate assessment of cellular involvement will thus improve substantially as the position of the section approaches the center of the bite site, and it is possible that numerous sections within the current study did not meet the requirement, especially as the larvae were rarely identifiable in stained sections. It cannot be assumed that the location of a bite site is in the center of a skin biopsy sample and the location of sectioning within the sample can to a certain extent be considered at random. Sectioning accurately through the bite site is thus partially left to chance and will very likely contribute to a large amount of sampling error. Such a sampling error is possibly a drawback of the very early sampling time point. The larvae are very small and the stage of feeding very primitive, causing difficulty in sectioning within a tick feeding lesion.

The tissue assessment in this study provides an indication of general tissue damage on a cutaneous level. The results showed strong evidence that cutaneous damage was considerably more pronounced within parasitized skin than the control samples. A lack of differences between the treatment groups disagrees with previous observations (Piper *et al.*, 2010; Marufu *et al.*, 2014) that susceptible breeds display more pronounced cutaneous changes than resistant hosts. Furthermore, the higher feeding success rate displayed by the *R. microplus* group (as suggested in chapter 3) and the high level of resistance displayed by the Brahman group would suggest that these treatment groups should be able to possibly be separated on a cutaneous level. It is possible, however, that the aforementioned sampling error could affect tissue assessment scores in a similar fashion. Cutaneous changes are also considered a non-specific reaction to any noxious stimuli (Szabó & Bechara, 1999; Monteiro & Bechara, 2008) and the significant association between tissue scores and cell count categories is evidence that damage to skin infrastructure is in a large part due to the host's response, as similarly reported by Tatchell & Moorhouse, (1970) for the infiltration of neutrophils. Damage due to tick mouthparts will likely be very little at this time point, so a difference between the tick species might be clearer at a later stage of infestation.

4.5 Conclusion

The lack of differences between breeds or tick species does not support the hypothesis that reactions are breed dependent and possibly tick species specific. The contribution of cellular influences in responses has been well documented, however, and a considerable amount of sampling error has likely had a significant influence on the observation of results. Possible differential reactions in parasite-host relationships that have had the advantage of co-evolution is therefore considered inconclusive. Regarding the 12-hour time point, the possible increase in the biological accuracy of elucidating the cellular response of the resistant host has a corresponding increase in risk of sampling error. The earlier the time point, the less pronounced the formation of the tick feeding lesion will be and the greater the risk of a section that is a poor representation of the tick-host interaction. An early sampling time point remains a recommendation, but studies that involve the differential leukocyte subclasses could be problematic unless a high level of accuracy can be obtained within histological sampling. A high level of accuracy is needed for more complicated investigations which, if possible, should include differential leukocytes and the involvement of the adaptive immune response. Enumeration of neutrophils, eosinophils, basophils, mast cells, monocytes and T-cells (CD^{3+} and MHC class II) is recommended at the 12-hour sampling time point.

4.6 References

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CHAPTER 5 - GENERAL CONCLUSIONS AND RECOMMENDATIONS

The Brahman breed displayed the highest level of resistance to tick infestation between the three breeds even though the Nguni's resistance may have been compromised by the poor condition of the animals used in the study. A body condition score of all animals is needed prior to the commencement of the study, as this would provide a more useful link to the animals' condition and their performance within the trial. The intensity of infestation needs to be increased substantially to produce more reliable results, or counts need to be performed following multiple infestations in to increase reliability of resistance assessments. A higher intensity should also amplify animal responses and possibly clarify differences between treatment groups. Based on this small study it would seem that the high level of cross resistance displayed by the Brahman breed suggests that resistance is not limited to parasite-host relationships that have a co-evolutionary history. It is possible, however, that the mechanisms of selection pressure between *R. microplus* and *R. decoloratus* was similar due to the ticks' related ancestry, and this would explain the indicine Brahman breed's high level of adaption to the African *R. decoloratus*. *Rhipicephalus microplus* had a substantially greater success rate compared to *R. decoloratus* across all breeds. The below expected success rate suggests *R. decoloratus* might not be suited for artificial infestation. A higher intensity will produce more reliable comparisons between tick species. The *R. microplus* species might have a higher ferocity than *R. decoloratus* which contributes to concerns regarding the Southern African invasion of the Asiatic intruder.

The intensity of infestation was too low or the sampling timepoints too early to produce RBCC, PCV and haemoglobin differences in the susceptible cattle. The Brahman breed displayed a lower MCV than the Nguni and Angus breeds. Despite the adjustment for control values, the difference related to breed as *microcytic hypochromic* anemia is not a valid explanation. The breeds were not similar regarding shifts in circulating leukocyte levels. Differential systemic differences in these cells at an early timepoint show that the cells play a key role in responses to infestation, and that responses are specific to breed.

The breeds and tick species groups did not differ in the amount of inflammatory cell infiltration observed in cutaneous bites sites, but the level was substantially higher in the parasitized skin of all breeds. These cells play a key role in the host's interaction to tick infestation and their presence at an early timepoint emphasizes that tick possible rejection occurs at a very primitive stage of infestation. The breed differences in the differential leukocyte levels in circulation emphasizes the need for the enumeration of the differential involvement of leukocytes within cutaneous responses. Histological assessments were similar between treatment groups, strengthening the view that cutaneous damage in parasitized tissue is non-specific. Sampling accuracy was possibly inadequate due to the small and local nature of bite sites at the 12-hour interval.

Albumin and blood urea nitrogen levels indicated altered protein metabolism in more heavily infested animals. Altered protein intake is associated with infestation. The secretion of a hepatoxic compound is possible, but liver damage and dysfunction was not severe. Different globulin levels between tick groups suggest that antibody responses to tick species are specific. Cholesterol and alanine amino transferase was associated with resistance, but their functional relationships with infestation remains unknown. Fibrinogen levels showed more severe inflammatory reactions in the Nguni and Angus breeds. An acute inflammatory response is considered an important contribution to their susceptibility.

This study reinforced that genotypes plays a key role in resistance. Both on a systemic and cutaneous level, the study has shown that the effect of ticks, as well as the hosts responses to it, is already visible at very early time points. It was shown that the effects of ticks are not limited to immunological parameters and that the effects of tick infestation are visible at low intensities, relative to artificial infestation.

Addendum A

Reactions and Reagents involved with the use of the VITROS® 350 Dry Slide Chemistry Analyzer

Biochemical Parameter	Reactions and Reagents
Total Protein	Copper tartrate diffuses to the spreading layer and reacts with protein which forms a violet complex measurable by reflectance spectrophotometry.
Albumin	Bromcresol green dye (BGD) diffuses to the spreading layer and its binding to albumin causes a change in the wavelength of the reflectance maximum relative to the unbound dye.
Globulin	Globulin is determined as the difference between total protein and albumin.
Alanine Transferase:	The enzyme transfers the amino group of L-alanine to α-ketoglutarate to produce pyruvate and glutamate. Lactate dehydrogenase then acts a catalyst for the conversion of NADH and Pyruvate to Lactate and NAD^+. The oxidation of NADH is measured by reflectance spectrophotometry and is proportional to enzyme activity.
Alkaline Phosphatase:	The enzyme catalyzes the hydrolysis of p-nitophenol phosphate to p-nitrophenol and the change in reflection density is used an n indicator of enzyme activity.
Gamma-Glutamyl Transferase:	The enzyme acts a catalyst for the transfer of the γ-glutamyl component of L-γ-glutamyl-p-nitroaniline to glycylglycine and p-nitroaniline.
Uric acid:	Uricase oxidases uric acid to produce allantoin and hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide oxidises a leuco dye to produce a colored dye.
Bilirubin:	Sodium Benzoate and caffeine enables the separation of unconjugated bilirubin from albumin, allowing for bilirubin (unconjugated and conjugated) to migrate to the reagent layer where it binds to a cationic mordant.
Cholesterol:	Lipoprotein complexes within the sample is dissociated in the presence of Trition X-100 surfactant in the spreading layer. Cholesterol ester hydrolase then catalyzes the hydrolysis of cholesterol esters to free cholesterol. Free cholesterol is then oxidized to cholestenone and hydrogen peroxide by cholesterol oxidase. Aided by a peroxidase, the hydrogen peroxide hydrolyses a leuco dye to produce a colored dye.
Creatine Kinase:	Creatine phosphate and ADP is converted to creatine and ATP in the presence of creatine kinase. Glycerol kinase catalyzes the phosphorylation of glycerol to L-α-glycerolphosphate by ATP. Dihidroxyacetone phosphate and hydrogen peroxide is produced through the oxidation of L-α-glycerolphosphate by L-α-gycerolphosphate oxidase. The hydrogen peroxide oxidises a leuco dye to form a colored dye.
Blood Urea Nitrogen:	Only fluid and non-protein components pass through to the underlying layer and ammonia is generated through the urease reaction. A semi-permeable membrane allows ammonia to pass to the underlying layer where it reacts to form a dye.
Creatinine:	In the reagent layer, creatinine is hydrolyzed to creatine. Creatine amidinohydrolase converts creatine to sarcosine and urea. The sarcosine is oxidized by sarcosine oxidase to produce glycine, formaldehyde and hydrogen peroxide. Hydrogen peroxide oxidizes a leuco dye to produce a colored dye.
Lactate dehydrogenase:	The conversion of pyruvate and NADH to lactate and NAD^+ is catalyzed by lactate dehydrogenase.

Addendum B

The active ingredients of the reagents as used in the SYSMEX Xt2000i for haematological analysis:

Red cell counts and platelets: The sample rotor valve measures 4.0 µL of blood which is diluted with 1.996 mL of CELLPACK (1:500) and proceeds to the RBC sample chamber. 11.7 µL of the diluted sample is slowly sent to the RBC/PLT detector, which counts RBC and PLT levels using Hydro Dynamic Focusing while the RBC pulse height is used to calculate heamatocrit.

Haemoglobin: The sample rotor valve measures 4.0 µL of blood which is diluted with 0.996 mL of CELLPACK (1:250) and proceeds to the flow cell. Simultaneously, red cells are hemolyzed through the dilution with of 0.5 mL of SULFOLYSER (SLS) (1:375) and the hemoglobin is converted to SLS-hemoglobin. A light emitting diode emits a 555nm beam of light that passes through the lens and into the heamoglobin cell that contains the sample. The difference of the light absorbance of the diluent with and without the samples is used to calculate the concentration of SLS haemoglobin.

The mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) are calculated from red blood cell count (RBCC), Haemoglobin (HGB) and packed cell volume (PCV) parameters using the following equations:

$$MCV\ (fL) = \frac{PCV(\%)}{RBCC\ (\times 10^{12}/L)} \times 10$$

$$MCHC\ (g/dL) = \frac{HGB(g/dL)}{PCV\ (\%)} \times 100$$

Leukocytes and its subclasses: The 4DIFF analysis procedure is used to differentiate between the respective white blood cell groups, including neutrophils, eosinophils, basophils, monocytes and lymphocytes. The sample rotor valve measures 20.0 µL of blood which is diluted with 0.980 mL of STROMATOLYSER-4DL and proceeds to the flow cell. Simultaneously, the sample is diluted with the addition of 40µL of STROMATOLYSER-4DS (1:52). A 22 second reaction time allows for red cell hemolysis and the staining of white cells after which 40µL of the dilution proceeds to the optical detector block. The sample is then analyzed with the use of flow cytometry with a semiconductor laser.

Ingredients of reagents used in Sysmex XT2000i

CELLPACK	Sodium Chloride: 0.64% Boric Acid: 0.10% Sodium Tetraborate: 0.02% EDTA-2k: 0.02%
STROMATOLYSER-4DL	Non-ionic surfactant: 0.18% Organic quaternary ammonium salt: 0.08%
STROMATOLYSER-4DL	Ethylene Glycol: 96.90% Methanol: 3.00% Polymethine dye: 0.002%
SULFOLYSER	Sodium Lauryl Sulphate: 0.17%

Addendum C

Standard histological processing procedure

STEP	REAGENT	TIME(MIN)	TEMP (°C)
1	70% Ethanol	30	40
2	80% Ethanol	30	40
3	95% Ethanol	45	40
4	95% Ethanol	45	40
5	100% Ethanol	45	40
6	100% Ethanol	45	40
7	100% Xylene	45	40
8	100% Xylene	45	40
9	Paraffin	30	58
10	Paraffin	30	58
11	Paraffin	30	58
12	Paraffin	30	58