COMPARISON OF GENETIC AND IMMUNOLOGICAL RESPONSES TO TICK INFESTATION BETWEEN THREE BREEDS OF SHEEP IN SOUTH AFRICA

by

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DECLARATION

I hereby declare that the thesis submitted for the PhD degree at the University of the Free State is my own work and has not been submitted at another university to obtain any qualification. I therefore cede copyright of the thesis in favour of the University of Free State.

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CHAPTER 1

General Introduction

Sheep farming is one of the major agricultural enterprises in South Africa. Sheep production has several advantages. Firstly, the high reproductive rate, and shorter gestation period make it possible for sheep to have three lambing opportunities in two years. Furthermore, sheep can produce more than one offspring at a time. Secondly, sheep can do well in more arid areas and under conditions where cattle cannot produce optimally in. Thirdly, due to their small size many of sheep breeds can be reared on smaller land areas than cattle. Sheep breeds kept for fibre production also produce wool as a high-value secondary product. Sheep meat is mostly consumed locally while wool is an export product, resulting in an increased stability of the enterprise. The above attributes render sheep production as important to rural sustainability in marginal pastoral areas, alleviating poverty in resource-poor families and meeting the increasing demand of animal protein. Sheep are mainly produced for meat and wool production, in addition skin, mohair and pelt are produced in South Africa (DAFF, 2013).

South Africa is blessed with a wide variety of indigenous, exotic and composite ovine genotypes which are maintained in communal, small-scale and commercial production systems. In 2013 the sheep population was estimated at over 21.247 million (DAFF, 2013).

Sheep farming, like other livestock farming enterprises is faced with challenges of parasites, among them, ectoparasites such as ticks. Ticks are a major constraint to livestock production globally, especially in the tropical areas and affect most domestic animals. They are blood feeding ectoparasites which are capable of transmitting disease-causing pathogens to their hosts if they are infected (Ostfeld *et al*., 2006). Ticks do not only transmit diseases but also cause damage to the skin of their hosts (Spickett *et al*., 2011), resulting in irritation (tick worry) of hosts, lower productivity, blood loss and udder damage (Cloete *et al*., 2013) while they also inject toxins into host animals (Spickett *et al*., 2011). Each engorging female tick corresponds to a body weight reduction of 1.37 ± 0.25 and 1.18 ± 0.21 g in *Bos Taurus* and *B. Taurus* x *B. indicus* crossbred cattle (Jonsson, 2006). In addition, ticks carry numerous diseases that affect as much as 80 % of the worlds' cattle population (Marcelino *et al*., 2012).

Therefore ticks can lead to significant economic losses (Jongejan & Uilenberg, 2004; Rajput *et al*., 2006). Economic losses attributable to ticks comprise of decreased productivity and mostly losses due to costs of controlling both tick infestation and tick-borne diseases. These economic losses may vary according to the environment where livestock production takes place. So far, the impact of ticks on sheep production has not been studied to the same extent as in cattle.

Even though sheep can survive in various and often challenging environmental conditions (Fourie & Horak, 2000), ticks pose a threat to their productivity and general welfare (Fourie *et al*., 1988). Ticks' prevalence in sheep has been reported in Africa, Asia and Europe (Bouhous *et al*., 2011; Moshaverinia *et al*., 2012; Grøva *et al*., 2014). In a study by Horak *et al*. (2006) on the *Ablyomma marmoreum* tick, 46% of the sheep examined were found to be infested with this tick species in most provinces of South Africa (Eastern Cape, Western Cape, Northern Cape, Mpumalanga, Limpopo, Free State and KwaZulu-Natal). The study did not indicate the number of ticks per province, neither did it indicate which provinces had higher or lower tick numbers. The question remains why the 54 % of sheep studied did not have ticks. Defensive mechanisms against tick infestation as discussed in the following paragraph might have played a role to accomplish this.

Various literature sources reported that host animals infested with ticks develop some defensive mechanisms to prevent excessive damage (Roberts, 1968) through homeostasis (Carvalho *et al*., 2010b). Some of the mechanisms employed are complex immune responses against tick feeding (Brossard & Wikel, 2007). Genetic variation of host animals is considered as one of the factors that influence immune responses to tick infestation (de Castro & Newson, 1993). Piper *et al*. (2009) confirmed the effect of genetic variation on immune response to tick infestation in Brahman and Holstein-Friesian cattle in a study reporting lower tick counts in Brahman than in Holstein-Friesian cattle. In addition they reported higher white blood cell counts in tick-infested Holstein-Friesians than in Brahmans, higher levels of IgG1 antibodies specific for tick antigen extracts in the Holstein-Friesian animals' sera than in the Brahman animals' sera. The breeds also had effects on the expression of some cytokines in tick infested cattle. The expression of Interleukin-2 (IL_2) and Tumor necrosis factor-alpha (TNF-α), among others, were higher in Brahman cattle peripheral blood leukocytes than in those of Holstein-Friesian animals (Piper *et al*., 2009).

Marufu *et al*. (2013) also reported differences in hypersensitivity reactions to tick extract between Nguni and Bonsmara cattle. This is an indication that genotype plays a role in the immune response to tick antigens.

The host's genetic variation in response to tick infestation and tick extract inoculations may be used to improve animal production. Selecting animal genotypes that are resistant to tick infestation may reduce the costs of controlling ticks. Immunological responses to tick infestations have been suggested as one of the determinants of the genotypes' resistance or susceptibility to tick infestation (Piper *et al*., 2008; Piper *et al*., 2009; Marufu *et al*., 2013). This is because immunological responses to infestations and infections are considered to be genetically mediated.

Given the association of the immunological responses to host's resistance to ticks, genomics is increasingly used to investigate the mechanisms of innate and acquired resistance to tick feeding (Valenzuela, 2002a). Research on ticks and immunological responses to tick infestation date back to the 1970's, when the histological examination of tick attachment sites suggested that acquired resistance is influenced by immunological factors (Allen, 1973). Comprehensive research has been conducted on the resistance of bovine hosts to tick infestation and it has often been demonstrated that different bovine breeds differ in their resistance to ticks (Seifert, 1971; Wang *et al*., 2007; Piper *et al*., 2008; Maharana *et al*., 2011; Marufu *et al*., 2014). There is paucity of information on genetic and immunological components of resistance to ticks and tick-borne diseases in sheep. This underlines the need to conduct studies to determine the genetic and immunological responses of different breeds to tick infestation.

The sheep breeds used in this study are the Dorper, Namaqua Afrikaner (NA) and South African Mutton Merino (SAMM). The Dorper and SAMM are commercial breeds which contribute respectively 19.3% and 18.1% to the weaning weight records in National Small Stock Improvement Scheme (NSSIS) (Olivier, 2014 reviewed by Cloete *et al*., 2014). The NA is an unimproved, indigenous fat-tailed breed being maintained only in conservation flocks (Cloete & Olivier, 2010; Qwabe *et al*., 2012).

1.1 Justification

There are reports of the emergence of the shift of some tick-borne diseases to previously unaffected areas, findings which are attributable to factors such as acaricide resistance, environmental changes and genetic changes in the vector-borne pathogens (Maharana *et al*., 2011). Thus, it is essential to have a thorough understanding of mechanisms employed by host animals to combat tick infestations. This knowledge will assist to develop rational management and breeding programs. Analyzing tick attachment sites can clarify how hosts defend themselves against tick challenge.

The current most intensively used tick control measure is based on chemicals (acaricides). The extensive use of acaricides to control ticks leads to some tick species developing resistance to these acaricides. Furthermore, acaricides are not a permanent solution in controlling ticks (Frisch, 1998). Chemical residues that remain in milk and meat, in addition to the potential of environmental pollution by used chemicals (Brossard, 1998; Regitano *et al*., 2008), underline the need to find alternative ways of controlling ticks in sheep. One of the more permanent tick control measures that may be useful in an integrated tick control programme is the breeding of animal hosts for tick resistance. Studies on variation in the sheep's genetic and immunological responses to tick infestations are limited. Breeding for tick resistance in sheep may be facilitated by studying components of genetic and immunological resistance to tick infestation. Pertaining to the immunological response aspect, histological parameters and cutaneous hypersensitivity response to tick larvae extract will be examined while the expression of cytokine genes will be investigated. Histological examination is used because some of the host skin reactions to tick attachment have been proven to differ between animals resistant and susceptible to tick infestation in cattle (Marufu *et al*., 2014). Similarly some cytokines have been reported to be highly expressed in cattle more resistant to ticks compared to those that are susceptible. Investigating these scenarios in sheep may yield useful information for future research and animal production improvement in this species.

The differences in tick counts between Dorper, NA and SAMM sheep at the Nortier Research Farm, Western Cape have been reported (Cloete *et al*., 2013). Therefore, the aim of this study was to investigate differences in genetic and immunological responses to tick infestation in

these breeds and to estimate additive and non-additive effects associated with tick resistance in sheep.

1.2 Objectives

1.2.1 Main Objective

The main objective of the study was to investigate differences in genetic and immunological responses to tick infestation in three South African breeds of sheep (NA, Dorper and SAMM).

1.2.2 Specific Objectives

The specific objectives of the study where three sheep breeds were involved were to:

- estimate genetic and crossbreeding parameters for tick count and weaning weight
- examine the histology of tick attachment and control sites
- select reference genes suitable for normalizing gene expression data in this study
- compare cytokines gene expression at tick attachment sites and control sites
- compare cutaneous hypersensitivity reactions to unfed larvae extracts of *Rhipicephalus evertsi evertsi* between NA, Dorper and SAMM sheep.

CHAPTER 2

Literature review

2.1 Introduction

This review chapter will consider the following aspects: sheep production in South Africa, a brief description of the breeds that are used in this study, tick species parasitizing sheep, the effects of tick infestation on animal productivity, health and general welfare of the animals. Furthermore tick resistance and methods used to measure tick resistance, genetic parameters of tick resistance, and responses of the host to tick infestation are reviewed. Finally cytokines and techniques used for gene expression studies are discussed and gaps in current knowledge are exposed.

The three breeds that are used in this study are two commercial breeds namely the Dorper and the South African Mutton Merino (SAMM) and one indigenous breed, the Namaqua Afrikaner (NA). It is necessary to know the origin and characteristics of these breeds as it may influence their adaptation to tick challenge. It could be argued that the origin of the animal leads to adaptability which also impacts on resistance to parasites or diseases. The reaction of the animal to parasitic challenges is associated with anatomical and physiological characteristics. The following paragraph briefly highlights tick species parasitizing sheep.

More than 850 tick species are reported world-wide. As a broad classification, *Ixodid* (hard) ticks and *Argasid* (soft) ticks form the two main families of ticks (Tongjura *et al*., 2012). The *Ixodid* ticks are of more economic importance to livestock as compared to *Argasid* ticks. *Ixodid* tick species are predominant, constituting about 80% of tick species in South Africa (Spickett *et al*., 2011). Despite the higher frequency of *Ixodid* tick species, six species have been found to be the predominant parasites of importance in South African sheep (Fourie *et al*., 1988). These are *Amblyomma hebraeum*, *Ixodes rubicundus*, *Rhipicephalus evertsi evertsi*, *Rhipicephalus glabroscutatum*, *Hyalomma truncatum* and *Hyalomma rufipes*. This

may not be the case in other countries in Africa as the prevalence of tick species is influenced by environmental conditions.

There are various production losses in sheep attributable to ticks: Firstly, ticks attach to the skin of the animal causing damage which results in significant losses to the leather industry as the skin value is reduced (Tongjura *et al*., 2012). Secondly, when ticks are attached to the animals, they suck blood which can lead to anemia in high infestations (Jonsson, 2006; Rajput *et al*., 2006). Thirdly, the attachment of tick provokes the immunological response of the host which may lead to some itching and inflammation causing irritation to the host: this may cause discomfort to the animal and reduce feed intake, which may lead to weight loss (Jonsson, 2006; Rajput *et al*., 2006). Fourthly, ticks act as vectors of pathogens and may transmit diseases, such as theilerioses and babesioses, which are protozoan diseases and rickettsial diseases (anaplasmosis and heartwater) (Jongejan & Uilenberg, 2004). Tick-borne diseases have been reported as one of the greatest challenges in livestock production (Gray *et al*., 2009). Udder and teat damage in female animals are also commonly recorded in cattle (Ndhlovu *et al*., 2009) and sheep (Cloete *et al*., 2013). Lastly, the farmers are compelled to routinely dip their animals to control ticks resulting in higher input costs. Ticks may also become resistant to the accaricides used, compromising the sustainability of livestock production (Ntondini *et al*., 2008).

The costs of combating ticks in cattle have been reported in various parts of the world (Meltzer *et al*., 1996; Mukhebi *et al*., 1992; Minjauw & McLeod, 2003). A cost burden of up to US$168 million annually have been reported for tick control in eastern, central and southern Africa (Mukhebi *et al*., 1992). The cost of controlling ticks and tick-borne diseases in the sheep industry have not been established and no reliable figures were found at the time of writing this manuscript.

Even though information on the economic losses due to ticks in sheep could not be sourced from the literature, it does not mean that ticks are not important in sheep. The prevalence of some tick species in sheep is evidence that researchers should not turn a blind eye on the importance of ticks in the sheep industry. In Iran sheep was the third most important livestock host species infested by ticks after cattle and goats (Sofizadeh *et al*., 2014). Similarly, a survey of livestock ticks in the North West province in South Africa by Spickett *et al*. (2011) showed that sheep were the second most important host species after cattle and they were

infested by the following tick species: *Hyalomma rufipes* and *Rhipicephalus (Boophilus) decoloratus*. Other tick species that infested sheep, even though at a lower percentage compared to cattle and goats, were *R. appendiculatus, R. evertsi evertsi* and *R. simus*.

2.2 Sheep production in South Africa

Sheep production forms an integral part of livestock production in South Africa. It is practiced throughout the country. However, it is more extensively practiced in more arid provinces, namely: Free State, Northern Cape, Western Cape and parts of the Eastern Cape (DAFF, 2012).

2.2.1 Breeds description

South Africa supports a wide variety of indigenous, exotic and composite ovine genotypes which are maintained in communal, small-scale and commercial production systems.

2.2.1.1 Namaqua Afrikaner (NA)

The NA sheep breed is one of the oldest indigenous sheep breeds and is well adapted to the harsh and challenging environment of the southwestern Cape. Its origin is not well-defined except that the breed is known to have migrated to Southern Africa together with the Khoi people and is considered one of the true indigenous breeds (Soma *et al*., 2012). NA sheep are fat-tailed with hairy coats, long-legged and are mainly produced to provide meat. This breed either has a black or red/brown head, with black headed ones being dominant (more than 60%) (Qwabe, 2011). It has a twisted tail which turns either right or left (Campbell, 1995). There is limited commercial use of the breed; it is at present mostly maintained for conservation purposes (Cloete & Olivier, 2010). Its limited commercial use may be attributed to a lower meat yield compared to the Dorper and SAMM breeds, among others contributed to a higher percentage of bone in the carcass (Burger *et al*., 2013).

2.2.1.2 Dorper

The Dorper sheep is a composite breed which was locally developed in the 1930's by the Department of Agriculture of South Africa from a cross between Dorset Horn rams and Blackheaded Persian ewes (Soma *et al*., 2012). It was officially accepted as a breed in 1950 (Fourie & Horak, 2000). The black-headed Dorper is large bodied with a black head and a white body, contributing about a quarter of the records to the National Small Stock

Improvement Scheme database (NSSIS; Cloete & Olivier 2010). The breed is well adapted to different climatic and grazing conditions and is considered to be very productive in terms of fertility and meat production, the lambs can have a carcass dressing percentage of up to 50% (Cloete *et al*., 2000). It has a thick skin which is covered with a mixture of hair and wool.

2.2.1.3 South African Mutton Merino (SAMM)

The SAMM is the dominant commercial dual-purpose breed in SA contributing about 18.1% of the weaning weight records to the NSSIS database in 2010 to 2011 as reviewed by Cloete *et al*. (2014). It can produce apparel wool of fairly good quality in addition to meat and is generally classified as a fine-wool sheep. This breed originates from Germany and was derived from the German Mutton Merino or Deutsche Merino Vleisschaf (Soma *et al*., 2012).

2.3 Tick species parasitizing sheep

Seven genera of ticks are the most important in domestic animals in Africa. Among them are *Amblyomma, Boophilus, Haemaphysalis, Rhipicephalus* and *Hyalomma* (Walker *et al*., 2003). There is general consensus that the most important and widely spread ticks in Africa are the genera *Amblyomma* and *Boophilus* (Abebaw, 2004; Fantahun & Mohamed, 2012). In their survey on the distribution of tick species in and around Assosa in Ethiopia, Fantahun & Mohamed (2012) found *B. decoloratus, A. coherence*, *R. evertsi evertsi* and *A. variegatum* to be the most prevalent in cattle. In Iran the dominant tick genera in domestic animals were *Rhipicephalus*, *Hyalomma*, *Haemaphysalis*, *Ixodes* and *Boophilus* (Sofizadeh *et al*., 2014). This is an indication that even though tick species' prevalence may be different from one area to the other, *Boophilus, Rhipicephalus* and *Amblyomma* species are widely distributed.

Globally, sheep are infested by 10 genera of ticks (Liebisch, 1997). There is contradictory evidence in the literature pertaining to the number of tick species that parasitize sheep in South Africa. Six out of the 25 tick species infesting sheep have been documented as important (Fourie *et al*., 1988; Fourie & Kok, 1995). These tick species are *Amblyomma hebraeum, Hyalomma marginatum rufipes, Hyalomma truncatum, Ixodes rubicundus, Rhipicephalus glabroscutatum* and *Rhipicephalus evertsi evertsi*. Fourie & Horak (2000) however reported 17 tick species parasitizing Dorper sheep in South Africa. Species of four common genera are found in sheep in South Africa and are briefly discussed below.

2.3.1 *Rhipicephalus species*

Rhipicephalus evertsi evertsi is considered one of the dominant *Rhipicephalus* species infesting Dorper sheep, especially adult ticks (Fourie & Horak, 2000). This is consistent with findings of Horak *et al*. (1991) indicating that *R. evertsi evertsi* was the most abundant sheep tick. Cloete *et al*. (2013) accordingly reported that about 50% of ticks detached from sheep at the Nortier Research Farm belonged to the species *R. evertsi evertsi.* This species was followed by *R. nitens* in various farms in the North-Eastern Orange Free State and the Eastern Cape provinces (Horak *et al*., 1991). The peak periods for *R. evertsi evertsi* abundance are March to June for immature stages and March to May for adult ticks with a minor peak of adult ticks in October and November (Horak *et al*., 1991). *Rhipicephalus evertsi evertsi* can cause paralysis in adult sheep but predominantly in lambs (Gothe & Bezuidenhout, 1986). The adult engorged ticks can only be toxic and thus cause paralysis if they have reached a weight of 15 to 21 mg (Gothe & Bezuidenhout, 1986). This species has been reported to prefer smooth skin, such as, under the tail, the inguinal region and perineum (Fantahun & Mohamed, 2012). Other *Rhipicephalus* species reported in sheep are *R. glabroscutatum* and *R. neumanni*. Adults ticks of both species prefer to attach between the hoofs of their hosts.

2.3.2 *Amblyomma species*

The species of *Amblyomma* especially, *A. hebraeum* are vectors of the pathogen that cause cowdriosis in sheep. According to Spickett *et al*. (2011), *A. hebraeum* made up 17 % of the total number of ticks collected in their study in South Africa. Adult ticks of this species were present throughout the year, with a peak in summer (November and December) in the North-eastern region. In the central regions higher numbers were recorded in autumn (between March and May) (Spickett *et al*., 2011).

2.3.3 *Hyalomma species*

The most dominant species in *Hyalomma* genus is reported to be *H. marginatum rufipes* in the Free State province (Fourie *et al*., 1988). However, seven years later Kok & Fourie (1995) documented that *H. truncatum* was the dominant species in the same province. The latter species was also the most important representative of the genus *Hyalomma* in the study of Cloete *et al*. (2013) on the South African west coast. These literature sources provide evidence that the dominance of tick species within the same genus may differ in the same

area at different seasons of the year and/or in different years. The *Hyalomma* species was reported to be found on sheep throughout the year. Mostly mature ticks were present on sheep, as the immature stages prefer small mammals as hosts (Horak *et al*., 1991). This was later confirmed by the findings of Horak & Fourie (1992). *Hyalomma* species preferred to attach at the anogenital or inguinal region of Dorper sheep. Fourie & Kok (1995) reported that more than 60% of *Hyalomma* species were found in the above-mentioned regions. The preference of attachment sites does not differ in Merino sheep as Kok & Fourie (1995) observed that *Hyalomma* species were attached to the axilla, inguinal and anogenital regions. However, no ticks were recorded on the anogenital region of lambs.

Hyalomma species can cause wounds and swelling in sheep because of their long mouthparts and their tendency of clustering at attachment sites. According to Kok & Fourie (1995) clusters of more than 50 ticks at an attachment site can occur in sheep. *Hyalomma trancatum* is the main cause of lameness in infested Merino lambs when the ticks attach to the interdigital clefts and feet of lambs (Kok & Fourie, 1995).

2.3.4 *Ixodes species*

Ixodes species are widely distributed in the provinces of Free State and Western Cape of South Africa (Walker, 1991). *Ixodes* species, in particular, *I. rubicuntus* which is referred to as the Karoo Paralysis tick, produces toxins that cause paralysis in sheep, which can lead to substantial production losses (Fourie & Horak, 2000). The probability of sheep being paralysed depends on the level of infestation.

2.4 The effects of tick infestation on animal productivity, health and general welfare

It has been established in cattle that tick infestation has a substantial impact on the productivity of animals. Scholtz *et al*. (1991) reported a reduction of up to 8.9 g weaning weight per engorged female tick recorded on the animal. This indicates that if tick burden is high there can be a marked loss in overall production. Similarly, Jonsson *et al*. (1998) confirmed the detrimental effect tick infestation can have on animal productivity by reporting reductions of 8.9 ml in daily milk yield and of 1.0 g in body weight in dairy cattle infested with ticks.

Ticks in sheep can cause body weight loss through several ways, which may adversely affect production. Ticks attach to the sheep and cause irritation, discomfort that end up affecting the normal grazing behaviour of the animal; hence the loss of weight or lack of weight gain (Hamito, 2010). Ticks also cause wounds in the skin of the animal, which are prone to secondary infection in addition to causing the animal to become anemic. When the ticks attach to the claws of sheep in aggregates, they cause lameness, especially in lambs (Kok & Fourie, 1995). Lameness in lambs affects normal grazing and causes weight loss. Subsequent ulceration caused by ticks attached to sheep has a negative effect on production. Cloete *et al*. (2013) published evidence that linked udder health in sheep to tick infestation (Figure 2.1). The damaged udder of the ewe may lead to the refusal of the ewe to allow suckling by lambs, resulting in weight loss or death. Moreover, ticks can also transmit toxins at the attachment site that may lead to paralysis in lambs (Gothe & Bezuidenhout, 1986). Tick infestation was associated with significantly higher zinc deficiency, emaciation, alopecia and hyperkeratosis in naturally infested sheep when compared to control sheep. The hematology parameters were also found to be affected in tick infested sheep (Mustafa, 2013). Therefore, a lack of tick control measures in a heavily infested area can lead to production losses.

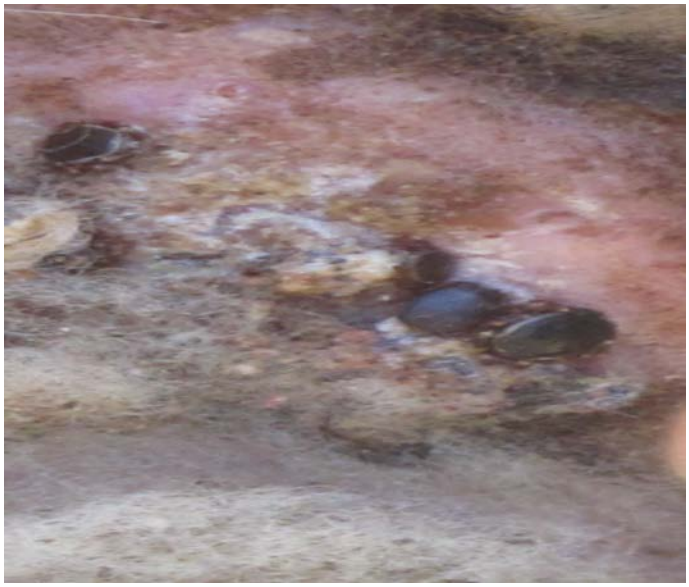

Figure 2.1 Udder damaged by tick infestation

Apart from impacting on general welfare of the sheep, ticks can also transmit disease-causing pathogens, which can also cause some serious losses in productivity (Yin *et al*., 2002; Abunna *et al*., 2012). Yin *et al*. (2002) also observed that *H. qinghaiensis* were capable of infecting sheep in China with a disease-causing pathogen, *Theileria spp*. The authors noted that 50 adult ticks of *H. qinghaiensis* infesting sheep are sufficient to cause the infection. The ticks that were used for infestation were collected from grasses in the pastures. These findings indicate that sheep kept under extensive grazing are vulnerable to disease-causing pathogens transmitted by ticks. Where the leather industry is important, ticks can cause a marked loss in monetary income resulting from skin damage during infestation (Gbolagade *et al*., 2009).

2.5 Tick resistance and methods used to measure tick resistance

Host resistance to ticks refers to a phenomenon of genetic adaptation that allows the host to be less susceptible to infestation (Raberg *et al*., 2007). It has been concluded that bovine host resistance to ticks is influenced by genes at several loci (Regitano *et al*., 2008). However, there are several defense mechanisms used by hosts to combat ticks. These mechanisms include grooming by the host, skin characteristics, specific immunological responses and other breed characteristics (Minjauw & de Castro, 2000). Resistant animals affect tick feeding by either preventing ticks to successfully attach or by reducing blood intake by the attached ticks and thus limiting successful engorgement (Tatchell, 1987). Resistance is manifested by a reduced tick count, a reduced number of engorged ticks, a lower egg production by female ticks, decreased viability of eggs and reduced susceptibility to tick-borne diseases (Wikel, 1996; Willadsen & Jongejan, 1999).

Differences in tick loads of different breeds of cattle have been well-researched (Scholtz *et al*., 1991; Silva *et al*., 2007). Previous studies have shown the genetic basis for variation in tick counts (Budeli *et al*., 2009). Although breed differences have been well-researched in cattle (Scholtz *et al*., 1991; Silva *et al*., 2007), there is little information regarding genetic differences in small ruminants. Cloete *et al*. (2013) observed differences in tick counts among the NA, Dorper and SAMM sheep breeds. The NA had generally lower tick counts on the udder and hind legs, while they also had less tick damage to their udders. Moreover, udder and hind leg tick counts as well as udder health scores were repeatable (0.58 and 0.75, respectively) with a significant between animal correlation amounting to 0.47 between the

traits. The significant breed differences, as well as the observed repeatability coefficients, thus suggest that tick resistance may have a genetic basis in sheep as well. Ovine host resistance to tick infestation is expected to be a valuable asset in an integrated tick control program.

Measuring host resistance to tick infestation is a complicated undertaking/assignment; currently two methods of measuring tick resistance in animals are commonly used. The first method used is counting the number of ticks infesting the animal (Budeli *et al*., 2009; Ayres *et al*., 2013). This method assumes that ticks have difficulty attaching and blood feeding from resistant animals, and as a result resistant animals will have a reduced tick burden compared to susceptible animals under similar tick challenge. The tick counting method is easy to use in naturally infested animals, where the assumption is that all the animals are exposed to the same tick challenge; thus the difference in tick count is due to resistance or susceptibility. The second method is counting the number of engorged ticks (Roberts, 1968; Jonsson *et al*., 2000). This method is feasible in artificial tick infestation studies because one has to know how many ticks the animals were exposed to and how many out of them failed to attach and engorge. This method assumes that resistant animals have better mechanisms of making blood feeding difficult for the ticks; hence the number of engorged ticks is lower compared to that for susceptible animals. Also, the lack of success in blood feeding may lead to some ticks detaching from the animals before they are fully engorged.

2.6 Genetic parameters for tick resistance

Various factors that influence tick burden in sheep include season of the year, different years and magnitude of tick challenge. Arnold & Travassos Santos Dias (1983) reported that tick numbers per sheep can go up to 10 depending on the season of the year and levels of tick challenge. However, breed differences are commonly regarded as the first indication of genetic variation for traits not yet assessed in studies on genetic (co)variance components. Cloete *et al*. (2013) recorded higher numbers of ticks on the SAMM breed and found that the indigenous NA ewes had a lower tick count compared to the commercial breeds (Dorper and SAMM) on the front and hind parts of the animals. This is an indication that the sheep breed can also influence tick burden as is the case in cattle. However, further studies are required. Since there is not much information on the genetic parameters for tick counts in sheep, the

discussion in this section will focus more on other livestock species to give an idea of the expected genetic variation in tick resistance in general.

2.6.1 Heritability estimates and repeatability

Host resistance to ticks has been reported to be influenced by genetic factors and therefore it can be transmitted to the offspring. Heritability estimates of tick resistance have been reported in different breeds of cattle (Davis, 1993; Burrow, 2001; Budeli *et al*., 2009). On average, the heritability estimate of tick resistance in cattle is 0.30, with the reported minimum amounting to 0.13 (Prayaga *et al*., 2009) and the maximum to 0.42 (Burrow, 2001). However, it is argued that the heritability of tick resistance is above 0.2 and that the minimum report (0.13) may be due to either the method used to measure tick resistance or to a low tick challenge (Porto Neto *et al*., 2011). Another possible reason of low heritability estimates is either low additive variance or high residual variance. Heritability estimates had the tendency of increasing with an increase in tick counts (Budeli *et al*., 2009). The moderate heritability estimates of tick resistance in cattle, linked to substantial phenotypic variation, indicates that this trait has substantial genetic variation and can therefore, respond to selection. Heritability for tick counts have been estimated at 0.32 to 0.59 using different analysis in Norwegian sheep (Grøva *et al*., 2014), suggesting that worthwhile genetic gains should be achievable. It is essential to know the magnitude of these estimates before deciding to select for this trait, or to include it in selection objectives.

A repeatability estimate of 0.45 was reported for tick counts in cattle (Mackinnon, 1990). By definition, repeatability is the total of genetic and animal permanent environmental (PE) effects and it is important for current flock gains. In the absence of animal PE variation, repeatability can also be seen as an upper boundary of heritability. If the observed repeatability estimates can indeed serve as a good indication of genetic variation, these results suggest that cattle can be selected to reduce tick counts. The repeatability of tick count in sheep has been estimated to be as high as 0.58 (Cloete *et al*., 2013), leading the authors to conclude that future generation and current flock gains are likely. Genetic variation for tick loads has been established in the Norwegian White sheep breed with the repeatability of tick loads ranging from 0.37 to 0.69 depending on the analysis used (Grøva *et al*., 2014).

Variance components form an important component of genetic parameters as it gives an indication of the ability of a population to respond to selection (Houle, 1992). The additive

genetic variance for tick counts in cattle has been found to range between 0.01 and 0.08 (Budeli *et al*., 2009). This value increased with an increase in tick counts, while the phenotypic variance decreased with increasing tick count in Bonsmara cattle (Budeli *et al*., 2009). The authors reported phenotypic variances of 0.41 to 0.67 and animal permanent environmental variance ranging from 0.00 to 0.03. They concluded that the permanent environmental variance was negligible and that there is adequate genetic variation in tick counts for worthwhile genetic progress in cattle. Variation among individual sheep for resistance to internal parasites such as nematode parasites has commonly been reported (Stear *et al*., 1999; Morris *et al*., 2009; Bishop, 2011). However, genetic variation in ovine host resistance to tick challenge studies are limited (Grøva *et al*., 2014).

2.6.2 Correlations between tick counts and growth as manifested by weaning weight

Alani & Herbert (1987) documented retarded growth and anemia on tick-infested lambs compared to non-infested lambs. More research on sheep parasites has been done on the genetic basis of resistance to internal parasites (Doeschl-Wilson *et al*., 2008; Karlsson & Greeff, 2012). There is, however, limited literature on the correlation of external parasite infestation with growth in sheep. Grøva *et al*. (2013) estimated the correlation of *Anaplasma phagocytophilum* infestation (which is transmitted by the tick species *Ixodes ricinus*) with growth of Norwegian lambs.

The only study that could be sourced from the literature reported that tick counts and weaning weights were uncorrelated for all practical purposes in cattle (Mackinnon *et al*., 1991), with phenotypic and genetic correlations of 0.04 and 0.02, respectively. The absence of a sizable correlation and a lack of comparable literature references suggest that more research has to be done.

2. 7 Physiological response to tick infestation

The skin of the animal is the first line of defense in the immunological response against external parasites. Tick-infested animal responds to infestation in various ways to prevent transmission of pathogens from the tick to the host. It is anticipated that these ways can be better understood by performing histological examinations of tick attachment sites. When a tick attaches, it destroys the skin tissue and blood vessels beneath the tip of its mouthpart, providing a pool of blood from which the tick can feed (Brossard & Wikel, 2004) resulting in

the formation of a wound or abscess (Gashaw & Mersha, 2013). This happens because a tick bite may lead to dermal necrosis, hemorrhage and inflammation and sometimes hypersensitivity reactions (Piper *et al*., 2010). Inflammation at the tick-bite site is thus an obvious sign of the response of the host to the infestation. The common lesions caused by ticks on the skin of hosts are crusts and scabs (Constantinoiu *et al*., 2010; Gashaw & Mersha, 2013). In some studies, tick attachment sites were characterized by papules and wheals, as well as hyperemic and edematous lesions (Chanie *et al*., 2010). The other features of tick-bite sites observed in previous studies are mild swelling, erythema, eosinophilic mass in the dermis, hyperplasia, cellular edema and necrosis in the epidermis (Szabo & Bechera, 1999; van Der Heijden *et al*., 2005; Constantinoiu *et al*., 2010).

No apparent damage to the epidermis of primary and secondary infested cattle were observed while the tertiary infested cattle had sub-epidermal edema and focal spongiosis with micro-vesicles in the epidermis (Allen *et al*., 1977). These findings were confirmed by Szabo & Bechara (1999) in guinea pigs. These are interesting findings as one would assume that naïve animals would be more hypersensitive to tick attachment and have more severe skin reactions. Francischetti *et al*. (2010) associated these findings with the fact that, at first exposure to ticks, only the innate immunity is involved by way of inflammation, while during secondary infestations both innate and acquired immune responses are invoked. Results reported by Wada *et al*. (2010) also concurred with this idea by showing that animals developed resistance after repeated tick infestation.

Apart from the destruction of skin tissue structure, tick bites also cause some cellular reactions at the site. It is believed that some cells in the epidermis, such as, Langerhans cells are involved in the production of antibodies against tick salivary antigens (Allen, 1994). There has also been a claim that the vesicles that form in the epidermis under the tick attachment site, are caused by the reactions between the antigens in tick saliva and the antibodies in the epidermis of sensitized hosts (Allen, 1994).

The hypersensitivity reactions observed at the tick attachment sites include basophil, mast cell degranulation and neutrophil and eosinophil infiltration (Preston & Jongejan, 1999), and these cells are all associated with resistance (Piper *et al*., 2010). The infiltration of cells leads to epidermal vesiculation, bulla formation and sometimes eventually to serous exudation. Mast cells presumably play a major role in tick resistance as they promote grooming in

animals and thus reduce tick burden. Verissimo *et al*. (2008) confirmed that there is a negative correlation between the number of mast cells in the skin and tick count. Breeds of cattle with higher mast cell counts had lower tick counts of *R. microplus*. Neutrophils are involved in local inflammatory response (Brossard & Wikel, 2004). Van der Heijden *et al*. (2005) recorded significantly higher numbers of total cells, basophils, mast cells and eosinophils at the site of the tick-bite lesion than in control samples taken away from the site (Figure 2.2). Furthermore, the attraction of leukocytes to the attachment site can either disturb tick feeding or induce grooming, which in turn leads to a reduced tick burden (Allen, 1994).

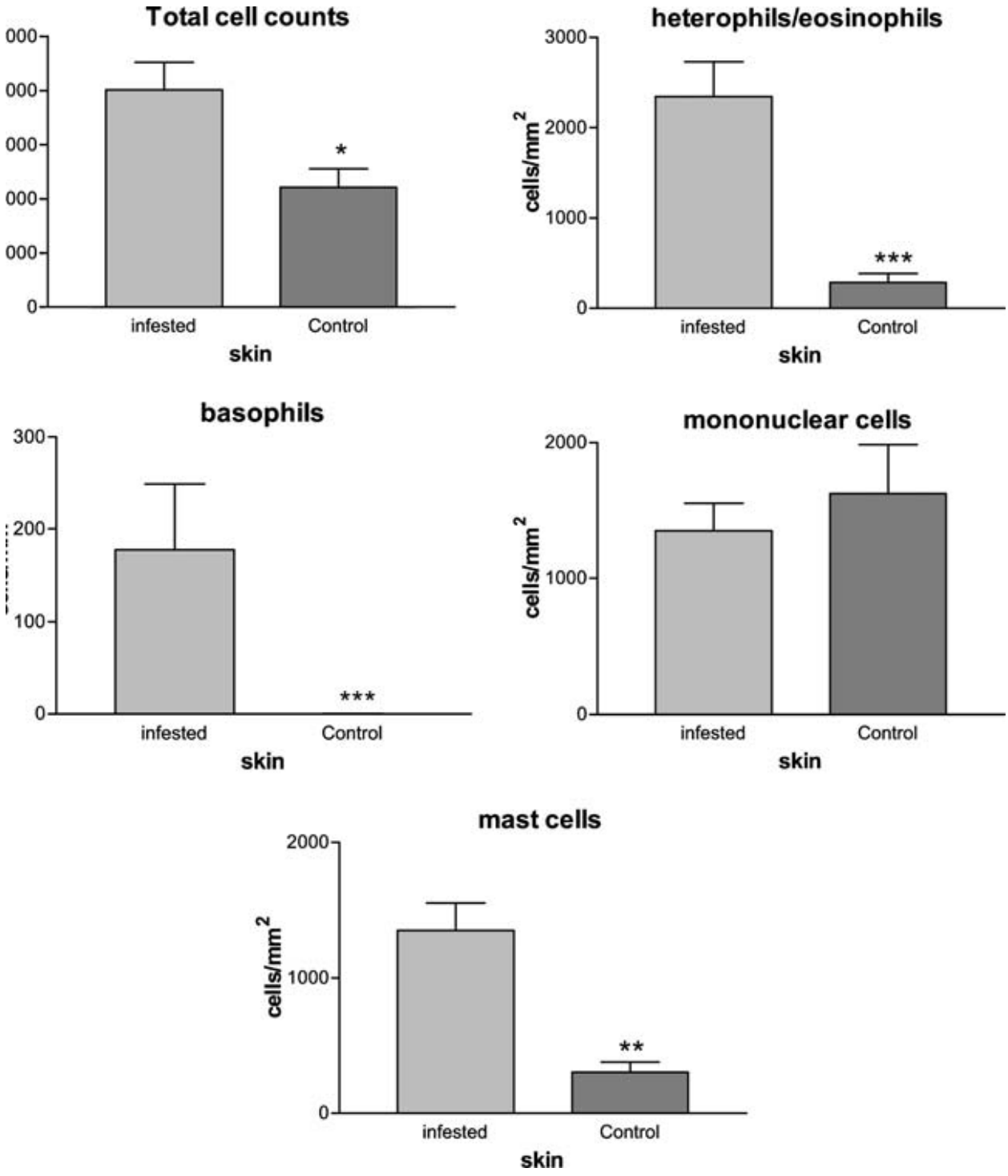

Figure 2.2 Bar graphs of total and differential cell counts at tick attachment sites of naturally infested capybaras and control sites (adapted from van Der Heijden *et al*., 2005)

Allen *et al*. (1977) reported that basophils were the first leukocyte cells to reach the tick-bite site. These basophils are regarded to play an important role in acquired tick resistance. Wada *et al*. (2010) found that, when basophils are removed from mice, the acquired tick resistance

is lost. These findings of Wada *et al*. (2010) concurred with observations from previous studies pertaining to the importance of basophils in acquired tick resistance (Allen, 1973; Brown & Askenase, 1981; Brown *et al*., 1982). Basophils are considered an essential source of cellular Th2-type cytokines (Karasuyama *et al*., 2011). Mast cells have also been associated with acquired tick resistance in mice (Matsuda *et al*., 1990). Wada *et al*. (2010) reported that, although both basophils and mast cells are required for tick resistance, the former contributed directly to antibody-mediated manifestation of tick resistance. Mast cells and basophils secrete histamine which is important in mediating inflammation and promoting immune responses such as swelling and redness (Nuttall & Labuda, 2004). Eosinophils were also found to be involved in tick resistance of guinea pigs and also in sheep during tertiary infestation (Abdul-Amir & Gray, 1987; Szabo & Bechara, 1999).

There is evidence that previous exposure of animals to ticks increased their reaction to future tick infestations. There were an increased number of basophils and neutrophils infiltrating the epidermis of previously infested animals compared to first and second time infested animals (Abdul-Amir & Gray, 1987; Szabo & Bechara, 1999; Boppana *et al*., 2005). Boppana *et al*. (2005) also observed infiltration of neutrophils, macrophages and lymphocytes around the tick attachment site in sheep infested with adult *Hyalomma anatolicum anatolicum* ticks. The importance of these leukocytes has also been observed in another study on sheep (Abdul-Amir & Gray, 1987). Neutrophils play an important role in preventing the transmission of pathogens from ticks to the host animal by phagocytizing particularly *B. burgdorferi* organisms (Nuttall & Labuda, 2004). They infiltrate the tick bite site in mass within a few hours after infestation (Menten-Dedoyart *et al*., 2012). The formation of tick feeding lesions and the development of tissue damage are also attributed to the neutrophils. Leukocytes were also found to increase in the blood of naturally tick infested sheep compared to non-infested sheep (Mustafa, 2013). These findings indicate that neutrophils, basophils and eosinophils are some of the main components of immunity acting against tick infestation.

Tick resistance through skin reactions during tick infestation has been studied in different species and was found to differ between species (Szabo & Bechera, 1999). Constantinoiu *et al*. (2010) and Piper *et al*. (2009; 2010) observed variation between and within cattle breeds in immunological responses to tick infestation at the tick attachment site. The variation observed included leukocyte infiltration in the epidermis and dermis of cattle. Their results suggest that there is variation in skin reactions towards tick infestation between different

breeds of cattle. Since South African sheep production systems use different breeds, there is need to investigate the variation in tick resistance of local sheep breeds.

2. 8 Genetic and Immunological resistance implicated by gene expression level

Host resistance to tick attachment is a complex series of reactions which is influenced by various components, such as the environment, physiology (reproductive status), gender, coat characteristics, age of the animal and climate (Marufu *et al*., 2011). In addition, there is also a genetic component contributing to immunological factors that influence it (Regitano *et al*., 2008, Anderson *et al*., 2013). The genetic variation between individuals in their resistance to pathogens has been established. Genetic variation studies for ovine resistance to lice and fly strike have also been done (Pfeffer *et al*., 2007; Scholtz *et al*., 2011).

Host resistance to tick attachment is regulated by immunological reactions; thus making immune resistance an important tool in protecting the host against ectoparasite infestation and burden (Wada *et al*., 2010). Immunological mechanisms that may be induced by tick feeding include antibodies, antigen-presenting cells, T-cells and cytokines (Wikel, 1982). It has been suggested that there are different immunity mechanisms by which different breeds resist tick challenge (Piper *et al*., 2009). An animal's immunity against ticks is promoted by repeated infestations. According to Brossard & Wikel (2004) and Maharana *et al*. (2011) both innate and specific acquired immune defenses are invoked against tick infestation. The host animal directs the immune responses at the tick salivary proteins to prevent the transmission of disease agents while simultaneously affecting tick feeding (Maharana *et al*., 2011). Narasimhan *et al*. (2007) observed that guinea pigs infested with ticks showed an immune response 24 hours after tick attachment. This observation suggests that 24 hours of tick infestation is sufficient to stimulate an immune response in the host animal. Anderson *et al*. (2013) reported fewer ticks in buffaloes with a stronger innate immunity than in buffaloes with a weaker innate immunity. These findings accord with the suggestion that, not only acquired immunity is involved in tick resistance, but the innate immunity as well.

Some manifestations of immunity to ticks have been reported in sheep. Barriga *et al*. (1991) found that native sheep demonstrated higher levels of resistance to tick infestation at the fourth infestation with *Amblyomma americanum* compared to the first infestation. This resistance was manifested by an extended period taken by the ticks to detach, lower weights of ticks during detachment, engorgement per day as well as the fertility and offspring

development of ticks. Barriga *et al*. (1991) found that the effect of immunity manifested well during the fourth infestation. The inverse relationship between manifestation of resistance and antibody responses allowed Barriga *et al*. (1991) to conclude that antibodies play a limited role in anti-tick protective immunity. Stuen *et al*. (2011) also found differences in immunological responses to infestation between two Norwegian sheep breeds infected with pathogens transmitted by ticks.

There are various ways of assessing the immunological responses to tick infestation in animals. Boppana *et al*. (2004) used circulating T- and B-lymphocytes to determine the response of the sheep immune system to *H. anatolicum anatolicum* infestation. Their results showed an increased CD4/CD8 and decreased T/B lymphocytes ratios in all infested sheep. The immune response of the host to tick infestation can be determined by assessing the expression of immune genes. Gene expression gives an idea of the genes and biological mechanisms used by host animals to respond to tick infestation (Porto Neto *et al*., 2011). The skin of the infested animal is the target for performing gene expression in most studies on tick resistance because this is the organ where ticks attach. Wang *et al*. (2007) reported differences in gene expression between cattle exhibiting high resistance to ticks compared to those that exhibited low resistance to ticks. During a follow-up study to confirm the findings of Wang *et al*. (2007), Piper *et al.* (2008) reported that differences in gene expression were in genes responsible for innate inflammation which included toll-like receptors (TLR5, TLR7 and TLR 9), chemokines (CCR1, CCL2 and CCL26) and cytokines (e.g. IL-1 β and IL-10). The cytokine genes have been reported to play a significant role in inflammation during infection (Zehnder *et al*., 2003). The down-regulation of some interleukins has been reported in resistant cows compared to susceptible animals (Regitano *et al*., 2008). Therefore, the quantification of cytokine gene expression may elucidate the different genetic and immunological responses in sheep.

2.8.1 Cytokines

Cytokines are immunomodulation molecules that are secreted by specific cells of the immune system and are involved in immunoregulation (Giulietti *et al*., 2001). Cytokines are considered as chemical mediators of inflammation and immunity (Nuttall & Labuda, 2004) and play a significant role in cell-mediated immunity and allergic responses (Science commentary, 2000). In addition, they are important for the development and functioning of

the innate and adaptive immune system (Turner *et al*., 2011). Cytokines have effects on other cells by transferring signals locally between cells; their action is autocrine and paracrine. This may be because the signals must be released around the pathogen-infected or the parasite attachment site cells before other immune molecules could follow the signal and arrive at the site. Cytokines direct the inflammatory response to the site of injury or infection (Cunha *et al*., 2004; Johnston & Webster, 2009). The host cells affected by the pathogen stimulate the release of the cytokines. This action recruits other immune cells to increase immune response at the infestation site.

There are many cytokines, which are divided into two main groups; inflammatory (e.g. Tumor necrosis factor-alpha (TNF-α) and Interleukin-1 (IL-1)) cytokines and anti-inflammatory (e.g Interleukin-10 (IL-10)) cytokines (Johnston & Webster, 2009). Cytokines are mainly produced by T-lymphocytes. The T-lymphocytes determine resistance and susceptibility to infections (Stenger & Rollinghoff, 2001). There are two subsets of T-lymphocytes; namely cells with CD4 surface molecules and cells with CD8 surface molecules. The CD4 cells are the main producers of cytokines, which can further be divided into Th1-cytokines and Th2-cytokines (Ferreira & Silva, 1999). Th1-cytokines are responsible for inflammatory responses during infection or parasite infestation, while Th2-cytokines are important for anti-inflammatory action. The former include interferon gamma and are important components of acquired resistance to ticks, whereas the Th2-cytokines include Interleukins (4, 5, 10 and 13) (Ferreira & Silva, 1999). Ferreira & Silva (1999) observed an up-regulation of Th2-cytokines (IL-4 and IL-10) in lymph nodes that drain tick attachment sites in tick-infested mice. Piper *et al*. (2009) reported T-cell-mediated responses to tick infestation in different breeds of cattle and that CD4 T-cells were significantly higher in the tick-resistant Brahman cattle compared to more susceptible Holstein-Friesian cattle in the peripheral circulation. Analysis of cytokines is vital for understanding immune response to parasites (Wikel, 1997).

2.8.1.1 Interleukin – 8 (IL-8)

Interleukin–8 (IL-8) is a chemokine, a subgroup of cytokines that has chemotactic activities (Vancova *et al*., 2010). This pro-inflammatory cytokine is produced by several immune cells and is responsible for the accumulation of neutrophils at the local site of infection or parasite infestation. Baggiolini & Clark-Lewis (1992) reported an increased number of neutrophils in

rabbits injected with IL-8 thus confirming the role of IL-8 as a chemo-attractant of neutrophils.

2.8.1.2 Interleukin-1 beta (IL-1β)

Interleukin-1 beta (IL-1β) functions more like tumor necrosis factor (TNF)-α cytokine because they are both pro-inflammatory. IL-1 β is essential in the initiation of an inflammatory response against ectoparasites in fish (Gonzalez *et al*., 2007).

2.8.1.3 Chemokine CC motif ligand 2 (CCL2) and Chemokine CC motif ligand 26 (CCL26)

CCL2 and CCL26 are referred to as chemotactic cytokines. These cytokines play an important role in the immune system. They have an ability to induce migration of leukocytes to the inflammatory sites and promote activation of leukocytes at these sites (Semple *et al*., 2010). These two cytokines were up-regulated in tick attachment sites of the Holstein-Friesian cattle (Piper *et al*., 2008).

2.8.2 Quantifying gene expression

There are various techniques used to assess gene expression. These include northern blotting, serial analysis of gene expression, microarray analysis, western blotting, solution hybridization (Schmittgen *et al*., 2000) and real time reverse transcription polymerase chain reaction (real-time RT-PCR). Real-time RT-PCR methodology has been proven to be effective in quantifying cytokine mRNA expression (Stordeur *et al*., 2002). Real-time RT-PCR is efficient because it is easy to conduct, while its accuracy, reproducibility, reliability, as well as rapidness are commendable (Pfaffl, 2001). It is also very sensitive and precise when compared to endpoint PCR (Schmittgen *et al*., 2000), which enables it to quantify small changes in gene expression (Giulietti *et al*., 2001) and is capable of quantifying mRNA from various sources.

2.9 Hypersensitivity reactions to ticks

Hypersensitivity reactions, which are the components of immunological response have been indicated to play a role in the resistance of animals to tick infestations (Allen, 1994). They are genetically mediated and can be inherited (Schurink *et al*., 2014). Hypersensitivity reactions can be of the immediate type or the delayed type. The immediate type is realized within a short period of time after the introduction of antigens to the animal skin while the delayed type is realized somewhat later. There is a delayed hypersensitivity reaction characterized by increased basophil and eosinophil infiltration. Because of these characteristics it has been referred to as cutaneous basophil hypersensitivity (CBH) reactions (Brossard & Wikel, 2004). Tick saliva is reported to induce CBH reactions in the skin of animals.

Various studies have been done to determine the association of hypersensitivity reactions with animal resistance to ticks. Hlatshwayo *et al*. (2004) reported different hypersensitivity reactions to *Amblyomma cajennense* whole extract in pre-infested and naïve rabbits. They indicated that pre-infested rabbits displayed both immediate and delayed hypersensitivity responses while naïve rabbits displayed only immediate hypersensitivity. Their results made them to suggest that delayed type reaction is associated with resistance to ticks in rabbits. Ferreira *et al*. (2003) also did not find any delayed hypersensitivity reactions in mice infested with *Rhipicephalus sanguineus* compared to the resistant guinea pigs infested with the same tick species in their study. Instead the immediate hypersensitivity reaction was observed in the mice while delayed hypersensitivity was observed in the guinea pigs. Research in cattle also confirmed that animals resistant to ticks display a delayed hypersensitivity type while those susceptible to ticks displayed immediate hypersensitivity reactions. Marufu *et al*. (2013) reported cutaneous delayed hypersensitivity reactions in Nguni (resistant) and immediate hypersensitivity reactions in the more susceptible breed, Bonsmara injected with *Rhipicephalus decoloratus* and *Rhipicephalus microplus* unfed larval extracts.

Variable hypersensitivity reactions have also been established between different animal species infected with the same tick species extract. Szabo *et al*. (1995) reported that guinea pigs and dogs intradermally injected with *R. sanguineus* extract on their ears demonstrated varying types of hypersensitivity reactions. The dogs showed immediate hypersensitivity while guinea pigs had low immediate type reactions and strong delayed reactions. Most of the hypersensitivity reactions assessed in previous studies were conducted by measuring ear

thickness to determine the extent of swelling induced by the introduction of antigens (Szabo *et al*., 1995; Marufu *et al*., 2013). However, Ferreira *et al*. (2003), in addition to using swelling as a measure of hypersensitivity, also looked at the cellular infiltration at inoculation sites in mice. Such a hypersensitivity study has not yet been done in sheep.

2.10 Conclusions

Sheep production forms an integral part of the South African livestock industry and it is practiced throughout the country. The Dorper, NA and SAMM breeds originate from different places but they are all adapted to varying South African environmental conditions. The Dorper and SAMM are good meat producers whereas the NA has a relatively low meat yield.

The literature indicates that *Ixodid* ticks are of economic importance to sheep production enterprises compared to *Argasid* ticks. In addition it was suggested that the genera of ticks known to commonly parasitize sheep are *Amblyomma, Hyalomma, Ixodes* and *Rhipicephalus*. However the literature lacks information on which tick species are prevalent in which Province.

It is apparent that tick infestation can affect productivity, health and general welfare of livestock. This is partly indicated by tick infestation affecting the udders of sheep on the Nortier Research Farm. However, research results on the impact of tick infestation to the productivity of sheep are lacking. In cattle genetic variation exist for tick resistance. Different genotypes or breeds display different immunological responses to tick infestation in cattle. Some of these immune responses have been associated with resistance to ticks but there is almost no comparable information on sheep. Host resistance to ticks can be measured by counting the number of ticks or by examining the ability of the ticks to attach, stay attached and engorge. Resistance to ticks proved to be genetically mediated and can be inherited but this is still to be ascertained beyond reasonable doubt in South African sheep.

Components of immunological responses contributing to resistance to ticks include cellular infiltration and differential expression of some cytokine genes. All these immunological responses have been studied in cattle but no comparable literature was found in sheep. Although most of the studies on gene expression cited in this literature review were conducted on cattle, the few studies on sheep also indicate that immune and non-immune

factors are associated with resistance to ticks. There is scope for research on sheep as far as resistance to ticks and the effects of tick infestation on production are concerned. This study will thus investigate the genetic and immunological basis of the ovine responses to tick infestation, genetic and non-genetic parameters of host resistance to tick infestation and the effects of tick counts on growth performance of sheep.

CHAPTER 3

Genotype effects, as well as genetic and environmental parameters for early live weight and tick count in an extensive sheep flock

3.1 Introduction

External parasites like ticks can impair sheep productivity and general welfare in free range, pastoral grazing systems. Negative impacts on sheep attributed to tick infestation include skin damage (Gothe & Bezuidenhout, 1986), tissue damage (MacIvor & Horak, 1987), necrosis (Howell *et al*., 1978), lameness and paralysis in lambs (Kok & Fourie, 1995) and udder damage in ewes (Cloete *et al*., 2013). Tick infestation has also been associated with zinc deficiency and emaciation (Mustafa, 2013), alopecia (Gbolagunte *et al*., 2009; Wood *et al*., 2010) and hyperkeratosis (Gbolagunte *et al*., 2009) in naturally infested sheep. Furthermore, ticks transmit disease-causing pathogens in sheep, which may cause serious losses in productivity (Yin *et al*., 2002; Abunna *et al*., 2012).

The detrimental effects of tick infestations to sheep production underline the need for tick control programmes in areas where tick infestation is a problem. So far, chemical control has been the first line of defence in tick control programmes. However, there are reports of ticks becoming resistant to acaricides (Nolan, 1990), while such chemicals are retained in milk and meat and also pose a risk of environmental pollution (Brossard, 1998; Regitano *et al*., 2008). Other tick control methods such as biocontrol methods, using parasitoids (Mwangi *et al*., 1997; Mwangi & Kaaya, 1997), pathogens (Kaaya *et al*., 1996; 2000; 2011) and predators (Kok & Petney, 1993; Petney & Kok, 1993; Dreyer *et al*., 1997); the development of tick vaccines (Willadsen *et al*., 1996; Willadsen, 2004; Torina *et al*., 2014) and tick-borne disease vaccines (De la Fuente *et al*., 2011) have been researched. However, it seems unlikely that any one of these will be sufficiently effective as the sole control agent for tick control. The future of tick control on domestic animals therefore relies on the integrated use of all available technologies such as chemicals, the management of acaricide resistance, tick vaccines and tick-borne disease vaccines as well as additive and non-additive genetic variation enabling host animals to resist tick infestation.

Exploiting the innate ability of animal hosts to resist tick attachment may be one of the tick control measures that can also be included in an integrated tick control programme. MacLeod (1932) as well as Fourie & Kok (1996) reported breed differences in tick count for sheep, suggesting a genetic basis for tick count. Genetic variation in host resistance to ticks has already been reported within breeds in cattle (Wambura *et al*., 1998; Burrow, 2001; Budeli *et al*., 2009). It is therefore necessary to investigate the genetics of host resistance to ticks in sheep as well. A literature survey found a single Scandinavian publication reporting additive heritability estimates of ~0.3 for tick counts in Norwegian sheep (Grøva *et al*., 2014). No account of non-additive genetic variation for tick count was found for sheep crossbred populations, although heterosis effects were reported for tick count in cattle (Ayres *et al*., 2015).

Comprehensive knowledge of ovine additive and non-additive genetic variation for tick count is vital to guide breeding programs. The resistance of sheep to ticks can be assessed by counting the number of ticks, as for other species such as cattle. It has been established in cattle that resistant animals have a lower average tick count than susceptible animals (Seifert, 1971; Piper *et al*., 2008; Marufu *et al*., 2011).

Against this background, this study analysed weaning weight and overall tick counts of purebred Namaqua Afrikaner (NA), Dorper and South African Mutton Merino (SAMM) lambs maintained on the Nortier Research Farm as well as those of crosses among these breeds. These breeds were compared under marginal, extensive conditions to gain insight in the adaptability and robustness of these breeds in a limiting production environment.

3.2 Materials and Methods

3.2.1 Animals, experimental site and recordings

The experimental animals used in this study were NA, Dorper and SAMM lambs maintained on the Nortier Research Farm from 2010 to 2015. Nortier is situated near Lamberts Bay on the west coast of South Africa (32˚02’ S and 18˚20’ E). The climate of the area is Mediterranean but the site is characterised by low rainfall of approximately 221 mm p.a. as

the long-term average, with 78% of the precipitation occurring during the winter months from April to September (Cloete & De Villiers, 1987). An extensive farming system was employed and ewes were mated annually from mid-January to the end of February to lamb during winter when sufficient grazing is available. Lambs were weaned during spring, when the nutritional conditions are expected to be maximal. Ewes and lambs utilized natural shrub grazing and received no extra feeding to supplement the available pasture. Ewes were randomly allocated to single-sire mating groups during January each year to create six genotypes, namely: purebred lambs of the three breeds forming the basis of the study, NA rams mated to Dorper ewes (NA x Dorper cross) and the reciprocal crosses between the Dorper and the SAMM (respectively the Dorper x SAMM and SAMM x Dorper). Owing to replacement needs, more ewes were purebred, resulting in less representation of crossbred lambs. Groups of ewes (Mean number ± SD = 31.9 ± 5.4) were mated for a 6-week period on paddocks of 15 ha each containing Veldt type 34, described as Strandveld by Acocks (1988). Dorper and NA ewes were mated to 3-4 rams each year. There were substantially fewer SAMM ewes available. A single SAMM ram was therefore used annually. One or two NA and Dorper sires were carried over from a given year to the next to provide sire linkages across years. The single SAMM ram was replaced every two years. All the Dorper and SAMM sires used were obtained from industry flocks to adequately link the study to industry genetic resources. NA rams were obtained from other conservation flocks, either maintained by research institutions or museums. The ewe flock had a normal age structure, and each breed was represented by ewe age groups from 2 to 7+ years of age. Ewes of the respective breeds were managed as a single flock, except at mating. Ethical clearance for the project was obtained from the Departmental Ethical Committee for Research on Animals (DECRA reference numbers R13/88) in the Department of Agriculture, Western Cape Government.

3.2.2 Design

The purebred genetic resources available on the research farm included two important commercial breeds (Dorper and SAMM), as well as the indigenous, fat-tailed NA. As the dominant meat breed in South Africa, the Dorper supplied 19% to the annual weaning weights recorded in the local performance testing scheme (Cloete *et al*., 2014). The SAMM accordingly represented the most important dual-purpose breed and accounted for 18% of the recorded weaning weights. In contrast, the NA is not a commercially developed breed and is

only found in a number of conservation flocks (Qwabe *et al*., 2012). This structure in the data allowed for the combination of the available resources to address specific outcomes, as follows:

3.2.2.1 Study 1: Breed effects and crossbreeding parameters for Dorper and SAMM lambs and their reciprocal cross

Data of purebred commercial Dorper and SA Mutton Merino lambs were combined with data of the reciprocal cross between these breeds. This outlay allowed the assessment of breed effects and the possible effect of non-additive genetic variation on weight traits and tick count in the commonly available commercial breeds. All these breed combinations were annually available throughout the experimental period from 2010 to 2015.

3.2.2.2 Study 2: Breed effects and crossbreeding parameters for Dorper and NA lambs and progeny of Dorper ewes mated to NA rams

Data of purebred commercial Dorper lambs were combined with data of the indigenous, fat-tailed NA and the NA x Dorper cross in this study. It should be noted that the more optimal design would have included the Dorper x NA cross as well, to ensure unbiased estimates of heterosis to be derived from the study. However, it is known that rams from commercial breeds sometimes achieve limited success when mated to indigenous, fat-tailed ewes under extensive conditions without human intervention to facilitate successful mating (Obst *et al*., 1980). Tail-docking of karakul ewes was also associated with a small but significant increase in lambing rate, as well as a propensity to lamb earlier in the lambing season (Shelton, 1990). This combination was therefore not included in the study. However, the existing experimental outlay should still allow an indication of the existence of non-additive effects on live weight and tick count in crosses of indigenous fat-tailed rams with commercial ewes.

3.2.3 Recording of data

All reproducing ewes were marked on their sides with spray-paint prior to lambing to allow the identification of individual lambs with their dams under extensive conditions without undue disturbance to the lambing flock. The lambing flock was inspected daily. During these

daily inspection rounds, newly arrived lambs were weighed to obtain their birth weights and identified with their dams within 24 hours of birth. This allowed the breed and identity of the sire to be obtained from mating lists for the respective years. Gender of lambs, age of dam and birth type were all recorded concurrently. The lambs were weaned in spring (October) at an average age of 122 ± 25 days in Study 1 and 121 ± 23 days in Study 2, and weaning weight was also recorded at this stage. An adequate tick challenge in adult ewes was present during spring (September-October) at the experimental site (Cloete *et al*., 2013).

Therefore, within a fortnight prior to or after weaning weights were recorded, all lambs were upended one at a time by one person while another person counted the attached ticks and recorded the numbers. Only total tick count was available throughout the recording period. The bulk of about 4000 ticks collected in a previous study from sheep at the experimental site after natural challenge and collected in December, May and September from mature breeding ewes belonged to three species, namely: *Rhipicephalus evertsi evertsi* (50.3%), *R. gertrudi* (26.4%) and *Hyalomma truncatum* (22.4%) (Cloete *et al*., 2013). It was thus assumed that all the experimental animals were subject to a mixed natural challenge, with the tick species named above being predominant.

In view of the report of Cloete *et al*. (2013), indicating that tick counts at specific attachment sites differed according to breed, ticks on the fat-tail of the NA lambs were counted separately for 169 NA lambs present in 2013-2015. In these years, the ticks were counted and recorded on five regions of the lambs, namely, head (included the ears and the neck), front region (breast, front legs, belly to the navel), middle region (belly area from the navel, udder and hind legs) and lastly the breech and perineum area, while ticks on the tails of the NA and NA x Dorper lambs were counted separately.

3.2.4 Statistical analysis

ASReml software (Gilmour *et al*., 2009) was used to analyse the fixed effects and to derive least squares means for the fixed effects influencing the respective traits and to estimate variance components in initial single-trait analyses. Individual tick counts were found to be exceedingly variable and skewed (Table 3.1), needing transformation prior to analysis. The transformation applied was the square root transformation after 0.50 were added to individual

records to account for zero counts. The number of purebred lambs ranged between 74 (SAMM) and 455 (Dorper) while crossbreds ranged from 50 to 90 per group (Table 2). Given the low number of lambs in almost all genetic groups, within group genetic analysis was not attempted. The first single-trait analyses involved fitting various combinations of fixed effects to obtain an operational model, including the effects of genetic group (as specified previously for Study 1 and Study 2), birth year (as specified previously), gender (male and female), dam age (2-7+ years) and birth type (singles and pooled multiples). All two-factor interactions between main effects were considered initially. The birth year x genetic group interaction was significant for square root transformed tick counts in Study 2 and was reported. Weaning age was also included as a linear covariate for weaning weight and square root-transformed tick counts, while the latter trait was analysed with and without the inclusion of weaning weight as a linear covariate. It was assumed that the scaling of lambs for size would account for larger lambs having larger attachment areas for ticks thus potentially hosting higher tick counts. Non-additive heterosis effects were estimated by linear contrasts comparing the performance on the crossbred animals with the midparent value of the pure breeds used in their formation.

Given the size of the data sets used in Study 1 (633-635 records) and Study 2 (892-895 records) it was decided not to partition the animal variances between direct and maternal effects for tick count (the trait with limited representation in the literature). Also, accurate genetic parameters are commonly available for weaning weight, using more representative industry data. It was thus not considered necessary to partition the random effect of animal into direct genetic, maternal genetic and maternal permanent environmental effects in the present studies. Furthermore, it would be difficult to accurately partition maternal genetic and maternal permanent environmental effects since the number of dams and grand-dams with data themselves were too low to allow for this. A single random terms were thus added to the operational model for each trait, resulting in the following model for analyses (in matrix notation):

$$y = Xb + Z_1a + e \qquad (1)$$

In these analyses, y was a vector of observations for either untransformed weaning weights or square root transformed tick count in Studies 1 and 2, and b and a vectors of fixed effects and

direct genetic effects, respectively. X and Z_1 represented the corresponding incidence matrices relating the respective effects to y and e was the randomly distributed vector of residuals. It was assumed that:

$V(a) = A\sigma^2_a$ and $V(e) = I\sigma^2_e$,

With A representing the numerator relationship matrix, I an identity matrix; σ^2_a and σ^2_e being the direct genetic and residual variances, respectively. Ratios corresponding to additive genetic and permanent environmental variances were computed from these estimates. These variances were expressed relative to the total phenotypic variance. The pedigree file for both studies included 2590 animals born from 2008 to 2015, the progeny of 42 sires and 568 dams.

3.3 Results

3.3.1 Study 1: Breed effects and crossbreeding parameters for Dorper and SAMM lambs and their reciprocal cross

3.3.1.1 Descriptive statistics for weaning weight and tick count

Weaning weight had a coefficient of variation (CV) of 24% (Table 3.1). Untransformed total tick count had a large CV of almost 95%. The square root transformation of total tick count substantially reduced the observed CV to well below 50%. No ticks were detected (i.e. tick counts amounting to zero) on 39 of 633 (or 6.2%) of the Dorper, SAMM and crossbred lambs considered in Study 1.

Table 3.1 Descriptive statistics for weaning weight, as well as untransformed and square root transformed tick count in Study 1, involving lambs of the Dorper and SA Mutton Merino breeds, as well as their reciprocal cross

Trait analysed	Number of observations	Mean	SD	CV (%)	Range
Weaning weight (kg)	635	32.3	7.8	24.1	10.5–59.0
Untransformed total tick count (n)	633	11.58	10.98	94.8	0–116
Square root transformed tick count (n)	633	3.185	1.396	43.8	0.71–10.8

3.3.1.2 Effects of genotype and non-additive genetic variation on weaning weight and tick count

Weaning weight was affected by genotype (P=0.034; Table 3.2). However, when the standard errors were considered, breed effects could not be considered as substantive. The contrast between the midparent value and crossbred performance still reached statistical significance and resulted in a heterosis estimate of approximately 4%. Square root transformed tick count was independent of breed effects. No evidence of heterosis for tick counts was evident in the two commercial breeds, geometric means ranging from 8.7 to 10.5. The proportion of lambs with zero tick counts were independent of genotype, and ranged from 0.04 in SAMM x Dorper cross lambs to 0.09 in Dorper x SAMM cross lambs (Chi^2=1.90; degrees of freedom=3; P>0.05).

Table 3.2 Least squares means (± SE) for weaning weight, as well as square root transformed total tick count with geometric means for back transformed tick counts as affected by breed for Dorper and SA Mutton Merino sheep and their reciprocal cross in Study 1

Effect and level	Number of observations	Weaning weight (kg)	Number of observations	Transformed total tick count (n)	Geometric mean (n)
Breed		*		0.49	
Dorper	455	33.9 ± 0.7	455	3.31 ± 0.10	10.5 ± 0.7
SA Mutton Merino (SAMM)	73	33.2 ± 1.4	74	3.04 ± 0.19	8.7 ± 1.2
Dorper x SAMM	54	34.2 ± 1.1	54	3.17 ± 0.20	9.5 ± 1.2
SAMM x Dorper	46	35.7 ± 1.3	50	3.29 ± 0.22	10.3 ± 1.5
Heterosis		4.1**		$1.7^{0.80}$	

* P<0.05; ** P < 0.001; actual significant levels for P>0.05

3.3.1.3 Effects of covariates, birth year, sex, age of dam and birth type on weaning weight and tick counts

The regression of weaning weight on age (±SE) amounted to 0.127 ± 0.013 kg. In contrast, tick count was unaffected by age (b±SE=-0.0023 ± 0.0028 ticks per day), but heavier lambs appeared to have higher tick burdens (b±SE=0.026 ± 0.009 ticks per kg weaning weight). Both traits were affected by birth year (P<0.01), means (±SE) ranging from 31.2 ± 1.6 kg in

2014 to 37.3 ± 1.4 kg in 2015 for weaning weight and from geometric means of 2.86 ± 0.6 ticks in 2013 to 17.3 ± 1.5 ticks in 2015. Male lambs were 9.5% heavier than females at weaning (respectively 35.8 ± 0.8 vs. 32.7 ± 0.8 kg), while the geometric mean of tick count for males was 31% higher than in females ($P<0.01$; geometric means respectively 11.4 ± 0.7 vs. 8.7 ± 0.6 ticks). Weaning weights of lambs increased from 33.0 ± 0.9 kg in the progeny of 2-year-old dams to stabilise at maxima of 34.1 ± 0.9 kg to 35.4 ± 0.8 kg in progeny of ewes between 4 and 7 years of age. Square root transformed tick counts was not affected by dam age and birth type ($P>0.05$). However, single lambs were 15% heavier than multiples at weaning ($P< 0.01$). Both lamb weaning weights and square root transformed tick counts were not affected by two-factor interactions between main effects included in the analyses.

3.3.1.4 Heritability estimates

Genetic parameters for weaning weight were not very informative in Study 1, given the size of the data set. Much more informative parameters can be sourced from the literature and the heritability of weaning weight was thus not reported. In contrast, there is a dearth of heritability (h^2) estimates for ovine tick count data. Single-trait h^2 estimates were quite independent of the inclusion of weaning weight as a covariate to analyse square root transformed tick count and remained stable at 0.11 ± 0.09. A model analysing across-genotype h^2 by excluding genotype and the putative effect of heterosis from the model of analysis yielded a slightly lower h^2 estimates of 0.08 ± 0.07.

3.3.2 Study 2: Breed effects and crossbreeding parameters for Dorper and NA lambs and progeny of Dorper ewes mated to NA rams

3.3.2.1 Descriptive statistics for weaning weight and tick count

Weaning weight had a somewhat higher CV of 27% in comparison to the value of 24% in the Table 3.1 for the two commercial breeds and the cross between them (Table 3.3). The standard deviation of untransformed total tick count exceeded the corresponding mean in the data set containing Dorper and NA lambs as well as the NA x Dorper cross. Like in Table 3.1, the square root transformation of tick count data markedly reduced the observed CV to approximately 55%. On average, 11.3% of lambs contained in the data set had tick counts of

zero. The average (± SD) tick count on the tails of NA lambs during 2013-15 amounted to 2.54 ± 5.06 ticks, while total tick counts in this genotype averaged 5.82 ± 7.58 over the same period. Thus nearly 44% of ticks on NA lambs were found on their tails.

Table 3.3 Descriptive statistics for weaning weight, as well as untransformed and square root transformed tick count in Study 2, involving lambs of the Dorper and Namaqua Afrikaner breeds, as well as the cross of Namaqua Afrikaner rams with Dorper ewes

Trait analysed	Number of observations	Mean	SD	CV (%)	Range
Weaning weight (kg)	895	29.4	7.8	26.5	7.5–59.0
Untransformed total tick count (n)	892	9.17	10.33	112.6	0–116
Square root transformed tick count (n)	892	2.66	1.45	54.5	0.7–10.8

3.3.2.2 Effects of genotype and non-additive genetic variation on weaning weight and tick count

Weaning weight and square root transformed tick count were both affected by genotype (Table 3.4). At 27.0 kg, purebred NA lambs were the lightest at weaning. Dorper and NA x Dorper lambs were respectively 26 and 23% heavier than their NA contemporaries and not different from each other (P>0.05). At respectively 4.9 and 4.4, the geometric means for tick count of NA and NA x Dorper lambs amounted to below half the magnitude of the geometric mean for tick count in Dorper lambs at 10.2 (P<0.05; Table 3.4). Significant (P<0.05) heterosis estimates were derived for both traits, amounting to 8.5% for weaning weight and -23% for square root transformed tick count. The proportion of lambs with zero tick counts were higher in the NA breed (0.16) and in the NA x Dorper cross (0.19) in comparison with purebred Dorper lambs at 0.06 (Chi^2=23.3; degrees of freedom=2; P<0.01). These results were derived from the analysis including lamb weaning weight as a covariate in the analysis on tick count. The exclusion of lamb weaning weight as covariate resulted in similar geometric means for tick count in NA and NA x Dorper lambs (respectively 4.68 ± 0.43 and 4.68 ± 0.76) compared to 10.7 ± 0.58 for Dorper lambs, and a slightly lower heterosis estimate of -19% for square root transformed tick count.

Table 3.4 Least square means (± SE) for weaning weight, as well as square root transformed total tick count with geometric means for back transformed tick counts as affected by breed for Dorper, Namaqua Afrikaner sheep, as well as the cross of Namaqua Afrikaner rams with Dorper ewes in Study 2

Fixed effect and level	Number of observations	Weaning weight (kg)	Number of observations	Transformed total tick count (n)	Geometric mean (n)
Breed		**		**	
Dorper	455	$34.0^{b} \pm 0.7$	455	$3.27^{b} \pm 0.09$	10.2 ± 0.6
Namaqua Afrikaner	347	$27.0^{a} \pm 0.8$	347	$2.33^{a} \pm 0.10$	4.9 ± 0.5
Namaqua Afrikaner x Dorper	90	$33.1^{a} \pm 0.9$	90	$2.21^{a} \pm 0.17$	4.4 ± 0.8
Heterosis		8.5**		-22.5**	

** P<0.01; * P<0.05; actual significant levels for P>0.05; ^{a,b,c,d} significance in columns (P< 0.05)

3.3.2.3 Effects of covariates, birth year, sex, age of dam and birth type on weaning weight and tick counts

The regression of weaning weight on age (±SE) amounted to 0.131 ± 0.011 kg in Study 2. In contrast, tick count was unaffected by age (b±SE=-0.0023 ± 0.0026 ticks per day), but heavier lambs appeared to have higher tick burdens (b±SE=0.022 ± 0.008 ticks per kg weaning weight). Both traits were affected by birth year (P<0.01), means (±SE) ranging from 30.1 ± 0.9 kg in 2012 to 32.2 ± 1.0 kg in 2015 for weaning weight and from geometric means of 1.63 ± 0.4 ticks in 2013 to 14.0 ± 1.3 ticks in 2015. As in Study 1, male lambs were 11% heavier than females at weaning (respectively 33.0 ± 0.6 vs. 29.7 ± 0.6 kg), while the geometric mean of tick count for males was 21% higher than in females (P<0.01; geometric means respectively 6.9 ± 0.5 vs. 5.7 ± 0.4 ticks). Weaning weights of lambs increased from 30.0 ± 0.7 kg in the progeny of 2-year-old dams to stabilise at maxima of 31.6 ± 0.7 kg to 32.2 ± 0.7 kg in progeny of ewes between 4 and 7 years of age. Square root transformed tick counts was affected by dam age (P <0.05), least squares means ranging from 5.0 ± 0.7 in the progeny of 6-year-old ewes to respectively 7.2 ± 0.7 and 7.4 ± 1.4 in progeny of 4- and 7-year-old ewes. As in Study 1, square root transformed tick count was unaffected by birth

type. However, single lambs were 16% heavier than multiples at weaning (P<0.01, respectively 33.7 ± 0.6 vs. 29.0 ± 0.6 kg).

Lamb weaning weights were not affected by two-factor interactions between main effects (P >0.05). In contrast, square root transformed tick counts were affected by a significant interaction between genotype and birth year (P=0.013). Geometric means back transformed from square root transformed values are presented in Figure 3.1 to depict this interaction. Conclusions derived for the square root transformed mean values would be similar and the geometric means were preferred as they are expressed on the observed scale and should therefore be easier to comprehend. The overall tick burdens of NA and NA x Dorper lambs were the lowest in absolute terms in all years and were well below corresponding means for Dorper lambs throughout (P<0.05). There were some reranking between years between NA lambs and NA x Dorper lambs, geometric means for NA x Dorper lambs exceeding those of NA lambs in 2010 and 2012 and *vice versa* in 2014 (P<0.05). Geometric means for tick counts of Dorper lambs exceeded those of NA lambs by between 42% in 2014 to more than three-fold in 2010-2012. Geometric means of tick counts in Dorper lambs were accordingly between 60% (2012) and 5-fold (2011) higher than those of NA x Dorper lambs.

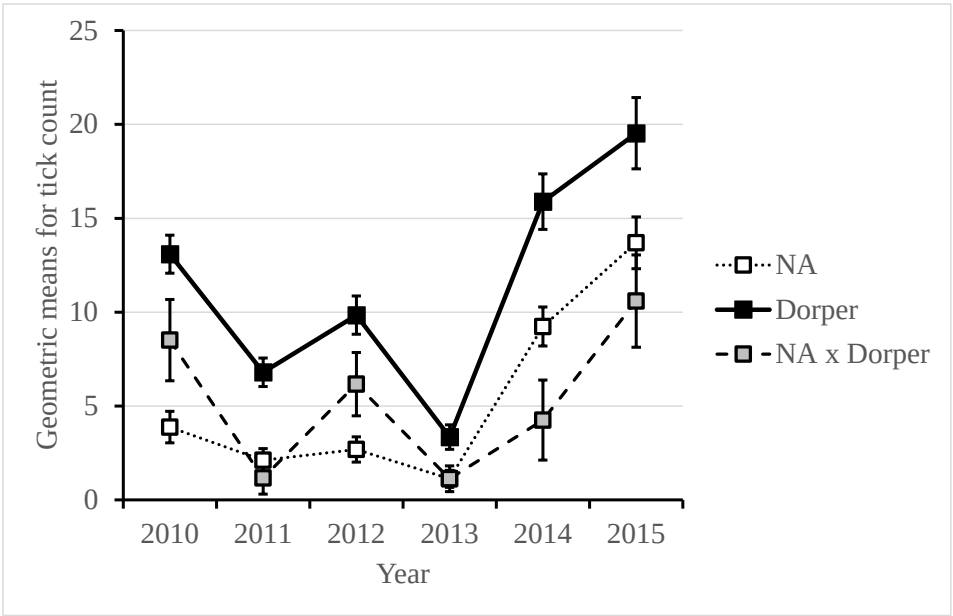

Figure 3.1 Least squares means (approximate SE from ASReml output as vertical bars about the means) depicting the genotype x birth year interaction for tick count in purebred Namaqua Afrikaner (NA), Dorper (D) and NA x Dorper lambs

3.3.2.4 Heritability estimates

As in Study 1, single-trait h^2 estimates were quite independent of the inclusion of weaning weight as a covariate to analyse square root transformed tick count and remained stable at 0.06 ± 0.05. A model analysing across-genotype h^2 by excluding genotype and the putative effect of heterosis from the model of analysis yielded a substantially higher h^2 estimates of 0.27 ± 0.07. These results seem to suggest that genetic variation in square root transformed tick count was primarily associated with differences among genetic groups in this study, while differences in tick counts between individual animals within genetic groups were not as important.

3.4 Discussion

The outcomes of Studies 1 and 2 were discussed together to allow direct inferences pertaining to the result of crossing commercial breeds with the outcome derived from using rams of an unimproved indigenous breed on ewes from a commercial breed.

3.4.1 Descriptive statistics for weaning weight and tick count

The CV of weaning weight amounted to 24-27%. These values were somewhat higher than the range of 16-18% reviewed by Safari *et al*. (2005) from >50 literature sources. However, it was quite consistent with values of 20-26% reported by Safari *et al*. (2007) for seven Australian resource flocks. In contrast, CV's of untransformed tick count were very high. Corbet *et al*. (2006) accordingly reported that tick count ranged from 1-150 in 622 cattle records, with a CV of 72%. The square root transformation of tick count data resulted in CV being reduced substantially to ~50%. These values were consistent with a report of Burrow (2001) where the CV of log transformed cattle tick counts amounted to 48%.

3.4.2 Effects of genotype and non-additive genetic variation on weaning weight and tick count

The results from Studies 1 and 2 suggested that results derived from the crossbreeding of commercial sheep differed substantially from those resulting from crossbreeding commercial ewes with unimproved indigenous rams. Breed effects for weaning weight in Study 1 involving commercial animals were modest compared to Study 2 where the unimproved NA was included. The effects of heterosis for weaning weight in Study 2 were more than double that of Study 1 at respectively 9% and 4%. However, both these estimates are well within the range of values of 3-10% reported by Fogarty (2006) as reasonable for heterosis of early live weights. It is published in the literature that the NA is by nature a slender and late maturing breed and comparable weaning weights were reported previously for this breed (Qwabe, 2011; Burger *et al*., 2013; Snyman *et al*., 2013). The lower weaning weights of NA lambs compared to the two commercial breeds were thus not entirely unexpected, as size was emphasised in commercial selection strategies while the NA is largely unimproved.

The contrast between the outcomes of Studies 1 and 2 is accentuated when tick count is considered. No evidence of heterosis was observed in Study 1 based on the crossing of commercial breeds. In fact, the mean tick count of reciprocal crossbred lambs was slightly higher (i.e. worse) in absolute terms than the midparent value derived from purebred Dorper and SAMM lambs. In contrast, the tick count of NA and NA x Dorper lambs were substantially reduced relative to that of Dorper lambs, resulting in substantial favourable (i.e. negative) heterosis estimates of 20-23%. MacLeod (1932) as well as Fourie & Kok (1996) previously reported breed differences in tick count for sheep, also suggesting a genetic basis for tick counts. This study confirmed earlier work, suggesting that NA ewes hosted fewer ticks than the commercial breeds included therein (Cloete *et al*., 2013). Breed differences in tick counts have also been established in cattle (Latif, 1984; Wambura *et al*., 1998; Marufu *et al*., 2011). Indigenous East African goats were better able to withstand ticks and abscesses caused by ticks than exotic Saanen goats (Schwalbach *et al*., 2003). Indigenous genetic resources, whether be it bovine, ovine or caprine, were often reported to be better adapted to local stressors such as ticks and tick-borne diseases (Mirkena *et al*., 2010). It is a pity that research on adaptive traits in indigenous genetic resources were often neglected in the past, given the role such material could play in adaptation to challenging environments (Van

Marle-Köster *et al*., 2015). No previous estimates of ovine heterosis to tick count were found in the literature. However, it is known that crosses of Bos indicus cattle breeds with Bos Taurus breeds do exhibit heterosis for resistance to tick infestation (Ayres *et al*., 2015). This study seems to suggest that a similar situation might prevail in sheep, as suggested by the results presented in Table 3.4. In contrast, the same benefits did not seem to accrue when commercial or improved breeds were crossed (Table 3.2).

It is notable that breed type interacted with birth year for square root transformed tick count in Study 2 (Figure 3.1), but not in Study 1. However, when scrutinising Figure 3.1, the generally lower overall tick count of NA and NA x Dorper lambs compared to Dorpers were quite consistent across years, although the exact magnitude of these differences changed from year to year. Such an outcome suggested an inference that adapted, indigenous ovine genetic resources and their crosses with commercial breeds may be quite competitive in challenging environments. The most obvious productive advantage associated with lower tick count in NA ewes in the sensitive udder and hind leg body region was an improved udder health, and particularly as breeding ewes grew older (Cloete *et al*., 2013).

3.4.3 Effects of birth year, gender, age of dam and birth type on weaning weight and tick counts

Birth year, gender, age of dam and birth type significantly influenced weaning weight of the lambs, both in Study 1 and Study 2. These results are not unusual, birth years on extensive pastures differ in terms of seasonal variation of climate and thus also in the availability and quality of nutrition as well as in disease and parasite challenge. Birth year effects on weaning weight are thus common in the scientific literature. The significant effect of sex and birth type on weaning weights has also been reported previously (Safari *et al*., 2007; Jalil-Sarghale *et al*., 2014). It is also not surprising to have a lower weaning weight for the progeny of 2-year-old dams and for weaning weight to increase with an increased dam age. Young dams are still growing and thus they have to share the nutrients they consume during their reproductive cycle between their own growth and the maintenance of pregnancy and lactation. Single lambs are advantaged relative to multiples because they do not have to share the uterus space of their dams during pregnancy and thus they have more space to grow. They also do not have to compete for milk after birth, resulting in higher weaning weights than

multiples. All findings pertaining to birth year, gender, dam age and birth type on weaning weight were thus consistent with the outcomes of previous studies (Ekiz *et al*., 2005; Safari *et al*., 2007; Rashidi *et al*., 2008; Jalil-Sarghale *et al*., 2014).

Birth year and sex had significant ($P<0.05$) effects on total tick count. Overall, mean tick count during 2015 was highest while lambs born in 2013 had the lowest tick load in both studies. As suggested previously, year effects are caused by a variety of factors, and are thus not unexpected. Male lambs had higher tick loads than females. No comparable literature could be sourced for sheep. However, several authors reported that male calves had a significantly higher tick load than females (Seifert, 1971; Utech *et al*., 1978; Burrow, 2001). The study by Utech *et al*. (1978) reported larger sex differences in summer than in winter, when the overall tick load was only about 50% of that recorded in summer. All tick counts in the present study were recorded in spring and seasonal effects are thus not present. The age of dam effect on tick count in Study 2 was unexpected, since no specific trend could be detected. This result thus seemed to be coincidental, and needs to be verified in future studies. Age of dam in Study 1 and birth type did not affect tick numbers ($P>0.05$). No comparable results on sheep were found in the literature to compare these results to.

3.4.4 Genetic parameters

There are better data sets to estimate environmental and genetic parameters on weaning weight of lambs. Attention will therefore only be given to the discussion of the heritability of tick count. Estimates of the heritability of tick count in sheep after accounting for the effects of breed and heterosis were low at around 0.10. There is not much information on genetic parameter estimates for tick count in sheep although reported breed differences did suggest some genetic variation available for exploitation (MacLeod, 1932; Fourie & Kok, 1996; Cloete *et al*., 2013). When compared to the cattle literature, the heritability for tick count of around 0.10 estimated in this study is within the range of the estimates reported by Budeli *et al*. (2009) for Bonsmara cattle but appreciably lower than other estimates by MacKinnon *et al*. (1991; 0.34) and Burrow (2001; 0.42). Accordingly, Grøva *et al*. (2014) reported moderate to high heritability estimates of 0.32 to 0.59 for tick count in sheep. The former estimate were consistent with the across-breed estimate of 0.27 in Study 2, but were much

higher than the within-breed estimates in both studies, as well as the across-breed analysis in Study 1.

3.5 Conclusions

Heterosis estimates for weaning weight were variable between Studies 1 and 2, but still within ranges reported in the literature. This study established significant variation in tick count between sheep genotypes when the indigenous NA breed formed part of Study 2. In contrast, Study 1 results involving commercial breeds did not support this generalisation. The crossing of indigenous NA rams with commercial Dorper ewes exhibited worthwhile levels of heterosis in Study 2. Indigenous ovine genetic resources may be instrumental in providing genetic material for adaptive traits in environments susceptible to high levels of tick infestation. Further research is required to elucidate the role that adapted indigenous ovine genetic resources may play in an integrated tick management strategy under conditions characterised by high levels of tick challenge.

CHAPTER 4

Cutaneous changes and cellular infiltration in response to tick attachment in Namaqua Afrikaner, Dorper and South African Mutton Merino sheep

4.1 Introduction

The skin is the interface of the tick with its host animal. What happens when a tick attaches may play a role in the host's resistance to tick infestation. Cutaneous changes and cellular infiltration at tick bite sites need to be investigated to understand the mechanism used by host animals to resist tick infestations. The responses of host animals to ectoparasites have previously been reported to include immunological and morphological features (Shu *et al*., 2009). Whether this also applies to *ovis aries* resistance to ticks is not known yet, as this species has not yet been subjected to comprehensive studies. However, it has been confirmed that there are differences in tick burdens of South African Mutton Merino sheep (SAMM), Dorper sheep and Namaqua Afrikaner sheep (NA) (Cloete *et al*., 2013). NA sheep had significantly lower tick counts on the udder and hind legs when compared to the other two breeds while the SAMM had the highest number of ticks. Udder health scores accordingly deteriorated (became worse) faster with an increased age in Dorper and particularly SAMM ewes compared to NA ewes. This research suggested that the NA is more resistant to tick infestation than the other two breeds while the SAMM is the most susceptible or the least resistant breed.

The differences between these breeds pertaining to their resistance or susceptibility to ticks may be better understood by examining cutaneous changes and cellular responses that take place at the tick bite site when the tick attach to the host. Tick infested sites have been characterized by higher numbers of mast cells and leukocytes such as neutrophils, basophils and eosinophils, compared to un-infested sites in cattle (Marufu *et al*., 2014). These findings indicate that cellular infiltration may play an important role in resistance or susceptibility to tick infestation and that breeds resistant to tick infestations show smaller skin changes at tick infested sites than those that are susceptible to tick infestations. Differences in cellular infiltration and skin changes at tick infested sites between different breeds reported in

previous studies on other species infer putative breed effects on the resistance or susceptibility to tick infestation in sheep. However, it needs to be recognized that the acquisition of whole skin samples using a disposable skin biopsy punch is an invasive sampling technique (Witcomb *et al*., 2014). Given the lack of published research on skin changes associated with tick attachment in sheep, it was considered as prudent to conduct a preliminary study on a reduced number of sheep. Adhering to the well-known principle "Reduction" forming part of the "Three R's" forming the basis of ethical animal experimentation (Russel, 2005) this preliminary study included the minimum number of sheep thought to possibly yield informative results.

Against this background, this study had the dual purpose of first establishing whether a minimum number of animals partaking in a study of this nature would allow the analysts to make robust recommendations pertaining to the impact of tick attachment to cutaneous skin changes in divergent sheep breeds. The second objective was to investigate the effect of sheep breed on cellular infiltration and skin changes associated with tick attachment and the differences of these reactions between tick attachment and control sites in a more comprehensive study involving a greater number of sheep.

4.2 Materials and Methods

4.2.1 Biopsy collection and fixation

4.2.1.1 *Preliminary study*

A total of 18 randomly selected mature ewes (6 ewes of each breed) exposed to natural tick challenge during summer (January 2014) at the Nortier research farm were used in this study. The experimental site is found near Lambert's Bay on the western seaboard of South Africa and was described by Cloete & De Villiers (1987). The climate at the experimental site is Mediterranean, with 78% of the annual precipitation of 221 mm expected between April and September. The animals grazed as a single flock and were presumably subjected to the same tick challenge. Summer was chosen as it is a season usually associated with an appreciable number of ticks on the animals, as reflected by breed-specific means ranging from 1.5-7.0 ticks on the udder, thighs and hind legs and 3.8-11.5 ticks on the breech, perineum and tail of individual ewes (Cloete *et al*., 2013).

The ticks at the experimental site consisted of a mixed challenge consisting of seven species, but with three species contributing >99% to ticks collected from sheep during December 2011, May 2012 and September 2012 (Cloete *et al*., 2013). These species (% contribution in brackets) were *Rhipicephalus evertsi evertsi* (50.3%), *R. gertrudi* (26.4%) and *Hyalomma truncatum* (22.4%).

No ticks were found on two NA ewes and one Dorper ewe, leaving 4 NA ewes, 6 SAMM ewes and 5 Dorper ewes to sample. Two skin biopsies were collected per animal; one at the tick attachment site and one at a non-attachment (control) site using the punch biopsy method. The punch biopsy involves taking a small disc of the full thickness of the skin. Only two tick species were recorded at the respective tick attachment sites, namely *R. evertsi evertsi* (7) and *H. truncatum* (8). These ticks consisted of 9 males and 6 females. Unfortunately these ticks were unequally distributed between breeds for species (NA – respectively 3 and 1; SAMM – respectively 3 and 3; Dorper – respectively 1 and 4) and sex (NA – respectively 3 and 1; SAMM – respectively 4 and 2; Dorper – respectively 2 and 3). The ticks detached from the experimental animals were all adults but their feeding status or weights were not recorded. Ticks were mostly detached from the tail in the NA and the udder, hind thighs or perineum in the other breeds, which have been tail-docked. The animals were administered 1 ml lidocaine (local anaesthetic) per sample site before six millimeter diameter disposable biopsy punches were used to collect skin samples. All samples were collected by an experienced veterinarian or by an experienced veterinary technician under the supervision of the veterinarian. Subsequent to collection, tissue samples were immediately put in saline buffered formalin to fix them. Ethical clearance for the project was obtained from the Departmental Ethical Committee for Research on Animals (DECRA reference numbers R13/88 and S13/95) in the Department of Agriculture, Western Cape government.

4.2.1.2 *Comprehensive study*

The results of the preliminary study showed a great variation in the response to tick bites within and between breeds. This underlined the need to do a more comprehensive study with an increased number of animals per breed. In this further study, a total of 89 breeding ewes (NA = 21, SAMM = 29 and Dorper = 39) kept under comparable conditions as those used in the preliminary study at Nortier farm was used. The skin biopsies were collected and processed for histopathology examination as in the preliminary study during late April 2015.

Previous results suggested breed-specific means ranging from 3.8-8.4 ticks on the udder, thighs and hind legs and 2.9-6.5 ticks on the breech, perineum and tail of individual ewes in autumn (Cloete *et al*., 2013). The attachment sites of ticks were duly recorded, while it was attempted to detach ticks from areas of the animals not covered by wool with the perineum, thighs, udder and the tail of the NA being identified as the premium sites. Care was taken to only use information of ticks detached from bare skin on the ventral part of the tail of NA ewes. A limited number of ticks were detached from the bare areas on the axillae in ewes without ticks attached to the premium sites. In addition, the level of engorgement of the detached ticks were also recorded. Furthermore, the ticks removed where biopsies were taken were collected for identification and weighing. The additional information recorded was meant to determine whether the species and engorgement level had an effect on the level of cellular infiltration and skin defects.

4.2.2 Tissue processing

Skin biopsies from both studies were processed using routine histological techniques at the University of the Free State and National Health Laboratory Services (NHLS). Briefly, an automated processor was used to process the tissues overnight; the tissues were dehydrated using ethanol and embedded in paraffin wax. The embedded tissues were subsequently cut into successive sections of 4 micrometer thickness each. These sections were then floated on a water bath and put on microscope glass slides. The slides were automatically stained with haematoxylin and eosin for general observation and with a Giemsa stain for cell counts. The stained slides were additionally processed through water, alcohol and xylene and a cover slip was finally placed over the section of each slide using an automated coverslip machine.

4.2.3 Slide observation

All slides of both studies were observed by an independent pathologist under an Olympus light microscope using a 40X objective. The conditions that were examined in the epidermis were hyperkeratosis, acanthosis, apoptosis, acantholysis, a prominent granular layer, crusts, necrosis and leukocyte migration (infiltration). The conditions examined in the dermis were oedema, collagen degeneration and dermal necrosis. The severity of changes in the epidermis and dermis were ranked on the scale of 0 to 3 (0 = absent or within normal range, 1 = a mild change was observed, 2 = a moderate change was observed and 3 = a severe change was

observed). Leukocyte infiltration was assessed by counting the number of leukocytes in 10 fields from each slide using a 100X oil immersion objective. The cells that were considered were mast cells, as well as the leukocytes eosinophils, basophils and neutrophils.

4.2.4 Data analysis

4.2.4.1 *Preliminary study*

In the preliminary study the data were analysed using Means, Frequency and analysis of variance (ANOVA) procedures in SAS software (SAS Institute Inc., 2013). Cell count data were highly variable and thus not distributed normally, and were transformed to square roots prior to analysis. Chi-square procedures were initially used to test for differences in frequencies of graded skin defects between infested and control sites within and across breeds. However, the within-breed analyses were complicated by more than 20% of cells that had counts below 5 resulting in unreliable outcomes. Fisher's exact test (FET) was subsequently used to compare the frequencies of skin defects (assessed as present or absent on the binomial scale) between infested and control sites within breeds. The presence of specific leukocytes in attachment and control sites (present or absent) was accordingly tested within breeds by the FET. Differences in means for neutrophils and eosinophils at the infested sites between breeds were tested by using one-way analysis of variance (ANOVA) procedures in SAS software (SAS Institute Inc., 2013). It was impossible to add information on tick species, tick sex and attachment site to the statistical model in a factorial design, since it resulted in the data becoming severely unbalanced. Differences in the means of mast cell and basophil counts between the infested and control sites within breeds were tested by using analysis of variance (ANOVA) t-test procedures in SAS software (SAS Institute Inc., 2013). The statistical model used was a 2 (site status) x 3 (breed) factorial.

The data for skin conditions at tick attachment and control sites were analysed by Chi-square procedures in the comprehensive study. Analyses involving graded skin defects, the attached tick species, the life stage of the tick attached, the attachment site and tick engorgement levels resulted in analyses with >20% of the cells represented by counts below five, compromising analyses. Against this background, data were pooled in a meaningful way to analyse the absence/presence of specific skin lesions, the genus of the tick attached

(*Hyalomma* vs *Rhipicephalus*), the perineum/tail vs. pooled other attachment sites and flat ticks vs. the other levels of engorgement.

4.2.4.2 *Comprehensive study*

Cell count data for the comprehensive study were not normally distributed and transforming the data by using square roots and $\log_{10}$ did not improve the distribution. Therefore the non-parametric, Kruskal-Wallis 1-way ANOVA test in SPSS software (IBM Corp. released 2015) was used to test differences in mast cell counts between tick infested and control sites within breeds. Most of the control sites in all breeds had no neutrophils, eosinophils and basophils, thus the control sites were excluded from further analyses of these cell types. Only attachment sites were thus analysed to determine the differences in neutrophil, eosinophil and basophil counts between breeds. The Kruskal-Wallis 1-way ANOVA test was used to do this analysis. The same test was also used to test the effects of the site where the tick was detached from the animal (perineum, tail, axillae or udder) and tick engorgement level (flat, slightly engorged, moderately engorged or fully engorged) on cell counts across breeds using only data from tick attachment sites across breeds. The Kruskal-Wallis 1-way ANOVA was also used to test for the effect of tick genera; *Hyalomma* and *Rhipicephalus* on cell counts. Spearman's ranking correlations between the detached tick weights and differential cell counts were also calculated. Chi-square procedures were used to test the differences in numbers of ticks detached from different animal body parts and tick distribution between breeds.

4.3 Results

4.3.1 *Preliminary study*

There were differences between the tick attachment and control sites for some of the histological skin conditions when they were scored as either absent or present. Higher levels of hyperkeratosis was found in SAMM ewes at attachment sites compared to control sites (5/6=0.833 vs. 0/6=0.000, Fisher's Exact Probability=0.02). The presence of acanthosis and oedema also approached significance between infested and control sites (4/6=0.667 vs. 0/6=0.000, Fisher's Exact Probability=0.06). The presence of oedema in the Dorpers differed significantly between infested and control sites (4/5=0.800 vs. 0/5=0.000, Fisher's Exact Probability=0.05). No significant differences were observed between attachment and control

sites in the 4 ewes representing the NA breed. Across breeds, histological skin conditions differed between attachment and control sites for crusts (6/15=0.400 vs. 0/15=0.000, Fisher's Exact Probability=0.02), acanthosis (8/15=0.533 vs. 1/15=0.067, Fisher's Exact Probability=0.01), hyperkeratosis (12/15=0.800 vs. 1/15=0.067, Fisher's Exact Probability<0.01) and oedema (10/15=0.667 vs. 1/15=0.067, Fisher's Exact Probability<0.01).

The presence/absence of eosinophils differed significantly ($P < 0.05$) between tick attachment and control sites within breeds in SAMM (6/6=1.000 vs. 0/6=0.000, Fisher's Exact Probability<0.01) and Dorpers (5/5=1.000 vs. 0/5=0.000, Fisher's Exact Probability<0.01). The presence/absence of neutrophils approached significance ($P<0.05$) between infested and control sites within the SAMM (4/6=0.667 vs 0/6=0.000, Fisher's Exact Probability = 0.06) and Dorper breeds (4/5=0.800 vs 0/5=0.000, Fisher's Exact Probability = 0.05). In NA animals there were no significant difference in the presence/absence of neutrophils and eosinophils between the attachment and control sites. Across breeds the presence/absence of eosinophils and neutrophils differed ($P < 0.05$) between the infested and control sites (15/15 = 1.000 vs 1/15 = 0.067, Fisher's Exact Probability < 0.01 and 12/15 = 0.800 vs 1/15 = 0.067, Fisher's Exact Probability <0.01, respectively). No eosinophils and neutrophils were found at the control sites of Dorper and SAMM ewes while a few eosinophils (13) and neutrophils (1) were observed at the control site of a single NA ewe.

Against this background, the control sites were excluded from further analysis on eosinophils and neutrophils. The data were characterised by extreme variation (for instance, raw counts reached values of 226 and 464 eosinophils in a Dorper and NA ewe respectively). Attachment sites were thus compared between breeds for eosinophil numbers using one-way ANOVA procedure on square root transformed counts. No indication of breed differences were found for either eosinophils or neutrophils (P=0.49; Table 4.1). When basophils were considered, a significant interaction was observed between breed and sampling site (P<0.01). Basophil numbers were significantly higher at tick attachment sites than at control sites in SAMM ewes while no differences was recorded in the other breeds. Overall, basophil counts were independent of breed (P=0.68), but basophil infiltration was increased at infested sites relative to control sites (1.45 ± 0.20 vs. 1.09 ± 0.13; P<0.05; Table 4.1). Mast cells were found at tick infested and control sites in all three breeds but the interaction between breed and sampling site was not significant (P=0.84). Means for square-root transformed mast cell

counts were independent of breed (P=0.17; Table 4.1). However, mast cell infiltration was increased at infested sites relative to control sites (3.35 ± 0.24 vs. 2.20 ± 0.22; P<0.01).

Table 4.1 Means (±SE) depicting breed effects for square root transformed eosinophil, neutrophil and mast cell counts, as well as for the interaction of breed and sampling site for basophil counts, in the exploratory study

	Dorper		**NA**		**SAMM**	
Eosinophils	8.199 ± 2.53		9.778 ± 1.34		11.025 ± 2.32	
Neutrophils	4.101 ± 2.77		7.293 ± 4.82		2.450 ± 0.84	
Mast cells	2.833 ± 0.31		3.501 ± 0.71		3.722 ± 0.31	
	Infested	Control	Infested	Control	Infested	Control
Basophils	1.393 ± 0.2	0.914 ± 0.13	1.055 ± 0.21	1.637 ± 0.15	1.908 ± 0.37**	0.707 ± 0.00**

** P<0.01; SAMM=SA Mutton Merino, NA= Namaqua Afrikaner

Images of representative tick attachment site samples are presented in Figures 4.1 to 4.3.

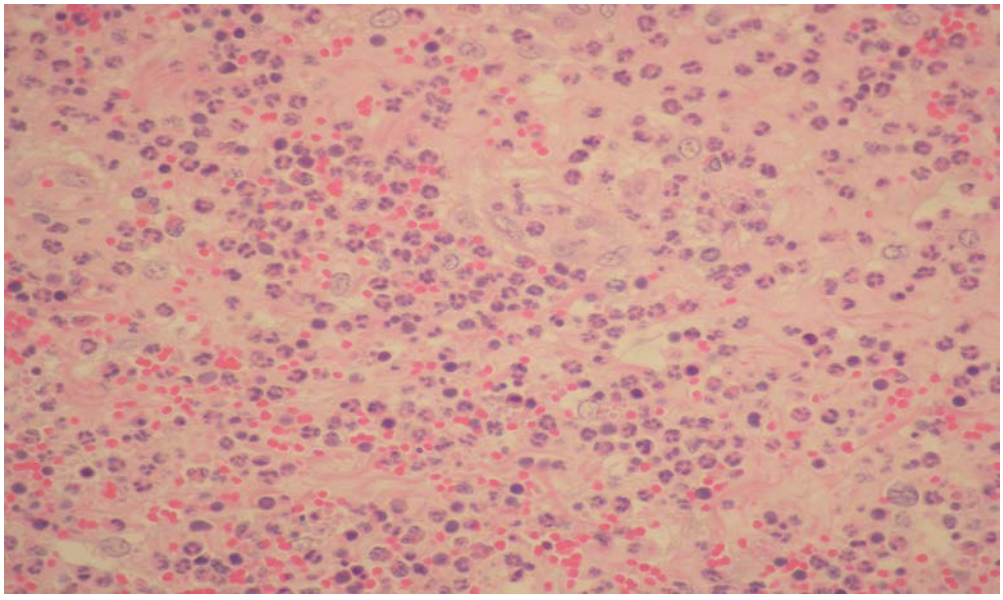

Figure 4.1 Representative tick infested site of NA showing numerous neutrophils (haematoxylin and eosin stain, 40X magnification)

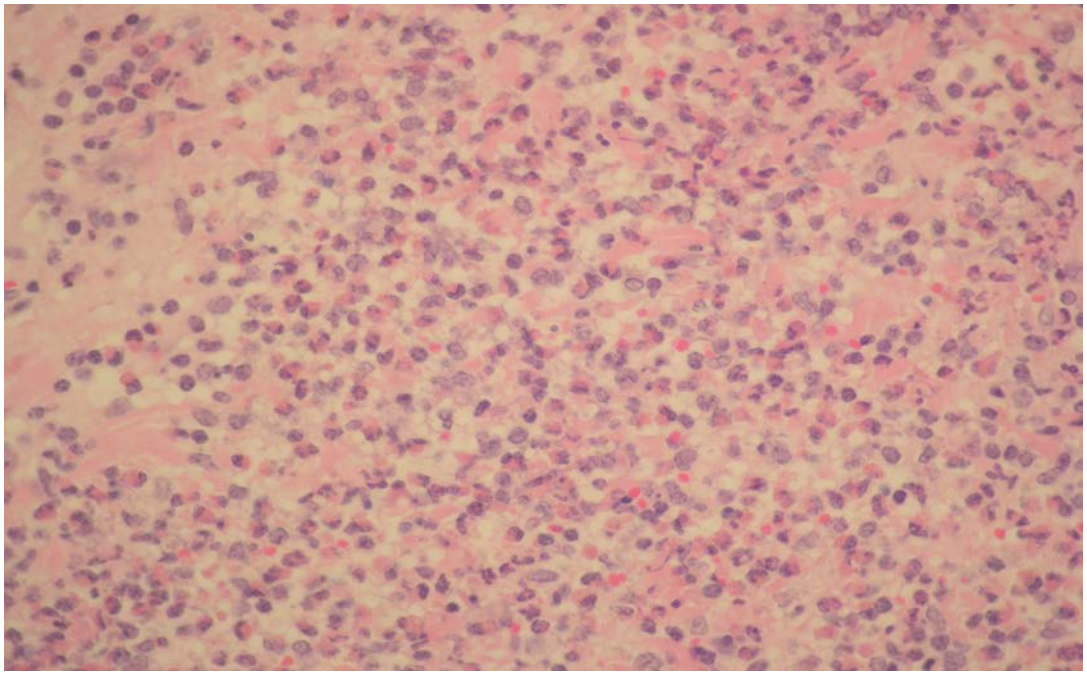

Figure 4.2 Representative tick infested site of SAMM showing eosinophils (Haematoxylin and eosin stain, 40X magnification)

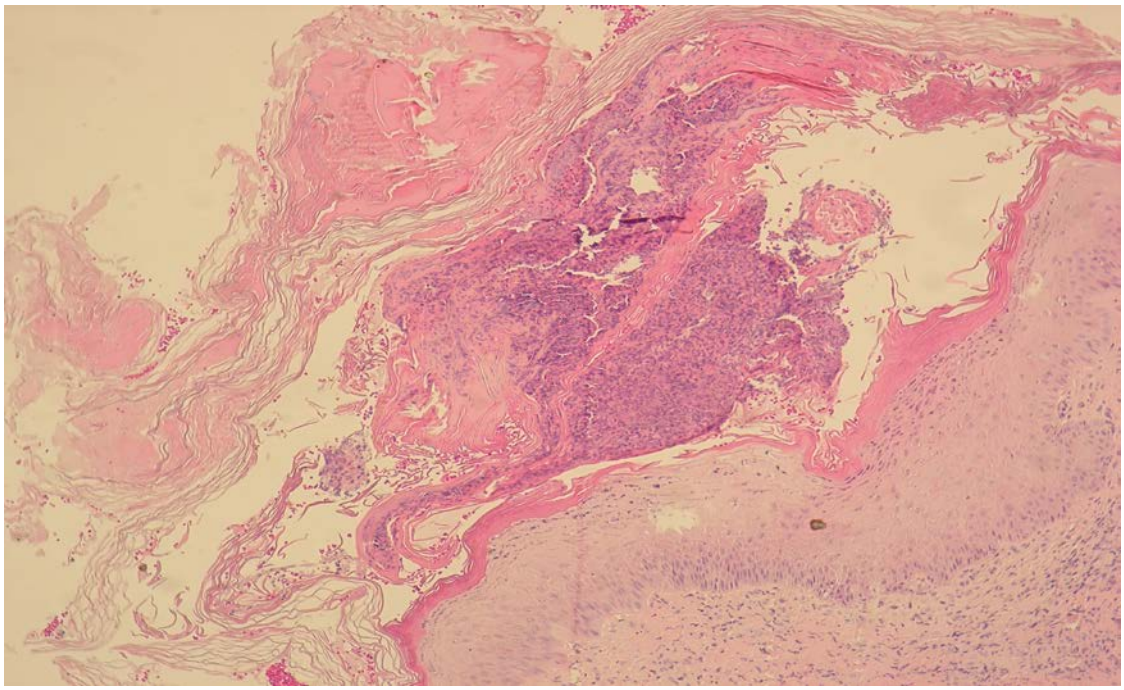

Figure 4.3 Representative tick infested site of a Dorper ewe showing crusting with numerous neutrophils in the keratin (Haematoxylin and eosin stain, 10X magnification)

4.3.2 *Comprehensive study*

4.3.2.1 Skin defects

The histological skin conditions studied were largely absent in the control skin samples (Table 4.2). The exceptions were for oedema, where the control samples of a single ewe of each of the three breeds were affected and hyperkeratosis, where control samples obtained from two NA ewes were affected. Samples from tick attachment sites of SAMM ewes were much less likely to display normal skin histology when compared to the corresponding control samples ($P<0.05$). Tick attachment samples of Dorper ewes were similarly less likely to display normal histology than control samples. The exception was apoptosis, where the proportion of tick attachment samples not displaying this condition only tended ($P=0.06$) to be smaller than the corresponding control samples. The situation seemed to be slightly different in the NA, where the proportion of skin samples displaying normal histology did not differ between tick attachment sites and control sites for spongiosis, apoptosis, necrosis and collagen degeneration (Table 4.2). Similar to the other breeds, a lower proportion of tick attachment samples displayed normal histology than control samples for the remainder of the histo-pathological conditions studied. It thus seemed that the skin histology of tick attachment sites were in most cases profoundly different from those of control samples in all breeds studied, although the impact of tick attachment seemed to be ameliorated in NA ewes for some of the skin conditions considered.

Overall, the proportions of tick attachment samples displaying normal histology ranged for 0.303 for hyperketatosis to 0.841 for apoptosis. No conclusive breed differences were found for the proportion of tick attachment site samples within the normal range for the histological indicators studied (Table 4.3).

Table 4.2 The proportion of tick attachment and control samples displaying normal histology for 29 SA Mutton Merino (SAMM), 39 Dorper and 21 Namaqua Afrikaner (NA) ewes, with corresponding Chi²-values after the Yates correction for continuity (critical Chi² for two degrees of freedom=5.74)

Breed and sample site	Condition[#]								
	Crusts	Spongiosis	Acanthosis	Apopthosis	Hyper-keratosis	Prominent granular layer	Necrosis	Oedema	Collagen degene-ration
SAMM[$]									
Attachment	0.448	0.643	0.250	0.750	0.241	0.536	0.690	0.379	0.621
Control	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.966	1.000
Chi²	19.4**	8.1**	31.3**	6.1*	14.9**	8.4**	20.0**	20.0**	11.2**
Dorper									
Attachment	0.615	0.846	0.385	0.872	0.385	0.744	0.846	0.487	0.769
Control	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.974	1.000
Chi²	16.2**	4.5*	31.8**	$3.4^{0.06}$	31.8**	9.3**	4.5*	8.0**	8.0**
NA									
Attachment	0.524	0.810	0.238	0.905	0.238	0.619	0.857	0.381	0.905
Control	1.000	1.000	1.000	1.000	0.905	1.000	1.000	0.952	1.000
Chi²	10.6**	$2.5^{0.11}$	22.7**	$0.5^{0.47}$	16.4**	7.6**	$1.4^{0.23}$	10.4**	$0.5^{0.47}$

*P<0.05; **P<0.01; Superscripts denotes actual significance for P>0.05; [#]All samples displayed normal histology for acantholysis and the data are not shown; [$]In some analyses only 28 SAMM ewes were represented (see Material and Methods)

Table 4.3 The proportion of tick attachment samples displaying normal histology for 29 SA Mutton Merino (SAMM), 39 Dorper and 21 Namaqua Afrikaner (NA) ewes, with corresponding Chi²-values after the Yates correction for continuity (critical Chi² for two degrees of freedom=5.74)

Condition[#]	Breed			Chi²-values
	SAMM[$]	Dorper	NA	
Crusts	0.448	0.615	0.524	$1.27^{0.53}$
Spongiosis	0.643	0.846	0.810	$2.84^{0.24}$
Acanthosis	0.250	0.385	0.238	$1.17^{0.56}$
Apopthosis	0.750	0.872	0.905	$1.46^{0.48}$
Hyperkeratosis	0.241	0.385	0.238	$1.31^{0.52}$
Prominent granular layer	0.536	0.744	0.619	$2.27^{0.32}$
Necrosis	0.690	0.846	0.857	$1.96^{0.38}$
Oedema	0.379	0.487	0.381	$0.51^{0.78}$
Collagen degeneration	0.621	0.769	0.905	$3.91^{0.14}$

Superscripts denote actual significance for P>0.05; [#]All samples displayed normal histology for acantholysis and the data are not shown; [$]In some analyses only 28 SAMM ewes were represented (see Material and Methods)

4.3.2.2 Cellular response infiltration

4.3.2.2.1 *Eosinophils*

The presence/absence of eosinophils differed significantly between infested and control sites within breeds; in SAMM (38/39 vs. 18/39, Fisher's Exact Probability<0.01), in NA (20/21 vs. 9/21, Fisher's Exact Probability<0.01) while there was no significant difference in Dorper (39/39 vs. 35/39, Fisher's Exact Probability=0.11). NA ewes tended to have the highest number of eosinophils at tick attachment sites compared to Dorper and SAMM ewes(Figure 4.4). This difference approached significance (P=0.096).

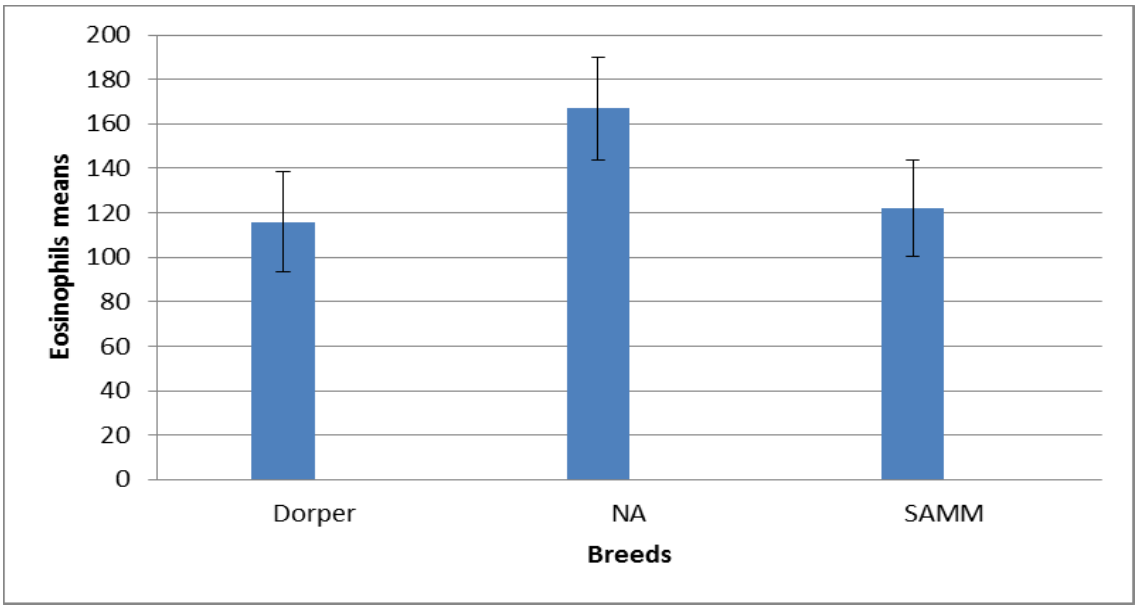

Figure 4.4 Means (standard errors as vertical bars around means) depicting eosinophil numbers at tick attachment sites for Dorper, Namaqua Afrikaner (NA) and SA Mutton Merino (SAMM) ewes.

4.3.2.2.2 *Neutrophils*

Ticks induced neutrophil infiltration where they attached in all the three breeds. The presence of neutrophils differed between infested and control sites in NA ewes (19/21 vs. 1/21, Fisher's Exact Probability <0.01), in SAMM ewes (28/29 vs. 5/29, Fisher's Exact Probability <0.01) but not in Dorpers. The SAMM breed had the highest number (P<0.05) of neutrophils at tick attachment sites, exceeding those counted in Dorper ewes by 2.6-fold and NA ewes by 2.4-fold (Figure 4.5).

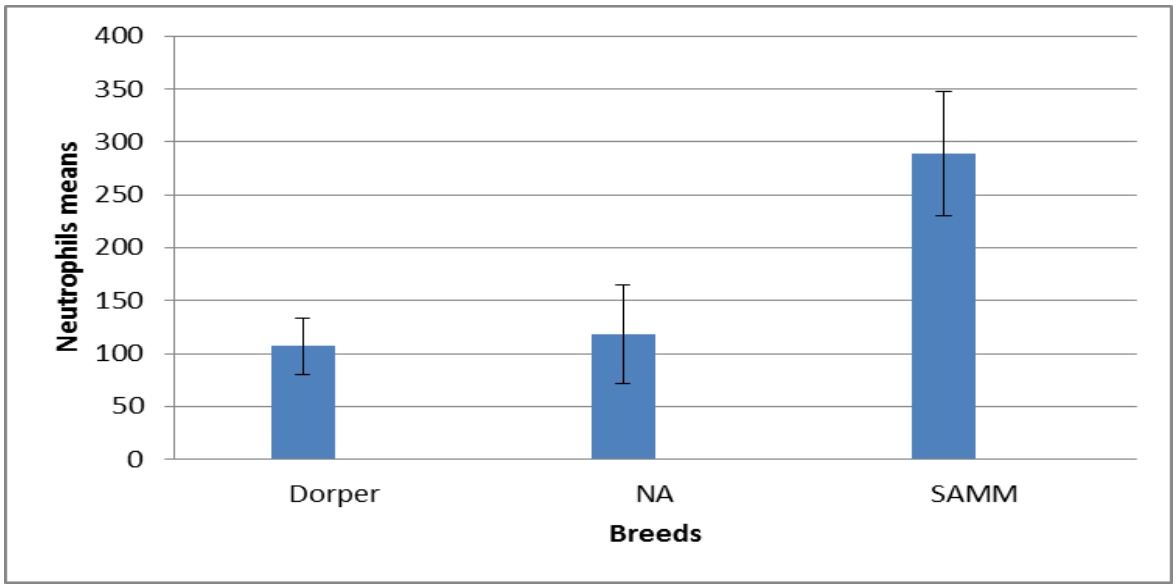

Figure 4.5 Means (standard errors as vertical bars around means) depicting neutrophil numbers at tick attachment sites for Dorper, Namaqua Afrikaner (NA) and SA Mutton Merino (SAMM) ewes.

4.3.2.2.3 *Mast cells*

As opposed to the preliminary study, the comprehensive study indicates that tick bites provoked infiltration of mast cells more in the NA than in the other two breeds, these differences approaching significance ($P=0.067$). Similarly to preliminary study, the number of mast cells at tick attachment sites were higher ($P<0.05$) than at control sites in all the breeds (Figure 4.6). Mast cell means at attachment sites were increased by 141% in the Dorper breed, 130% in the NA breed and 94% in the SAMM breed compared to control sites.

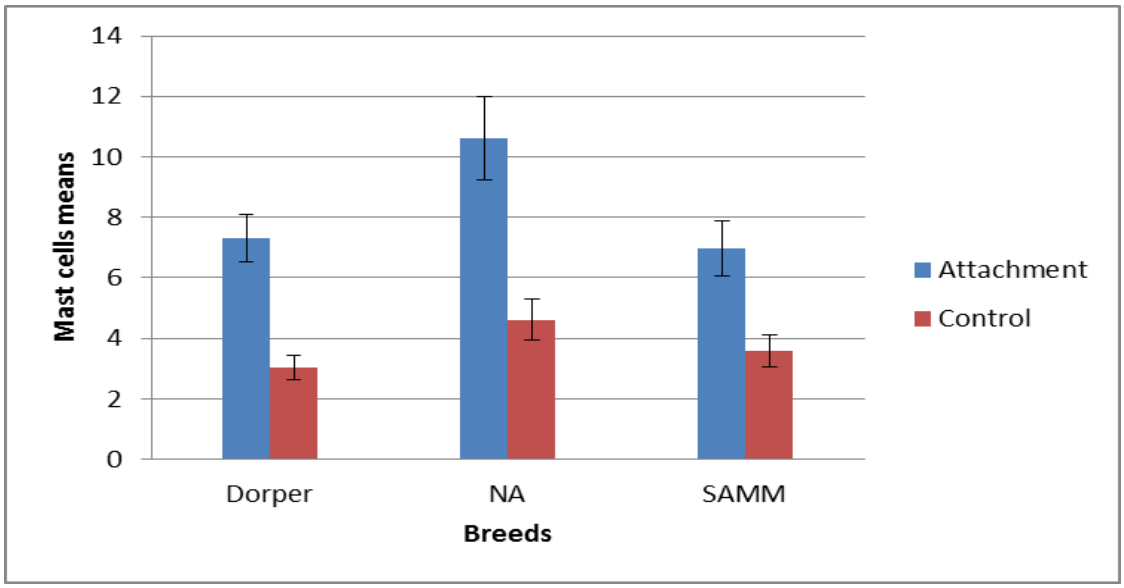

Figure 4.6 Means (standard errors as vertical bars around means) depicting mast cell numbers at tick attachment and control sites for Dorper, Namaqua Afrikaner (NA) and SA Mutton Merino (SAMM) ewes.

4.3.2.2.4 *Basophils*

Basophils were more recruited to infested sites ($P<0.05$) than at control sites across breeds. The presence of basophils differed between infested and control sites in Dorper ewes (18/39 vs. 1/39, Fisher's Exact Probability<0.01) and NA ewes (17/21 vs. 2/21, Fisher's Exact Probability <0.01) while in SAMM ewes there were no difference between tick attachment and control sites. Tick attachment induced basophil infiltration more ($P<0.05$) in the NA breed compared to the Dorper with NA ewes having basophil numbers 2-fold higher than the Dorper while there was, in contrast, no significant difference between the NA and SAMM breeds (Figure 4.7).

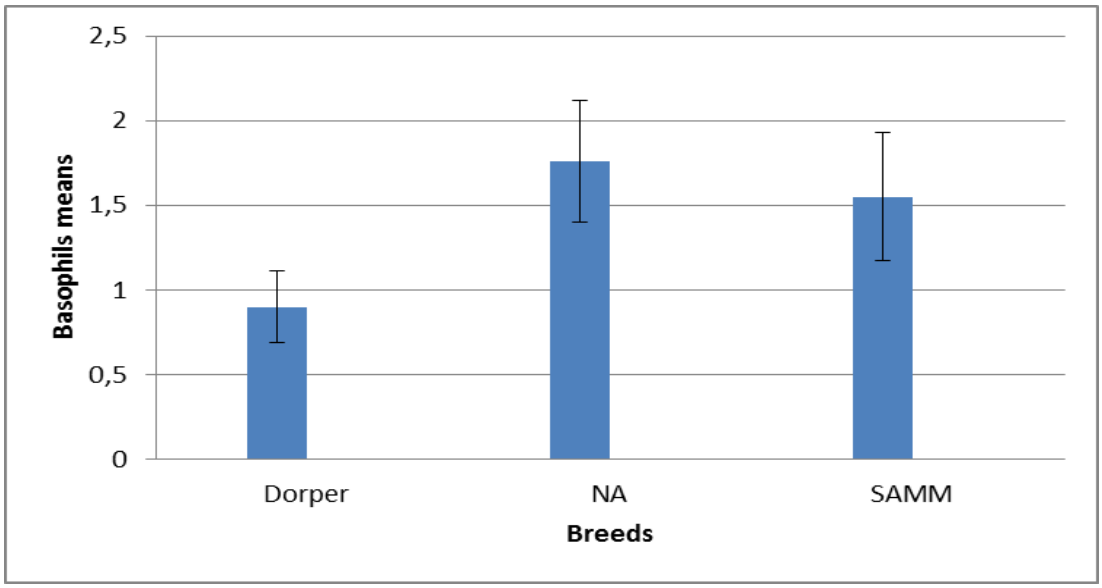

Figure 4.7 Means (standard errors as vertical bars around means) depicting basophil numbers at tick attachment and control sites for Dorper, Namaqua Afrikaner (NA) and SA Mutton Merino (SAMM) ewes.

4.3.2.3 Effects of tick genus, attachment site, tick engorgement status, and tick sex on cell counts

Low cell counts required the meaningful pooling of data to assess the distributions of tick genera, attachment sites, tick engorgement status and tick life stage for Chi-square analyses. There were strong evidence that a higher proportion of ticks of the genus *Hyalomma* was detached from SAMM ewes compared to NA ewes ($P<0.05$; Table 4.4). Adjusting the critical Chi-square to $P=0.0167$ (instead of 0.05) to account for multiple testing between breeds did not affect this inference (actual $Chi^2=6.17$; $P=0.013$). The proportion ticks of the genus *Hyalomma* detached from Dorper ewes was intermediate and not different from either of the other breeds. The vast majority of ticks were detached from the perineum-tail site in all three breeds, and no breed differences were found. The proportions of flat and female ticks detached accordingly did not differ between breeds (Table 4.4). The bulk of ticks in all breeds were designated as flat. The body site from which the ticks were detached did not have an effect on the cell count. Similarly, tick engorgement level and life stage did not have effect on the cell count.

Table 4.4 The distribution of the dominant tick species between breeds and the body locations from which ticks were detached, ticks engorgement levels and tick life stage in the comprehensive study

Contrast analysed	**Breed**			Chi²
	Dorper	**NA**	**SAMM**	
Hyalomma vs. *Rhipicephalus*	$0.727^{a,b}$	0.385^{a}	0.857^{b}	6.643*
Perineum-tail[#] vs other sites	0.872	0.810	0.931	$0.758^{0.68}$
Flat vs. all engorged	0.744	0.688	0.793	$0.040^{0.96}$
Female vs. male[$]	0.333	0.555	0.444	$1.562^{0.45}$

[a,b] Denote significant (P<0.05) differences in rows; *P<0.05; Superscripts denote actual significance for P>0.05; [#]Ticks (n=14) detached from the smooth ventral part of the tail of NA ewes were added to 3 detached from the perennial area; [$] Respectively 2, 1 and 2 nymphs were detached from Dorper, NA and SAMM ewes, rendering an analysis on life stage inaccurate owing to too many cells with counts below 5

Tick genus had significant effects on the infiltration of neutrophils (P=0.0014) with more neutrophils infiltrating sites where *Hyalomma* ticks attached compared to where *Rhipicephalus* ticks attached. In contrast, tick genus had no significant effect on the infiltration of mast cells (P=0.75), basophils (P=0.64) and eosinophils (P=0.87) (Table 4.5).

Positive Spearman's correlations were found between detached tick weight and the number of mast cells and neutrophils while other cells were not affected by tick weight (P<0.10; Table 4.6). Whether the tick weights depended on tick species could not be affirmed due to unequal numbers representing the respective tick species. Eosinophil numbers were significantly positively correlated with the numbers of neutrophils, mast cells and basophils. Basophil numbers were also positively correlated with the numbers of neutrophils and mast cells.

Table 4.5 Means and standard errors for cell counts induced by different tick species across sheep breeds in the comprehensive study

Tick genus	**Eosinophils**	**Neutrophils***	**Mast cells**	**Basophils**
Hyalomma	123.65 ± 17.54	231.77 ± 41.98	7.63 ± 0.78	1.35 ± 0.26
Rhipicephalus	137.70 ± 21.80	102.03 ± 30.03	8.20 ± 0.90	1.45 ± 0.27

* $P<0.001$

Table 4.6 Spearman's ranking correlations (below the diagonal) among tick weights and leukocyte cells counts as well as among differential leucocyte counts in the comprehensive study, using data from tick attachment sites

	Tick weight	**Eosinophils**	**Neutrophils**	**Mast cells**
Eosinophils	0.049			
Neutrophils	0.206*	0.297**		
Mast cells	0.203*	0.252**	-0.038	
Basophils	0.017	0.429**	0.239**	0.452**

** $P<0.05$; * $P<0.01$

4.4 Discussion

4.4.1 *Preliminary study*

4.4.1.1 Skin defects

All the breeds responded similarly to tick attachment as far as skin reactions/changes are concerned. Tick attachment induced conclusive skin reactions/changes in Dorper and SAMM ewes while they appeared to be less obvious in NA ewes. Crusts were absent at the tick bite sites

of NA ewes in contrast to the other breeds while necrosis was not found in all the breeds. However more animals are needed to be studied before inferences may be made.

4.4.1.2 Cellular infiltration

The cellular infiltration at tick infested sites and control sites in NA animals differed with those of SAMM and Dorper ewes. SAMM and Dorper ewes had significantly higher mast cells, eosinophils, neutrophils and basophils at the tick attachment sites than at control sites while no significant difference was observed in NA animals. No differences were found in cellular infiltration at tick attachment sites between breeds. However, the NA breed tended to have more neutrophil infiltrating at the tick attachment sites.

Furthermore it had been suggested that, although not significantly, the breed perceived to be resistant to ticks (the NA) had a lower number of basophils at attachment sites than at control sites while the most susceptible breed (SAMM) had more basophils at tick attachment sites than control sites. The high standard deviations, some of which were higher than the corresponding means of cell counts, are consitent with Walker & Fletcher (1986)'s study which indicated that standard deviations exceeded cell count means at tick attachments sites in rabbits and calves. These high standard deviations put a limitation to the ability of analysts to conclusively differentiate between breed-specific cell counts where small numbers of animals are involved (like in this preliminary study).

The preliminary study allowed some conclusions to be drawn pertaining to differences in skin defects and cell counts between tick attachment and control sites, but it would not support inferences pertaining to some skin defects and cell counts between breeds. More data were needed, as it was evident that the "Reduction" principle could not be applied to the level it was done in the preliminary study and still expect coherent and accountable results. Thus the comprehensive study had to be carried out.

4.4.2 *Comprehensive study*

4.4.2.1 Skin defects

The skin changes at tick attachment sites observed in this study have been reported in previous studies on cattle (Gashaw & Mersha, 2013; Marufu *et al*., 2014; Gholizadeh *et al*., 2015). Crusts were present in the NA in the comprehensive study. This therefore, suggest that the absence of crusts in the preliminary study was not an indication of differential hypersensitivity reactions in NA ewes, but rather of the limited number of NA ewes. Necrosis was found in all breeds. The absence of acantholysis at the tick infested sites in all breeds in this study is contrary to the report of Piper *et al*. (2010) which indicated the presence of this skin change in susceptible Holstein-Friesian cattle. This implies that the mechanims used by cattle against ticks probably differ in other animal species.

This study affirmed the presence of significant differences between attachment and control sites in the presence of all skin defects, barring acantholysis, in Dorper and SAMM ewes. In contrast, no signficant differences between attachment and control sites were found for four skin defects in NA ewes, namely; spongiosis, apoptosis, epidermal necrosis and collagen degeneration. The results of this study accord with the findings of Piper *et al* (2010) which indicated the more pronounced skin changes in lowly resistant Holstein-Friesian cattle than in more resistant Brahman cattle. Overall, this study did not find significant differences in the presence of skin defects at tick attachment sites between the breeds although NA ewes tended to have lower levels of spongiosis and collagen degeneration at tick attachments sites than Dorper and SAMM ewes. The NA, according to previous studies (Cloete *et al*., 2013), was associated with lower tick numbers and can be considered as a more resistant breed to tick infestation.

4.4.2.2 Cellular infiltration

Infiltration of eosinophils, neutrophils, mast cells and basophils were observed at the tick attachment sites of all breeds. In the comprehensive study, with more ewes per breed, there was significantly higher cellular infiltration at tick attachment sites than at control sites in all breeds.

Piper *et al*. (2010) found higher levels of cellular infiltration at tick infested sites of lowly resistant Bos Taurus cattle compared to more resistant Brahmans.

Generally, the current study found that eosinophil, neutrophil and basophil cells were largely absent at most of the control sites of the SAMM and Dorper animals and very few were observed at control sites of NA ewes. These results suggest that these cells are part of the ovine immune response and are recruited during infestations.

There were significant differences ($P<0.05$) in infiltration of eosinophils between tick infested and control sites in all three breeds. Eosinophils tended ($P=0.096$) to infiltrate tick attachment sites more in the resistant breed, namely the NA compared to the susceptible breeds, the Dorper and SAMM. These results agree with the findings of Carvalho *et al*. (2010) who reported recruitment of eosinophils in greater numbers to tick infested sites in resistant cattle than in those susceptible to ticks. The SAMM has been reported to have the highest number of ticks at some body locations in previous studies (Cloete *et al*., 2013) which suggested that it is susceptible to tick infestations. However, according to some previous studies (Tatchell & Moorhouse, 1968; Piper *et al*., 2010), the infiltration of eosinophils at the tick infested sites is typical of susceptible animals. Higher numbers of eosinophils at tick attachment sites are reported to increase tissue fluids which is to the advantage of ticks.

The tendency of Dorpers to have the lowest number of eosinophils in this study disagrees with the suggestion that higher numbers of eosinophils is common in animals susceptible to tick infestation (Tatchell & Moorhouse, 1968). The Dorper breed has previously been reported to have higher numbers of ticks than the NA on the front legs as well as the udder and hind legs (Cloete *et al*., 2013). Intense infiltration with eosinophils is considered to be a Type 1 hypersensitivity response in animals susceptible to tick infestations (Constantinoiu *et al*., 2010). The infiltration of the eosinophils have been reported in the skin of louse infested lambs and the infiltration were reported to be significantly higher in previously infested than in naïve lambs (Shu *et al*., 2009). Carvalho *et al*. (2010) also reported lower infiltrations of eosinophils at the tick bite sites of susceptible cattle compared to more resistant breeds. The findings of the current study suggests that eosinophils play a role in resistance to tick infestations and that the three breeds use different mechanisms.

The breeds under consideration differed ($P<0.05$) in their extent of neutrophil infiltration at tick attachment sites. The SAMM breed had significantly more ($P<0.05$) neutrophils at tick attachment sites compared to the other two breeds. This study accords with conclusions from previous studies (Turni *et al*., 2002; Constantinoiu *et al*., 2010) suggesting that neutrophils do not play a major role in resistance to ticks in cattle. According to Turni *et al*. (2002) and Constantinoiu *et al*. (2010) neutrophils are involved in forming feeding lesions of ticks and the development of tissue damage. The infiltration of neutrophils is reported to lead to oedema at the tick attachment site. Animals of all breeds included in the present study showed mild to severe levels of oedema at tick bite sites, with severe oedema observed in 4 SAMM ewes.

The results of this study indicated that higher numbers ($P<0.05$) of mast cells were found at tick attachment sites of all breeds compared to control sites. NA ewes tended to have higher ($P=0.067$) mast cell numbers compared to Dorper and SAMM ewes at tick attachment sites. This corresponds to the lower tick counts found in the NA breed compared to the Dorper and SAMM breeds. The results may imply that these cells play a role in the resistance or susceptibility of the animals to tick infestations. Previous authors have accordingly reported similar results in some breeds of cattle (Filho *et al*., 2006; Verissimo *et al*., 2008).

Mast cells are resident cells which are common in all tissues but more in the tissues exposed to the external environment such as the skin (Metcalfe *et al*., 1997). They are considered as guards against infections and infestations. Mast cells contain histamine which is associated with an itch scratch reaction at a tick bite site, so preventing tick attachment. It is therefore not surprising that they occur at all sites sampled. This study verified that higher absolute numbers of mast cells of NA ewes could possibly be related to more easily harnassed innate defense mechanisms to tick bites in this breed, as suggested by the previously reported differential tick counts in favour of the NA on some body locations (Cloete *et al*., 2013). These results correspond with comparable results in some cattle breeds (Filho *et al*., 2006; Verissimo *et al*., 2008). Mast cells have been negatively associated with tick counts, that is the animals that have high counts of these cells tend to have lower tick counts (Filho *et al*., 2006). In contrast, Verissimo *et al*. (2008) reported a lower number of mast cells in tick infested resistant Gyr Zebu cattle compared to tick infested susceptible European cattle.

Basophil numbers were more at tick infested sites than at control sites in all three breeds with significant ($P<0.05$) breed effects on the infiltration of basophils at tick attachment sites. The resistant breed, the NA had significantly more basophils at tick attachment sites compared to Dorpers, while no significant difference was found between the NA and SAMM breeds. The greater number of basophils at tick attachment sites in the resistant NA breed compared to Dorpers concur with findings of Carvalho *et al*. (2010) in cattle breeds. Basophils have been indicated to play a role in the resistance of animal hosts to tick infestation (Gill & Walker, 1985). Surprisingly the most susceptible breed, the SAMM did not have significantly fewer basophils than the NA at tick attachment sites. This may imply that there are other factors that were not studied here that make SAMM more susceptible to tick infestation despite the breed's ability to recruit basophils at attachment sites.

The dominance of neutrophils at the tick attachment sites of the SAMM in this study is consistent with previous studies (Gill & Walker, 1985; Walker & Fletcher, 1986; Bowles *et al*., 1992). Walker & Fletcher (1986) reported the infiltration of neutrophils at tick attachment sites of Bos taurus calves and rabbits. Gill & Walker (1985) reported neutrophils as the dominant cells at tick attachment sites in rabbits. In contrast, eosinophils were dominant at tick attachment sites in NA and Dorper ewes. Gill & Walker (1985) also documented increased eosinophil counts at tick attachment sites in rabbits during tertiary infestation.

The cutaneous cellular infiltration in all three breeds is indicative of a hypersensitivity immune response at tick attachment sites. NA ewes also demonstrated an extent of delayed type hypersensitivity response in their cellular infiltrations. Type I hypersensitivity responses, also known as immediate hypersensitivity, is characterised by the proliferation of mast cells and eosinophils which occured in all breeds. On the other hand, inflitration by neutrophils are characteristics of a type III hypersensitivity response/reaction. The findings of the present study are consistent with a paper by Allen (1994), indicating that resistance to ectoparasites is mediated by immunological factors and is associated with hypersensitivity reactions. James & Nattrass (2001) also reported that sheep demonstrated Type I hypersensitive responses against ectoparasites in their study on sheep biting lice. They also indicated that the immediate reactions were higher in sheep more resistant to lice than in susceptible sheep. A similar pattern was

observed in the present study. The hypersensitivity responses seem to be associated with resistance to ectoparasites. However, there is a controvesy in the literature with regard to which type of skin reactions (immediate hypersensitivity, delayed hypersensitivity and hyposensitivity) is more likely lead to susceptibility or resistance to tick infestation.

The body location of the attachment sites sampled from did not influence cellular infiltration significantly, as was also reported by Piper *et al.* (2008) for cattle. Even though they compared the effect of sampling site on gene expresion in skin, their results are comparable with those of the current study. The tick engorgement level also did not have an effect on cell numbers at tick attachment sites. Even so, this result has to be interpreted with caution as the impact of different tick species might have overshadowed the effect of tick engorgement level. Tick genera had a significant effect on the infiltration of neutrophils while no significant effects were found for other cell types at tick attachment sites. Tick weights were positively correlated with mast cells and neutrophil cells.

There is great variation in the response of sheep to tick attachment within and between breeds. All the breeds had variable skin changes in response to tick bites. The results of this study is consistent with James & Natrass (2001)'s findings which indicated significant variability in the susceptibility of sheep to lice. The comprehensive study with increased animal numbers enabled analysts to draw more robust conclusions from the data presented.

4.5 Conclusions

There is marked variation in immunological responses to tick attachment within and between sheep breeds. There are differences between the infested and control sites in most of the skin changes (defects) considered except for four skin defects in NA. All the breeds had similar frequencies of skin defects at tick attachment sites. There were differences between attachment and control sites in the number of leukocytes infiltrating the skins of the experimental animals. The infested sites were more likely to be infiltrated by mast cells, basophils, eosinophils and neutrophils within as well as across breeds. The NA and SAMM breeds tended to demonstrate greater cellular infiltrations of specific leukocytes at tick attachment sites compared to Dorpers. The extent of cellular infiltration differed between the three breeds at the tick infested site.

Basophils, mast cells and eosinophils are increasingly recruited at tick infested site in NA ewes compared to Dorper and, occasionally, SAMM ewes. These results suggest the importance of these cells in sheep resistance to tick infestation. Tick genera influenced the recruitment of neutrophils to tick attachment sites. Tick gender, sampling site as well as tick engorgement level had no effect on the number of immunological cells. Further studies should be done with one tick species at a time using artificial infestation to better comprehend the species-specific impact of tick attachment to animals belonging to divergent sheep breeds.

CHAPTER 5

Selection of reference genes for normalizing gene expression at tick bite and control sites of sheep skin

5.1 Introduction

Gene expression studies using the real-time qualitative polymerase chain reaction (RT-qPCR) technique are becoming popular in life sciences as the scientists endeavour to understand the genes manifested during different conditions, for instance in diseased tissues, parasite infested tissues/cells and different development stages of organisms (Wang *et al*., 2007; Piper *et al*., 2008). RT-qPCR is preferred for gene expression because of its accuracy, sensitivity, reliability and a short turnover time. Even so, to get reliable and accurate results in gene expression studies it is recommended that suitable reference genes (commonly known as housekeeping genes) have to be used to normalize gene expression data.

For a gene to be considered a reference gene it should not be expressed variably under different experimental conditions or treatments (i.e. equally expressed or stable) (Vandesompele *et al*., 2002; Kozera & Rapacz, 2013). Most of the genes used as reference genes are genes that have vital functions in the body cells of the organism (Thellin *et al*., 1999). Normalization of gene expression data aims to eliminate or minimize variation caused by external factors such as RNA quality and quantity, different concentrations and different PCR efficiencies (Huggett *et al*., 2005). Therefore, for accurate and reliable gene expression results, suitable reference genes for each particular experiment need to be analysed and the most stable ones selected.

There are several common reference genes used to normalize gene expression data in the literature and most of them are used without validation for the specific experimental set up. Some of the commonly used reference genes are *GAPDH, 18S, TBP, B2M, ACTB, SDHA* and *YWHAZ* (Vandesompele *et al*., 2002; Jain *et al*., 2006). This is done in spite of the reports that the reference genes may not be stable under different experimental conditions, different tissues,

species, breeds and even individuals within breeds. The aim of this study was to identify and select suitable reference genes for a study of gene expression at tick-bite and control sites in the skin of three South African sheep breeds; Namaqua Afrikaner (NA), Dorper and South African Mutton Merino (SAMM).

5.2 Materials and methods

5.2.1 Skin biopsy collection

One (1 ml) Lignocaine (lidocaine) local anesthetic was administered per sample site to the animals before sampling. The whole skin biopsies of 4 mm diameter were collected from 15 animals (NA = 4, Dorper = 5 and SAMM = 6). The animals were maintained in a single flock at the Nortier Research Farm near to Lamberts Bay in the Western Cape and were obtained from the resource population described by Cloete *et al*. (2013). Two samples were taken per animal, one at a tick bite site and one at a tick free control site, using 4 mm disposable skin biopsy punches (VKruuse, distributed by Kyron Laboratories). Individual skin samples were immediately put in 5 ml RNAlater tubes and refrigerated in a small, portable refrigerator in the weighing room on the farm connected to the main power supply. The samples were transported to the laboratory in the same refrigerator, connected to the battery of the vehicle used for transport.

5.2.2 RNA extraction

RNA was extracted/isolated from the 30 skin samples (15 tick bite sites and 15 control sites) at the molecular laboratory in the Plant Sciences department, University of the Free State, Bloemfontein, South Africa. The skin tissue was removed from the RNAlater and put in a 2 ml Eppendorf tube containing 500 µl Trizol reagents and two small stainless steel beads. The tissue was disrupted using the tissue lyser. Then the disrupted tissue was homogenized to reduce the viscosity of the cell lysates and then another 500 µl Trizol was added before incubating at room temperature for 10 minutes. A volume of 200 µl chloroform was added to the tube and mixed by inverting the tube 15 times and then incubated at room temperature for 5 minutes prior to centrifuging the tubes at 12 000 g for 15 minutes at 4 ^{o}C. After centrifuging there were three layers in the tube, the top aqueous layer (clear appearance) with RNA, middle layer (white

precipitate appearance) with DNA and the bottom layer (pink reagent colour) with proteins. A volume of 500 µl of the cleared supernatant was transferred to tubes containing isopropanol and mixed well before incubated at room temperature for 10 minutes. The tubes were centrifuged for 10 minutes at 12 000 g at 4 ^{o} C to pellet the RNA and the supernatant was removed from the pellet. A concentration of 70% ethanol was added to the pellet and mixed well by inverting the tubes several times, the tubes were centrifuged at 7 500 g for 10 minutes at 4 ^{o} C and the supernatant was removed from the pellet using a water jet pump. The tubes were incubated on the bench for 5 minutes and 50 µl Diethylpyrocarbonate (DEPC) treated water added to each pellet. The tubes were incubated on ice for 1 hour and afterward a 200 µl pipette was used to draw the liquid up and down to dissolve the RNA. The tubes were centrifuged for 5 minutes at 12 000 g at 4 ^{o}C to pellet any undissolved RNA. Lastly the supernatant was transferred to a newly labeled Eppendorf tube.

5.2.3 Reference gene selection

Five genes were selected from the literature and tested for their stability in the current experimental conditions. The selected genes are commonly used in gene expression studies (Wang *et al*., 2007; Piper *et al*., 2008; Sigl *et al*., 2012). Initially seven reference genes, *18s*, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *actin beta* (*ACTB*), *beta-2-microglobulin* (*B2M*), *TATA box binding protein* (*TBP*), *tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta* (*YWHAZ*) and *succinate dehydrogenase complex, subunit A* (*SDHA*) were selected for analysis. All the primers used were obtained from the literature and have been validated in other studies. However, when doing the standard curves to determine the efficiency of the primers, the *ACTB* and *TBP* primers did not have good efficiency and were nonspecific, this was indicated by melt curves displaying more than one peak and thus these genes were excluded from the analysis. The remaining five genes (*18S, GAPDH, YWHAZ, B2M* and *SDHA*) had good efficiencies and had specific melt curves with only one peak (Figure 5.1).

5.2.4 RNA concentration and quality determination

RNA concentration was determined using the Nano-drop machine, 1 µl of RNA was measured after measuring 1 µl of a blank solution in the Nano-drop machine. The quality of the RNA was determined by running 1.2 % agarose gels. RNA showed clear 18s and 28s bands on the gel indicating that it was not degraded.

5.2.5 Optimization of reference genes primers and amplification efficiency

The primers were optimized to obtain their annealing temperature in a C1000^{TM} Thermal Cycler CFX96^{TM} Real-Time System (BIO-RAD). Per each reaction 2 µl of RNA of the target gene was mixed with 1 µl of primer pair, 5 µl of KAPA SYBR Fast Master Mix One-step, 0.2 µl KAPA RT Mix and 1.8 µl of DEPC treated water. The RT qPCR conditions were; 42 ^{o}C for 5 minutes for incubation, 95 ^{o}C for 5 minutes, 95 ^{o}C for 10 seconds for denaturation, gradient for 30 sec for annealing temperature, 72 ^{o}C for extension and melting curve to evaluate specific amplification. The RT qPCR ran for 40 cycles. The annealing temperatures for each primer pair are given in Table 1. The standard curve was derived for each gene to determine the efficiencies of the primer pairs. All the primer pairs of the five genes had efficiencies within the recommended efficiency range of 90 – 110% as shown in Table 2. Similarly, the slopes of the standard curves of all the genes fell within the recommended range of -3.1 to -3.58.

Table 5.1 Sequences of the primers used, product size and annealing temperature

Gene	**Sequence**	**Product size**	**Temp (ºC)**
YWHAZ	Forward: TGTAGGAGCCCGTAGGTCATCT Reverse: TTCTCTCTGTATTCTCGAGCCATCT	102	59
SDHA	Forward: CATCCACTACATGACGGAGCA Reverse: ATCTTGCCATCTTCAGTTCTGCTA	90	64.5
B2M	Forward: ATCCAGCGTATTCCAGAGGTC Reverse: AATCTTCTCCCCGTTCTTCAG	138	59
18S	Forward: GAGAAACGGCTACCACATC Reverse: GCTATTGGAGCTGGAATTAC	185	57
GAPDH	Forward: AAGTTCAACGGCACAGTCAA Reverse: ACCACATACTCAGCACCAGC	181	59

Table 5.2 Appropriate literature sources and efficiency of the primers used for the reference genes

Genes	**Source**	**Efficiency (%)**	**R^2**	**Slope**
YWHAZ	Garcia-Crespo *et al*. (2005)	104.5	1.00	-3.22
B2M	Tian *et al*. (2013)	102.7	1.00	-3.26
SDHA	Garcia-Crespo *et al*. (2005)	102.6	1.00	-3.26
18S	Tian *et al*. (2013)	95.7	0.993	-3.430
GAPDH	Tian *et al*. (2013)	98.9	0.967	-3.349

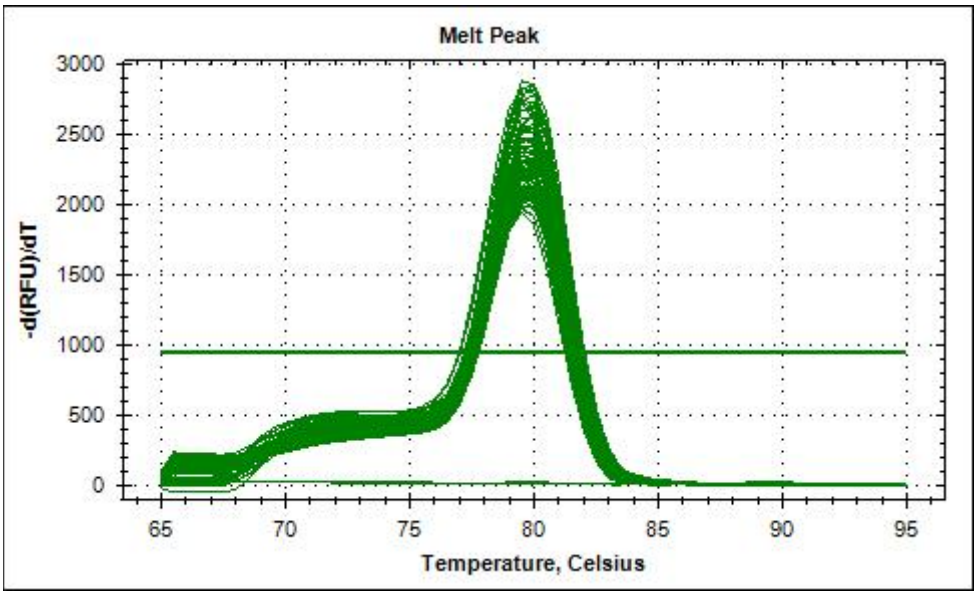

Figure 5.1 Melt curve of *YWHAZ* with one peak and all the amplifications above the threshold line except for the no template control (NTC). A single peak indicates the specificity of the primers and that a single amplicon is amplified.

5.2.6 Data analysis

The data were analysed using the geNorm program in qbase PLUS version, 2.6.1 (Biogazelle, Belgium, info@biogazelle.com).

5.3 Results

The raw data, expressed in threshold cycle (CT) values indicated variable expression of the studied reference genes. Highly expressed genes have low CT values while the less expressed have high CT values. The average CT values for the reference genes were 14.58 for *18s*, 21.67 for *B2M*, 23.75 for *GAPDH*, 24.22 for *YWHAZ* and 25.92 for *SDHA*. The gene 18s was the most variably expressed with a range of close to 20 CT while *SDHA* was the least variably expressed with the range of less than 10 CTs (data not shown).

The results in Figure 5.2 show that the commonly used reference genes have high expression stability value (M), which suggests that they are not most stable. Two reference genes of the five studied had M values below one, which is the threshold value for gene stability in heterogeneous samples. The *18s* gene had the highest M-value (1.72) followed by *GAPDH* while *SDHA* was the

most stable with an M-value of 0.965 followed by *YWHAZ* and *B2M*. *GAPDH* and *18s* proved not to be the preferred normalizing genes.

Figure 5.3 shows the optimal number of reference genes used for normalization. A threshold value of 0.15 was set by geNorm. If the value Vn/n+1 is below this threshold there is no need to use n+1 reference genes. For example, V3/4 means the comparison of the normalisation factors (NFs) from three and four genes respectively. In Figure 5.3, V2/3 was 0.34, V3/4 was 0.33 and V4/5 was 0.47. This suggests that increasing the number of reference genes from two to three reduced the variation but increasing them from three to four did not. At least three reference genes are thus needed to normalize gene expression data at tick-bite and control sites in the skins of sheep under the experimental conditions of this study.

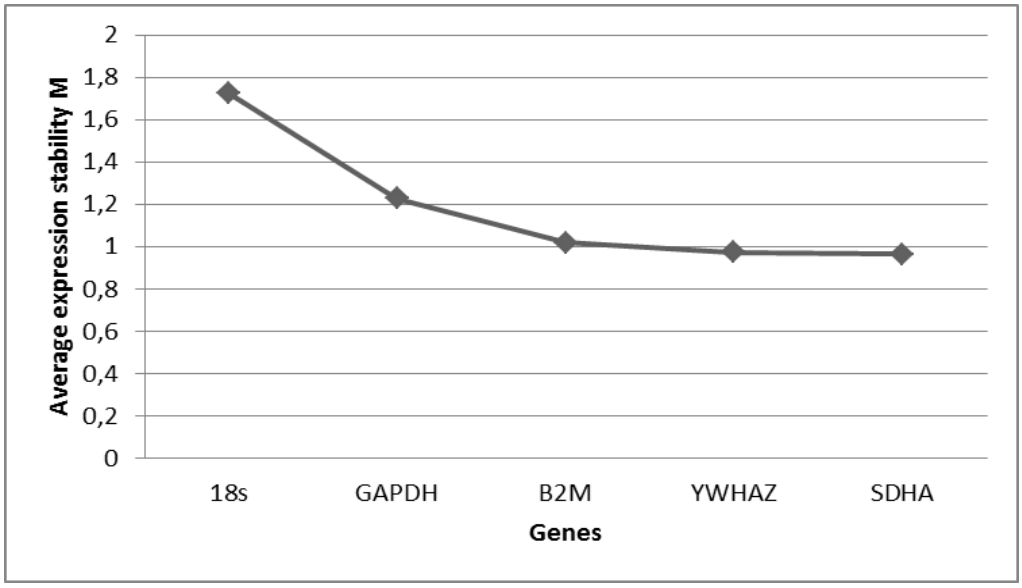

Figure 5.2 The average expression stability (geNorm stability value) of the reference genes. The genes are listed from least stable to most stable (left to right).

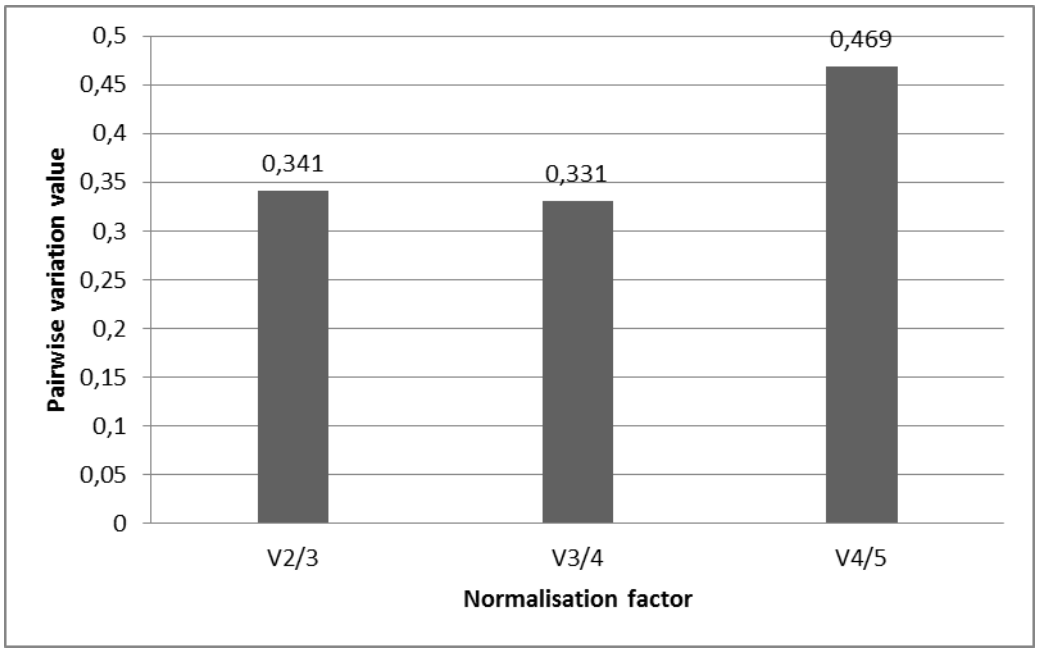

Figure 5.3 The determination of the optimal number of reference genes for normalization. The normalization factor (NF) is calculated from at least two genes by the geNorm program. The mean pairwise variation (V) is determined between two sequential NFn/NFn+1, where n is the number of reference genes needed for normalization of the gene expression data.

5.4 Discussion

The reference genes needed to study tick-bite sites in sheep can be ranked according to their stability (from most stable to least stable); *SDHA*, *YWHAZ*, *B2M*, *GAPDH* and *18s*. *SDHA* proved to be stable and is thus a good reference gene in sheep skin gene expression studies. The stability *SDHA* in this study concurs with the report of Turabelidze *et al*. (2010). However Turabelidze *et al.* (2010) reported *SDHA* to be more stably expressed in normal mouse skin while it was one of the least stable reference genes in mouse with wounded skin. Similarly McCulloch *et al.* (2012) reported *SDHA* as the most stably expressed reference gene in porcine articular cartilage. However the M value for *SDHA* in their study was higher than the M value in the current study. This result suggests that this gene was more stable in the current study.

The *YWHAZ* gene also proved to be suitable for normalizing gene expression data in sheep skin. Garcia-Crespo *et al*. (2005), who first published the primer sequence of this gene, accordingly indicated that this gene was stable in most ovine tissues studied. Even so, the M values recorded

by Garcia-Crespo *et al*. (2005) for this gene are lower than those observed in this study, indicating some variation in stability between the two studies for this gene. In contrast, Nygard *et al*. (2007) reported *YWHAZ* among the least stable reference genes.

The *B2M* sequence has been ranked as one of the most stably expressed reference genes in previous studies (Turabelidze *et al*., 2010; Wang *et al*., 2014). In contrast, other authors (Nygard *et al*., 2007; Guo *et al*., 2010; Tian *et al*., 2013) reported that it was less stable than genes they studied.

Interestingly two commonly used reference genes (*GAPDH* and *18s*) were not stable in this study on ovine skin. *GAPDH* was similarly not stably expressed in some previous studies (Svobodová *et al*., 2008; Wang *et al*., 2014). Nygard *et al*. (2007) and Schlotter *et al*. (2009) also found *GAPDH* to be amongst the least stable reference genes in different tissues of pigs and in dog skin, respectively. In contrast, Guo *et al*. (2010) and Tian *et al*. (2013) ranked *GAPDH* as one of the most stable reference genes. Surprisingly, primer sequences for *GAPDH* and *18s* were first designed and published by Tian *et al*. (2013) for use in the skin of fine wool sheep and these genes were stably expressed in their study. This study confirms that not all commonly used reference genes are suitable for all experimental conditions.

The expression stability M values for *GAPDH* and *B2M* in this study are somewhat higher than those recorded by Garcia-Crespo *et al*. (2005) in different ovine tissues and Wang *et al*. (2014) in pig blood. The low number of reference genes analysed was a limitation because genes with high M values could not be eliminated. The removal of the gene with highest M value is reported to improve the stability of those remaining (Vandesompele *et al*., 2002). The M-values for *GAPDH, B2M, YWHAZ* and *SDHA* were lower than those reported by Nygard *et al*. (2007) in pig tissues, suggesting that these genes were more stable in the present study than in the latter study.

5.5 Conclusions

The geNorm analysis has recommended that at least three reference genes with the lowest M-values be used. *SDHA, YWHAZ* and *B2M* were the most suitable reference genes for normalizing gene expression data in sheep skin. These findings will assist in normalizing data in gene expression studies at tick-bite and control sites of NA, Dorper and SAMM sheep skin. This study

also suggested that no reference gene is stably expressed in all tissues and different experimental conditions. The results stress the importance of validating reference genes for different tissues, contrasting experimental conditions and in different breeds.

CHAPTER 6

Gene expression of cytokine genes at the tick attachment site of Namaqua Afrikaner, Dorper and South African Mutton Merino sheep

6.1 Introduction

Cytokines are harnessed as part of local immunological responses by animals to combat local infections and/or infestations (Straubinger *et al*., 1997). Such localized immunological responses contribute to the host animals' innate defense to ticks. Cytokines are immune response components important in innate immunity and inflammatory response by acting in various ways; the autocrine way, paracrine way or endocrine way (Francischetti *et al*., 2010).

There are several types of cytokines classified according to their activity, including pro-inflammatory cytokines and chemokines. Pro-inflammatory cytokines invoke allergy and inflammation reactions (Dinarello, 2000; Burgess *et al*., 2010). Examples of pro-inflammatory cytokines are interleukin 1 beta (IL-1β), interleukin 10 (IL-10) and interleukin 8 (IL-8) (which is also a chemokine) (Dinarello, 2000). Examples of chemokines are chemokine C-C ligand 2 (CCL2) (also known as Monocyte Chemotactic Protein (MCP-1)) and chemokine C-C ligand 26 (CCL26).

Chemokines are a type of cytokines which have several functions in the immunity reaction. They are known as chemo-attractants, owing to their involvement in chemotaxis, and with the migration of inflammatory cells, such as neutrophils to the site of infection/infestation or injury (Wikel, 2013). The movement of the cells to affected sites follows the gradient of the chemokines (Gonzalez *et al*., 2007). The production of chemokines is influenced by feedback from some pro-inflammatory cytokines at affected sites.

Cytokines play an important role in host resistance or susceptibility to tick infestations in cattle (Gonzalez *et al*., 2007; Wang *et al*., 2007; Piper *et al*., 2008). The author could not source similar studies on sheep in South Africa or elsewhere. Therefore the aim of this study was to investigate

whether there are differences in expression of four selected cytokine genes, namely, *IL-1β*, *CCL2*, *CCL26* and *IL-8*, between tick attachment sites and nearby control sites of Namaqua Afrikaner (NA) (an indigenous fat-tail breed, which is speculated to be more resistant to tick infestation) and ewes of two commercial breeds, the Dorper and South African Mutton Merino (SAMM) (which are speculated to be more susceptible to tick infestation).

6.2 Materials and Methods

6.2.1 Location and management of study animals

The animals used in this study were maintained at the Nortier research farm. The farm is situated at 32º 02'South and 18º 20'East, and is about 10 km north of Lambert's Bay in the Western Cape. Average annual precipitation amounts to 220 mm, 78% of which is recorded during winter. The ovine genetic resource on the farm consists of NA, Dorper and SAMM sheep, and was described by Cloete *et al*. (2013). The animals were maintained under extensive production conditions and utilized indigenous shrub pastures typical of the Strandveld of the western seaboard (Cloete & De Villiers, 1987). A total of 6 mature unmated ewes of each breed were identified as experimental animals for an initial exploratory study in the absence of literature on immune responses to tick attachment in sheep. Of these, 1 NA ewe died during the duration of the experiment, resulting in 5 NA ewes still being available for study purposes. This study was followed by a more comprehensive study involving breeding ewes from the resource flock. These ewes were mated in January-February 2015 to lamb during June-July 2015. Sampling details of both studies are provided below.

6.2.2 Skin biopsy sampling

During the exploratory study, ewes were upended and inspected for ticks in summer (January) and late winter (August) of 2014. Tick attachment sites were identified with a permanent marker pen on each animal, while a nearby control site (unaffected by tick activity) was also marked for each attachment site. One (1 ml) Lignocaine (lidocaine) local anesthetic was administered to each of these sites before sampling. Whole skin biopsies of 4 mm diameter were collected from 15 ewes with adequate tick numbers. Four NA, five Dorpers and six SAMM ewes were sampled in summer. Ticks were not found on 2 NA and 1 Dorper ewes at that stage. Disposable skin

biopsy punches were used to collect two samples per animal, one at the tick attachment site and one at the control site (VKruuse, distributed by Kyron Laboratories). Skin samples were individually submerged in 5 ml RNase free cryo-tubes (Greiner bio-one) containing 4 ml RNAlater RNA stabilization Reagent (Qiagen Gmbh) within a minute of collection and the tubes were tightly closed. The tubes containing the skin samples were immediately put in a small camping refrigerator connected to the main power supply in the weighing room of the farm. The samples were transported to the laboratory still in the same small refrigerator connected to the cigarette lighter of the light delivery vehicle used for transport. The samples were kept in a 4 ^{o}C refrigerator upon arrival at the laboratory and the RNA was extracted within seven days. The same procedure of sampling was repeated in the same animals in late winter of the same year (2014). Fifteen ewes were used; four Dorpers, five NA and six SAMM. All the conditions for the late winter sampling were similar to that of the summer sampling except that the RNAlater^{TM} tubes (Qiagen) were used for the late winter sampling instead of cryo-tubes. Ethical clearance for the project was obtained from the Departmental Ethical Committee for Research on Animals (DECRA reference numbers R13/88 and S13/95) of the Department of Agriculture, Western Cape government.

Based on the outcomes of the exploratory study, skin biopsies were collected from 40 Dorper, 26 NA and 29 SAMM ewes (95 sheep in total) during April 2015 in the more comprehensive study. These ewes were potentially pregnant at the time of sampling. One skin biopsy was removed at the tick attachment sites and one at a nearby control site (where there is no tick attached), as in the exploratory study. As on the previous sampling occasions, the samples were collected by a qualified veterinarian and animal health technicians under the supervision of the veterinarian. The subsequent treatment of the samples was as described above. Ticks detached from where biopsies were taken were also collected and submerged in 70 % alcohol for identification and weighing. The engorgement level and the sheep location (sampling site) from which the ticks were detached were recorded.

Upon arrival at the laboratory the skin biopsies were kept stored in a -20 ^{o}C fridge until the extraction of RNA. The RNA was extracted from the biopsies within a month from collection.

6.2.3 RNA extraction

RNA of all tick attachment and control samples was extracted at the molecular laboratory in the Department of Plant Sciences, University of the Free State, Bloemfontein, South Africa using Trizol reagent (Life technologies). Skin tissue samples were removed from the RNAlater™ and put in 2 ml Eppendorf tubes containing 500 µl Trizol reagents. The tissue was disrupted by putting two stainless steel beads in the tubes containing Trizol reagent and the tissue was lysed using Tissuelyzer II (Qiagen). Later the tissues were homogenized to reduce the viscosity of the cell lysates produced by disruption using IKA homogenizer. The RNA was extracted according to the manufacturers' protocol. Briefly, after homogenization another 500 µl of Trizol reagent was added before incubating at room temperature for 10 minutes. 200 µl chloroform was added to the tube and mixed by inverting the tube 15 times and then incubated at room temperature for 5 minutes prior to centrifuging the tubes at 12 000 g for 15 minutes at 4 ^{o}C. After centrifuging there were three layers in the tube, the top aqueous layer (clear appearance) with RNA, middle layer (white precipitate appearance) with DNA and the bottom layer (pink reagent colour) with proteins (Figure 6.1). The 500 µl of the cleared supernatant was transferred to isopropanol containing tubes and mixed well before incubation at room temperature for 10 minutes. The tubes were then centrifuged for 10 minutes at 12 000 g and 4 ^{o}C to pellet the RNA and the supernatant removed from the pellet using a water jet pump. 70 % ethanol was added to the pellet and mixed well by inverting the tubes several times. The tubes were then centrifuged at 7 500 g for 10 minutes at 4 ^{o}C and the supernatant was removed from the pellet using a water jet pump. The tubes were opened and incubated on the bench for 5 minutes and 50 µl DEPC treated water was added to each pellet. The tubes were incubated on ice for 1 hour and afterward 200 pipette was used to draw the liquid up and down to dissolve the RNA. The tubes were centrifuged for 5 minutes at 12 000 g at 4 ^{o}C to pellet any undissolved RNA. Lastly the supernatant was transferred to a new labeled 1.5 ml Eppendorf tube and stored at -20 ^{o}C until use.

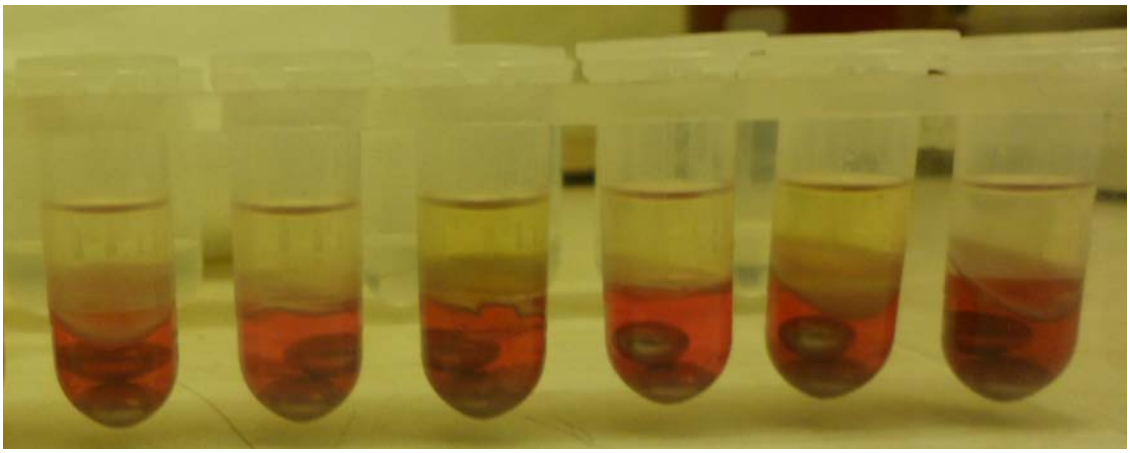

Figure 6.1 Three layers were discernible in the tubes, the top aqueous layer with RNA (clear appearance), middle layer with DNA (white precipitate appearance) and the bottom layer with proteins (pink reagent colour).

6.2.4 RNA concentration and quality Determination

RNA concentrations were determined using the nano-drop machine. One µl of RNA was measured after measuring one µl of DEPC treated water as a blank solution in the Nano-drop machine. The quality of the RNA was determined by running a denaturing agarose gel electrophoresis, involving 0.5 g agarose powder dissolved in 5 ml 10X N-morpholino propane sulfonic acid (MOPS) buffer and 43.3 ml DEPC water. The mixture was put in the microwave to melt the powder and subsequently placed in a 65 ^{o}C in a water bath for 30 minutes. A total of 1.7 ml (0.41 M) formaldehyde was added to the solution in the fume hood and mixed with the stirrer. The gel was left to set for about 15 to 20 minutes. In the meantime, the running buffer was prepared by diluting 30 ml 10 X MOPS in 270 ml DEPC water. The RNA was prepared as follows before being loaded in the gel; 18 µl of RNA buffer was put into RNase free labeled 1.5 ml tubes, 1 µl RNA added to it and then denatured at 65 ^{o}C for 15 minutes in the water bath. The solution was then snap-cooled on ice before adding 2 µl of loading buffer and loading on the gel. The RNA was separated in the gel at 100 V for 30 minutes (Figure 6.2).

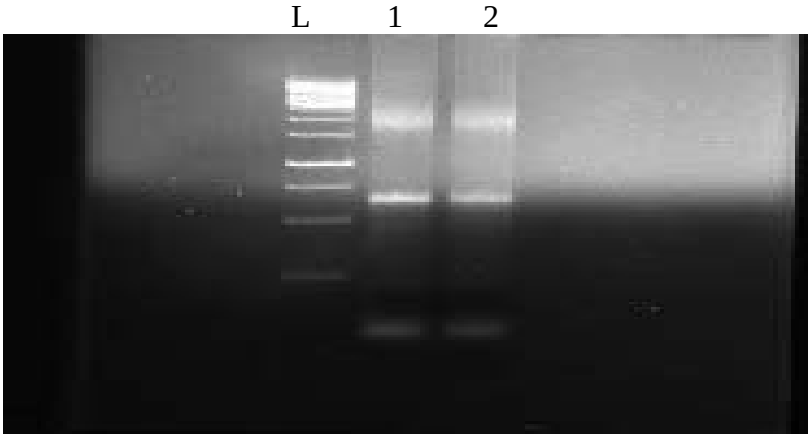

Figure 6.2 A photograph of an agarose gel clearly showing representative RNA bands in the lanes 1 and 2, lane L is a 1 kb ladder.

6.2.5 Design and Optimization of primers

The two primers (*CCL2* and *CCL26*) were designed using the IDTDNA program and synthesized by Inqaba biotech in Pretoria, South Africa. The other four primers (including two reference genes) were previously published (sources provided in Table 6.1). Initially five genes were to be expressed but the primers of the fifth gene did not amplify with good efficiency. Five more gene primer sets were optimized to select one primer pair for the fifth gene. None of these amplified with good efficiency and only four target genes were studied. The primers were optimized to obtain their annealing temperature in a C1000^{TM} Thermal Cycler CFX96^{TM} Real-Time System (BIO-RAD). During each reaction 2 µl of 5ng/µl RNA was mixed with 1 µl of primer pair, 5 µl of KAPA SYBR Fast Master Mix One-step, 0.2 µl KAPA RT Mix and 1.8 µl of water, consequently having a total of 10 µl volume of the reaction mixture per tube. The real-time qPCR conditions were; 42 ^{o}C for 5 minutes for incubation, 95 ^{o}C for 5 minutes, 95 ^{o}C for 10 seconds for denaturation, gradient for 30 sec for annealing temperature, 72 ^{o}C for extension and melting curve to evaluate specific amplification. The real-time qPCR ran for 40 cycles. The real-time qPCR product was run on a 1.2 % agarose gel to verify that there is only one band per sample, indicating that there is only one product that amplified. Figures 6.3 and 6.4 show the bands of *CCL26* and *IL-8*, respectively.

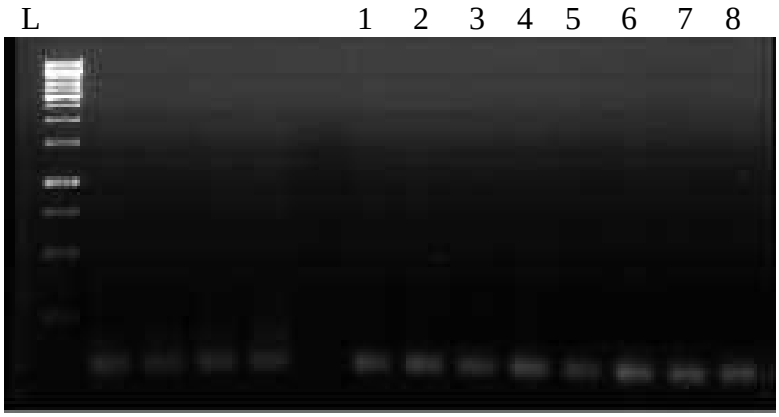

Figure 6.3 DNA fragment bands on agarose gel after optimizing *CCL26*. Lanes 1,2,3,4,5,6,7 and 8 depict optimization at 65 ºC, 64.5 ºC, 63.3 ºC, 61.4 ºC, 59 ºC, 57 ºC, 55.7 ºC and 55 ºC, respectively while lane L is a 1 kb ladder.

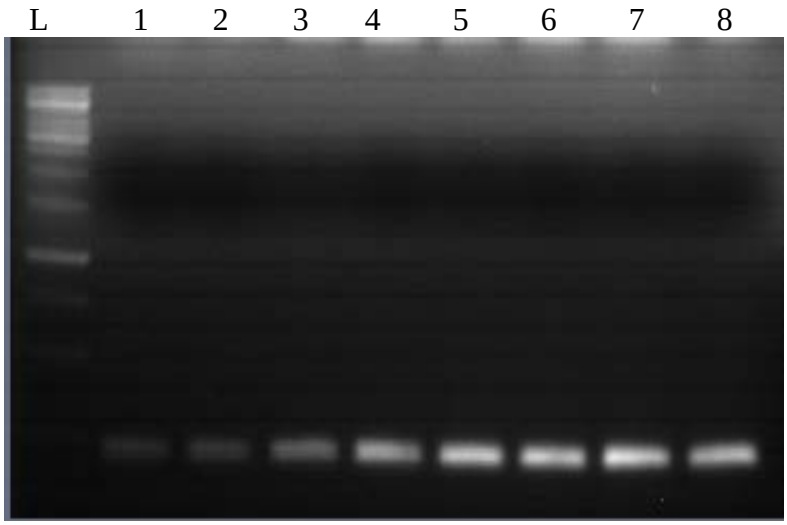

Figure 6.4 An agarose gel picture showing amplified products of *IL-8* in gradient. Lanes 1,2,3,4,5,6,7 and 8 depict optimization at 65 ºC, 64.5 ºC, 63.3 ºC, 61.4 ºC, 59 ºC, 57 ºC, 55.7 ºC and 55 ºC, respectively while lane L is a 1 kb ladder.

Table 6.1 A summary of the specific primers used, references, primer sequences and annealing temperatures

Gene	**Source**	**Primer pairs**	**Annealing temp (ºC)**
IL-8	Egan *et al*. (1996)	Forward: ATGAGTACAGAACTTCGA Reverse: TCATGGATCTTGCTTCTC	55.0
IL-1β	Herman *et al*. (2010)	Forward: CAGCCGTGCAGTCAGTAAAA Reverse: GAAGCTCATGCAGAACACCA	57.0
CCL2	Present study	Forward: CCAGCAAGTGTCCCAAGA Reverse: AGATGGTTTATGGCGTCCTG	55.7
CCL26	Present study	Forward: TCCCTATGGCTTCCCTTCTT Reverse: TACTGGAAACAGCAGAACTTGG	55.7
18S	Tian *et al*.(2013)	Forward: GAGAAACGGCTACCACATC Reverse: GCTATTGGAGCTGGAATTAC	57.0
GAPDH	Tian *et al*. (2013)	Forward: AAGTTCAACGGCACAGTCAA Reverse: ACCACATACTCAGCACCAGC	59.0

6.2.6 Determination of primer's efficiency

The efficiencies of the primers were determined by running the standard curve reactions. The serial dilution of 5-fold RNA concentrations was used for the standard curve. The reaction mixture preparations and real-time qPCR conditions were similar to those used for optimization except that the annealing temperatures varied for each primer as given in Table 6.1. The efficiencies and slopes of the primers are given in Table 6.2.

Table 6.2 A summary of primers, references, efficiency coefficients, R^2 values and slopes

Gene	**Source**	**Efficiency (%)**	**R^2**	**Slope**
IL-8	Egan *et al*. (1996)	102.8	0.961	-3.257
IL-1β	Herman *et al*. (2010)	97.4	0.917	-3.385
CCL2	Present study	90.5	0.988	-3.572
CCL26	Present study	93.8	0.936	-3.480
18S	Tian *et al*. (2013)	95.7	0.993	-3.430
GAPDH	Tian *et al*. (2013)	98.9	0.967	-3.349

6.2.7 Gene expression quantification

The expression of three genes, *IL-1β*, *CCL2* and *CCL26* were quantified in both summer and late winter 2014 in the exploratory study, while *IL-8* was only quantified in late winter 2014. This is because by the time the primers for *IL-8* were optimized and their efficiency determined the RNA samples for summer sampling was already degraded and did not give reliable results. All four genes were analysed in samples acquired during the comprehensive study in April 2015. Gene expression was quantified in real-time qPCR using C1000^{TM} Thermal Cycler CFX96^{TM} Real-Time System (BIO-RAD). The reaction mixture was prepared as in Section 6.2.5 above. Each sample was analysed in triplicate and two reference genes (*GAPDH* and *18S*) were also quantified according to the above-mentioned procedure. These reference genes had been validated previously by Tian *et al*. (2013) and have been found to be suitable for normalizing gene expression data in sheep. For the comprehensive study three reference genes were validated and selected to be suitable for normalizing gene expression data in the studied sheep (Chapter 5 of this thesis). The standard curve was also included in the reaction plate in each gene analysis to give the efficiency of the primers per reaction. The real-time qPCR conditions were similar to those in Section 6.2.5.

6.2.8 Data analysis

All the outliers pertaining to cq-values of technical replicates from each sample were excluded from the analysis for quality control purposes. The replicates were considered outliers if they differed by more than 0.5 from other sample replicates. The software used required that the variation between technical replicates should not be more than 0.5. Furthermore all samples with only one technical replicate amplifying were excluded. The data were analysed by averaging the cq-values of the triplicates for each sample and analyzing the gene expression relative to the two reference genes (*18S* and *GAPDH*) using the qbase+ program (Biogazelle). These reference genes were only used to normalize data in the preliminary study conducted in the summer and late winter of 2014. As a result of high variation observed in the latter study, it was assumed that the reference genes used for normalizing the data could be approved upon. Thus an additional study was carried out to validate and select reference genes specifically suitable for this study (Chapter 5 of this thesis). The results of this study suggested that *GAPDH* and *18S* were not the best reference genes, as their expression varied between samples. The best reference genes for sheep skin gene expression as suggested by the results presented in Chapter 5 were *SDHA*, *YWHAZ* and *B2M*. The results further indicated that the variation can be reduced by using at least three reference genes to normalize the gene expression data. Three reference genes instead of two were thus used to normalize the data of the comprehensive study, namely *SDHA*, *YWHAZ* and *B2M*.

All the statistical analyses were done using SAS (SAS Institute Inc. 2013) In the exploratory study, the difference of means between seasons, between breeds and the interaction between breed and season was tested using the Proc Mixed procedure in SAS while the effect of status was tested using the GLM procedure in a 2 (sample site: attachment site or control) x 3 (breed: SAMM, Dorper or NA) factorial design. The data from the comprehensive study were analysed using the GLM procedure in SAS in a 2 (sample site; attachment or control) x 3 (breed; SAMM, Dorper or NA) factorial design. A one-way ANOVA analysis was used to test for the effect of tick species, the attachment site (perineum, tail and udder), engorgement level and tick life stage on gene expression at tick attachment sites. The following models were used for analyzing the data for the exploratory study (1) and the comprehensive study (2), respectively:

$y_{ijk} = \mu + \alpha_i + \gamma_k + (\alpha\gamma)_{jk} + u_i + e_{ijk}$ (1)

Where:

Y = data reflecting the expression of the *IL-1β* or *CCL2* or *CCL26* genes; μ = the effect of the overall mean; α_j = the fixed effect of the j^{th} breed (j=SAMM, Dorper or NA); γ_k = the fixed effect of the k^{th} season (k=summer or late winter); $(\alpha\gamma)_{jk}$ = the fixed effect of breed x season; u_i = the random effect of the i^{th} animal and e_{ijk} = randomly distributed errors associated with the data recorded.

$Y_{ijk} = \mu + \alpha_i + \beta j + (\alpha\beta)_{ij} + e_{ij}$ (2)

Where;

Y = data reflecting the expression of the *IL-1β* or *CCL2* or *CCL26* genes; μ = the effect of the overall mean; α_j = the fixed effect of the j^{th} breed (j=SAMM, Dorper or NA); βj = the fixed effect of the sampling site (attachment or control site); $(\alpha\beta)_{ij}$ = the fixed effect of breed x sample site and e_{ij} = randomly distributed errors.

Pearson correlations were calculated between gene expression and tick weights.

6.3 Results

6.3.1 *Exploratory study*

6.3.1.1 *IL-1beta*

Season affected the expression of *IL-1β* at tick attachment sites ($P<0.05$) but *IL-1β* expression was independent of breed and the interaction of breed with season. Across seasons, *IL-1β* was more expressed ($P<0.05$) during late winter than during summer at tick attachment sites. There was no significant difference in the expression of the gene ($P=0.45$) at the tick attachment sites. This gene expression was also not significantly different between tick attachment and control sites.

6.3.1.2 *CCL2*

Chemokine C-C motif ligand 2 (*CCL2*) expression was independent of breed, season and the interaction between them (P=0.21, P=0.13 and P=0.18, respectively). Similarly no significant difference was found in *CCL2* gene expression between the tick attachment and control sites.

6.3.1.3 *CCL26*

The breed, seasons and their interaction had no effect on the expression of *CCL26* (Table 6.3). Although NA had an absolute higher expression of the gene at tick attachment site, there was no significant difference at both tick attachment and control sites.

6.3.1.4 *IL-8*

The interleukin-8, which is a pro-inflammatory cytokine and also a chemokine had no significant expression difference at tick attachment sites between the breeds (P=0.88) when assessed in the winter of 2014. Similarly, the expression of *IL-8* at tick attachment sites did not differ with that at the control sites (P=0.164).

Table 6.3 Least squares means (±s.e) depicting the relative expression (normalized with two reference genes) of the *IL-1 β*, *IL-8*, *CCL2* and *CCL26* genes at the tick attachment sites and control sites in three sheep breeds. *IL-8* was only recorded during late winter 2014.

Breed	***IL-1β***	***CCL2***	***CCL26***	***IL-8***
Dorper-attachment	3.208 ± 1.01	2.137 ± 0.5	1.540 ± 0.4	0.504±0.35
Dorper-control	1.783 ± 0.6	1.950 ± 0.5	1.953 ± 0.5	0.374±0.35
NA-attachment	1.560 ± 1.01	1.055 ± 0.5	1.039 ± 0.5	0.932±0.35
NA-control	0.568 ± 0.7	1.101 ± 0.6	0.708 ± 0.6	0.121±0.41
SAMM-attachment	1.681 ± 0.8	0.960 ± 0.4	0.857 ± 0.4	0.776±0.32
SAMM-control	0.997 ± 0.47	1.303 ± 0.4	1.269 ± 0.4	0.419±0.32

6.3.2 *Comprehensive study*

6.3.2.1 *IL-1β*

Overall, the expression of the two Interleukin genes (*IL-1β* and *IL-8*) analysed in this study were higher ($P<0.05$) across breeds at tick attachment sites compared to control sites. Within breeds, the NA and SAMM had *IL-1β* being upregulated at tick attachment sites compared to control sites. In contrast, *IL-1β* was equally expressed at both sites in Dorper ewes. The expression of *IL-1β* was significantly higher at the tick attachment sites of NA ewes compared to Dorper ($P<0.05$) and tended to be higher than in SAMM ewes ($P=0.096$) (Figure 6.5). The interaction of breed with site (tick attachment or control) also approached significance ($P=0.052$).

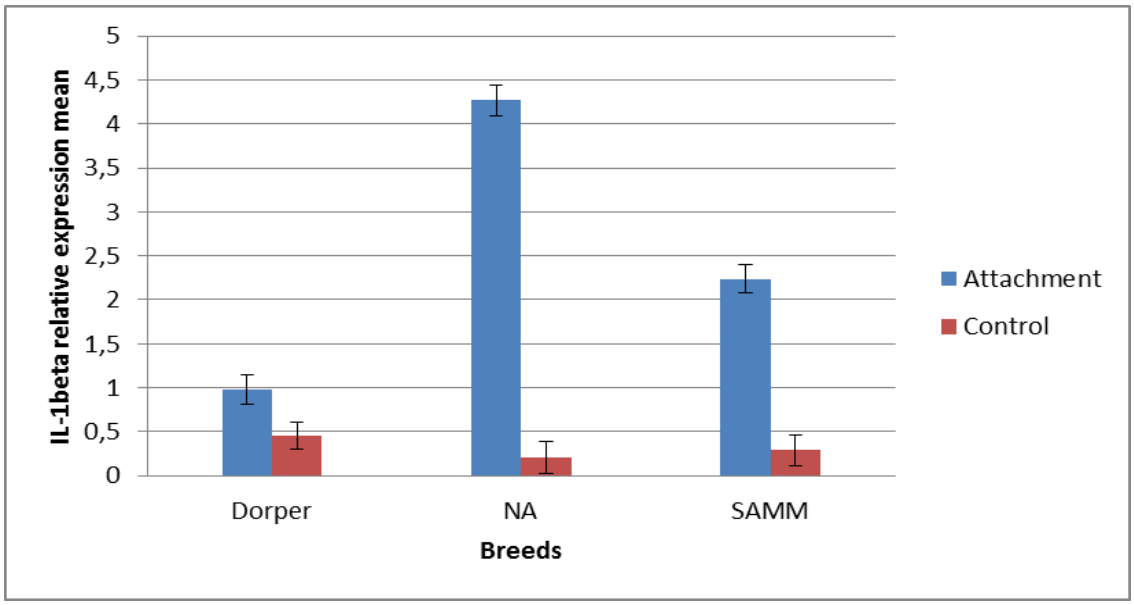

Figure 6.5 Least-squares means depicting the effects of the breed x sampling site interaction on relative expression (normalized with three reference genes) of the *IL- 1β* gene. Vertical lines about means represent SE's.

6.3.2.2 *IL-8*

There was no significant difference (P=0.155) in the expression of *IL-8* gene at control sites between breeds (Figure 6.6). Across breeds, *IL-8* expression was higher (P<0.05) at tick attachment sites than at control sites. Within breeds, the difference in gene expression between tick attachment and control sites was significant (P<0.05) in NA ewes and approached significance (P=0.096) in SAMM ewes, while there was no difference in Dorper ewes. Furthermore, the interaction of breed with sampling site did not affect the expression of *IL-8* in sheep skin.

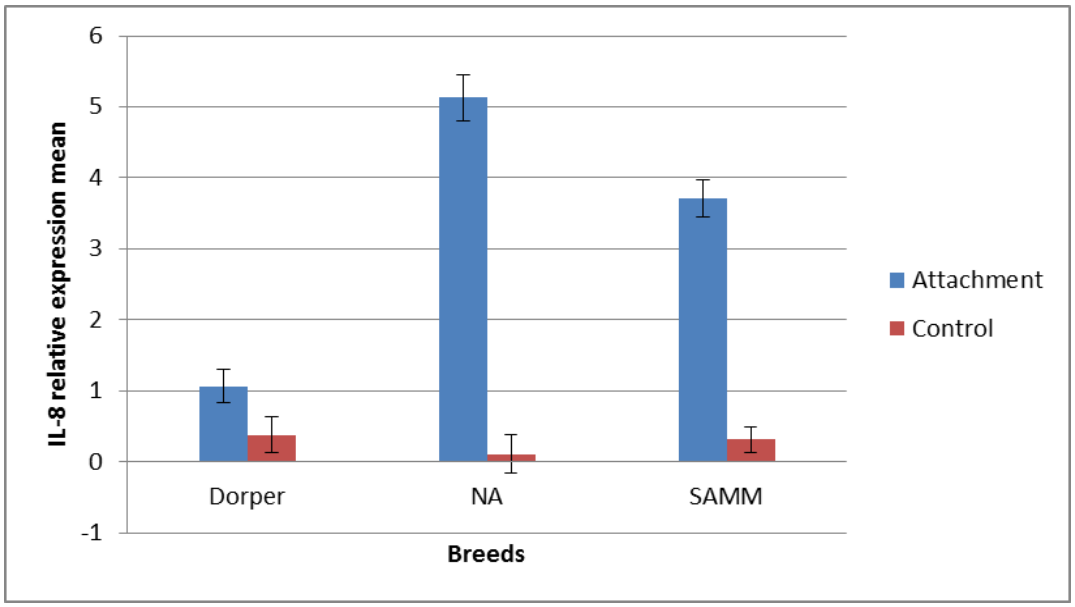

Figure 6.6 Least-squares means depicting the effects of breed x sampling site interaction on the relative expression (normalized with three reference genes) of the *IL-8* gene. Vertical lines about means represent SE's.

6.3.2.3 *CCL2* and *CCL26*

Breed and sampling site interacted (P<0.05) for *CCL2* expression (Figure 6.7). This interaction could be attributed to marked up-regulation of *CCL2* expression at tick attachment sites in NA ewes (P<0.05). In contrast, *CCL2* expression in SAMM and Dorper ewes were independent of sampling site (P>0.05).

Breed and sampling site did not affect the expression of *CCL26* gene (P>0.05) while the interaction of the breed and sampling site tended to affect gene expression (P=0.099). Still, the gene was more highly expressed at the tick attachment sites of NA ewes than at control sites (P<0.05). The gene was similarly expressed at tick attachment sites and control sites in the other breeds (Figure 6.8).

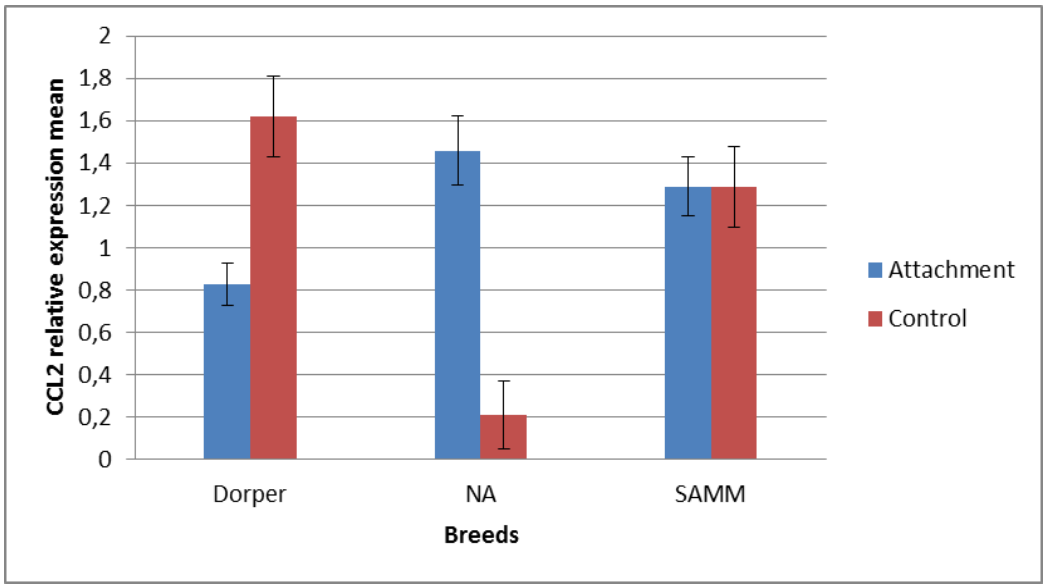

Figure 6.7 Least-squares means depicting the effects of breed x sampling site interaction on the relative expression (normalized with three reference genes) of the *CCL2* gene. Vertical lines about means represent SE's.

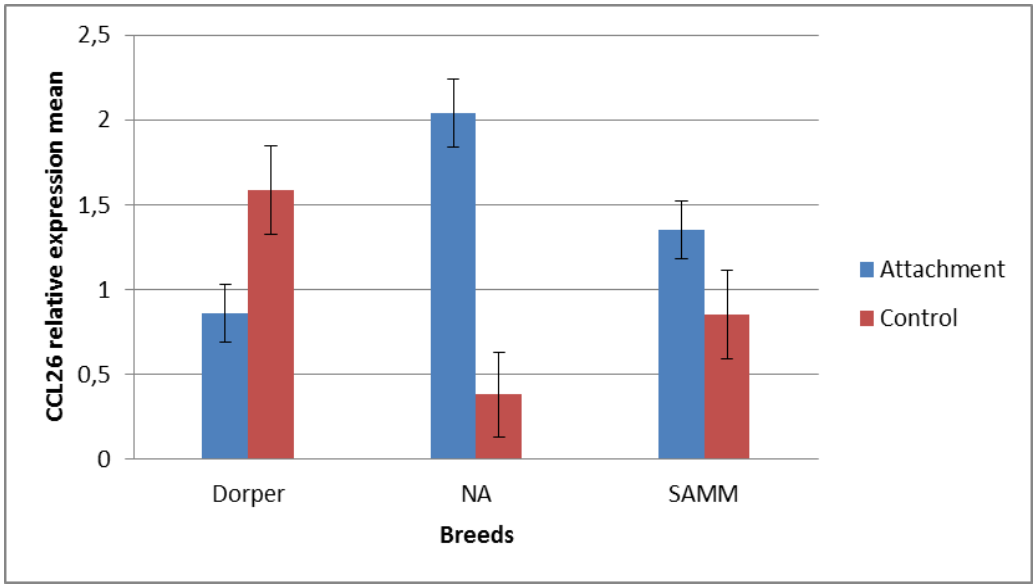

Figure 6.8 Least-squares means depicting the effects of breed x sampling site interaction on the relative expression (normalized with three reference genes) of the *CCL26* gene. Vertical lines about means represent SE's.

6.3.2.4 Effect of tick species, tick engorgement level, tick life stage and the host body location from which the ticks were detached on gene expression

The tick life stage (nymph, female and male) affected (P<0.05) the expression of *IL-1β* when tested across the breeds. The difference was between nymphs and adults (males and females) with the gene being expressed more where nymphs attached compared to where adult ticks attached. The means were 0.306 ± 0.16, 0.170 ± 0.15 and 1.498 ± 0.29 for females, males and nymphs, respectively. The expression of *IL-8*, *CCL2* and *CCL26* was unaffected by the life stage of ticks (P=0.105, P=0.083 and P=0.176, respectively). Nevertheless, the effect of tick life stage on *CCL2* approached significance (P=0.08), suggesting that this gene is highly expressed where the nymphs attached compared to where adult ticks attached. The *CCL2* expression means were 0.051 ± 0.12, -0.044 ± 0.10 and 0.771 ± 0.09 from where females, males and nymphs attached, respectively. The expression of all genes was generally independent of the body location from which the ticks were detached, the tick engorgement level and tick species.

The correlations of tick weight with the expression of *IL-1β* and *IL-8* were small in magnitude (-0.15 to -0.06) and not significant (P=0.170 and P=0.564, respectively). Correlations of tick weights with the expression of *CCL2* and *CCL26* were also small in magnitude (0.199-0.200), and also not significant (P=0.115 and P=0.096). However, tick weights tended to correlate with the expression of *CCL26* (P=0.096).

6.4 Discussion

The exploratory study did not provide any clear evidence of differential gene expression in the respective breeds, possibly owing to the small numbers of animals involved. Different seasons in the exploratory study did not influence gene expression except for *IL-1β* at tick attachment sites. However, the comprehensive study enabled the detection of significant differences between tick attachment and control sites and between the breeds. The results of both comprehensive and preliminary studies are being discussed together.

6.4.1 *IL- 1β*

Overall, *IL-1β* was up-regulated (P<0.05) at the tick attachment sites, although site effects did not reach significance (P=0.267) in Dorper ewes in the comprehensive study and all breeds in the

exploratory study. The up-regulation of *IL-1β* at tick attachment sites relative to control sites in NA and SAMM in the comprehensive study is consistent with previous studies on different species, including cattle, sheep, fish, (Bridle *et al*., 2006; Piper *et al*., 2008; Burgess *et al*., 2010; Heinze *et al*., 2012; Brannan *et al*., 2014; Hermance & Thangamani, 2014). The expression of *IL-1β* at tick attachment sites in NA and SAMM ewes was significantly higher than at the control sites while in Dorper the difference was not significant. Piper *et al*. (2008) also reported a significantly higher expression of *IL-1β* at tick attachment sites than at control sites in cattle. Nevertheless, the authors reported significantly higher expression at the tick attachment sites than at control sites in susceptible Holstein-Friesian cattle. The lower expression level of *IL-1β* at tick attachment sites in Dorpers compared to other breeds in the comprehensive study may be related to results of Ramachandra & Wikel (1995). The latter authors reported that the expression of this gene is more suppressed by the saliva of ticks in susceptible than in resistant animals.

The significantly higher expression of *IL-1β* at tick attachment sites in the NA breed compared to Dorpers in the comprehensive study concurs with findings of Piper *et al*. (2008) whom indicated significant differences in the expression of *IL-1β* at tick attachment sites between breeds of cattle. Nevertheless, no significant difference in *IL-1β* expression was found between NA and SAMM ewes. McGuire *et al*. (2004) and Glass & Jensen (2007) also reported that there was no significant difference in the expression level of *IL-1β* between cattle breeds that were either resistant or susceptible to protozoan parasites. Although they were investigating gene expression in cattle infected with protozoan parasites, while the effect of tick infestation on gene expression is investigated in this study, it is assumed that the underlying biological mechanisms for eliciting the immune response to parasitic challenge are the same. The significantly higher expression of *IL-1β* at tick attachment sites in NA than in Dorper ewes indicated that this gene may play a role in the resistance to ticks in NA ewes.

The relative expression of the *IL-1β* varied greatly between individual ewes within breeds (results not shown). Piper *et al*. (2008) and Piper *et al*. (2010) also reported such marked variation in cattle. This observation might be attributed to several factors. Firstly, the animals were naturally infested in this study, which means ticks attached at different times. Therefore it is assumed that skin biopsies were sampled from attachment sites at different times post

infestation. This potentially had an effect on the level of gene expression since the expression level of *IL-1β* differed according to the interval the host animal was exposed to the parasitic challenge (Burgess *et al*., 2010). Previous studies confirmed that the level of *IL-1β* expression is low in mite (*Psoroptes ovis*) infected host sheep compared to controls at 1 hour post-infestation, peaks up at 3 hours post-infestation before lowering down by 6 to 24 hours post-infestation (Burgess *et al*., 2010). Gene expression levels are inherently variable by nature (Piper *et al*., 2008).

The results of the comprehensive study confirmed that there were differences ($P<0.05$) in *IL-1β* expression between the NA (resistant breed) and Dorper (susceptible breed) and that the susceptible SAMM breed also tended to have higher expression of *IL-1β* ($P=0.09$) than Dorpers at tick attachment sites. The exploratory study indicated a lack of significance in *IL-1β* expression between breeds, thus accepting the hypothesis that there is no difference in *IL-1β* expression level between different breeds of sheep infested with ticks. However, the tendency of NA to have a higher expression than other breeds in exploratory results as well as the breed difference in the comprehensive study indicate that *IL-1β* may account for breed-related differences in resistance or susceptibility to tick infestation in sheep.

6.4.2 *IL–8*

The up-regulation of the *Interleukin–8 (IL-8)* cytokine at the tick attachment sites of all three breeds accorded with findings of previous studies done on sheep infested with the sheep blowfly, *Lucilia cuprina* (Egan *et al*., 1996) and *Psoroptes ovis* mites (Burgess *et al*., 2010). The expression of this gene was significantly higher at tick attachment than control sites in NA ewes, while it did not differ significantly between attachment sites and control sites in the Dorper and SAMM breeds. Contrary to the findings of Wang *et al*. (2007) which indicated the down-regulation of *IL-8* in both high and low resistant cattle, the results of this study shows the up-regulation of *IL-8* in perceived resistant breed of sheep. There was no significant difference in *IL-8* expression at tick attachment sites between breeds. However, NA ewes had the highest absolute *IL-8* expression at the tick attachment sites compared the other two breeds, both in the comprehensive and exploratory studies.

The expression of this gene has also been confirmed to be suppressed by ticks (Vančová *et al*., 2010). As it is one of the pro-inflammatory cytokines, tick saliva suppresses its expression so that the inflammatory response is prevented. However, the results from this study suggest that this defensive mechanism of ticks against the secretion of this gene at the tick attachment sites in NA ewes naturally infested by ticks were not achieved.

6.4.3 *CCL2*

CCL2 was significantly up-regulated at tick attachment sites in NA ewes, while in other breeds sample site did not affect its expression. The preliminary study had failed to find significant difference in *CCL2* expression between both the tick attachment and control sites as well as between breeds. This might have been due the small sample size used in exploratory study. The lack of significant difference in *CCL2* expression between breeds is contrary to findings of previous studies on cattle (Piper *et al*., 2008; Burgess *et al*., 2010; Heinze *et al*., 2012). Piper *et al*. (2008) reported the up-regulation of this gene in the Holstein-Friesian breed (susceptible to ticks) in comparison to the resistant Brahman breed. These results suggest that the mechanisms used by cattle to combat ticks may differ from those used by sheep.

CCL2 is a chemokine gene and previous studies reported the existence of anti-chemokine activity in the saliva of ticks, which assists the ticks to cope with the immune response evoked by their hosts (Vančová *et al*., 2007). The strength of the anti-chemokine activity varies with the interval of infection/infestation, as well as with the sex and species of the tick involved. Kramer *et al*. (2011) accordingly reported the down-regulation of *CCL2* gene expression in cells treated with tick saliva. The up-regulation of this gene at tick attachment site in NA suggests that the immune response of NA ewes was capable of overcoming the tick's defense mechanisms.

6.4.4 *CCL26*

NA ewes also appeared to be able to overcome the defense mechanisms of ticks against *CCL26* production at the tick attachment site, as indicated by the significant up-regulation of this gene in this study (both comprehensive and exploratory) in this breed only. There were no significant differences between the tick attachment and control sites in SAMM and Dorper ewes. Wang *et*

al. (2007) reported a down-regulation of this gene at the tick attachment sites in Hereford x Shorthorn cattle compared to control sites.

The higher expression of *CCL26* at tick attachment sites compared to control sites in NA ewes compared to other breeds might infer that this gene may contribute to host resistance or susceptibility to tick infestation in this breed. Wang *et al*. (2007) observed the down-regulation of this gene at 24 hours post infestation in Hereford x Shorthorn cattle. The lack of significant differences in the expression of this gene at tick attachment sites between breeds is in contrast with the report by Piper *et al*. (2008).

6.4.5 Effect of tick species, tick engorgement level, tick life stage and the host body location from which the ticks were detached on gene expression

The lack of animal body sampling site effects on gene expression recorded in this study concur with findings of Piper *et al*. (2008). This suggests that in future biopsies for gene expression can be taken from any region of the animal body without seriously biasing the results. Contrary to Vančová *et al*. (2007)'s findings the present study suggested that gene expression is independent of tick gender. The tick life stage only affected the expression of *IL-1β*, where nymphs elicited higher expression than adult ticks (males and females).

Across breeds, *CCL2* was more highly expressed where *H. rufipes* attached compared to where *H. truncatum* and *R. evertsi evertsi* attached. Hajnická *et al*. (2005) similarly reported that anti-chemokine activity varied between tick species. No information on the effect of tick engorgement levels and the relationship between tick weight and gene expression were found in the literature.

6.5 Conclusions

Overall, the pro-inflammatory cytokine genes (*IL-1β and IL-8*) were more highly expressed at tick attachment sites than at control sites while no significant differences in chemokines (*CCL2* and *CCL26*) were found between tick attachment and control sites. Although there was no significant difference in other cytokine gene expression between the three breeds, NA ewes expressed *IL-1β* more at tick attachment sites compared to Dorpers. The NA breed was also more likely to upregulate the expression of the *CCL2*, *CCL26* and *IL-8* genes at tick attachment sites

compared to control sites than the other breeds. This indicates that *IL-1 β*, *CCL26* and *IL-8* may play a part in resistance or susceptibility of sheep to tick infestation. *CCL2* was down-regulated at tick attachment sites in SAMM and Dorper ewes. *CCL26* was also down-regulated in Dorpers and not significantly up-regulated in SAMM ewes. These differences in expression of the two chemokines between the resistant breed (NA) and more susceptible breeds (SAMM and Dorper) imply that the NA breed could be able to overcome the anti-chemokine activity of tick saliva and reduce the number of ticks attaching as a result. The results of this study gave an indication that cytokines are involved in immune responses to tick challenge and laid a foundation for further studies using specific tick species.

CHAPTER 7

Cutaneous hypersensitivity reactions against unfed larvae extract of *Rhipicephalus evertsi evertsi* in South African Mutton Merino, Namaqua Afrikaner and Dorper sheep

7.1 Introduction

There is evidence of varying levels of ovine host resistance to tick infestations. An indigenous breed, the Namaqua Afrikaner (NA) has been reported to be more resistant to tick infestations than two commercial breeds, the Dorper and South African Mutton Merino (SAMM) (Cloete *et al*., 2013). Genetics and immunological factors are indicated to play a role in cattle resistance to ticks, thus this also need to be investigated in sheep. Since *R. evertsi evertsi* is one of the tick species that are dominant in Nortier farm, constituting up to 50% of approximately 4000 ticks detached from sheep (Cloete *et al*., 2013), it was chosen to be used in this study.

Among immunological responses elicited by ticks are hypersensitivity reactions. Hypersensitivity stems from mononuclear leukocyte initiated inflammatory reactions, which are key mechanisms of defence against parasites and infections (Larsen *et al*., 1995). Per definition, hypersensitivity can be of a delayed type or of an immediate response type. Delayed hypersensitivity reactions occur after 24 hours post infection while the immediate response occurs as early as 15 minutes post infection or post antigen introduction (Dryden, 2014). Delayed hypersensitivity reactions are based on cell-mediated immune reactions (Larsen *et al*. 1995). Delayed hypersensitivity reactions have been associated with resistance to tick infestation in tick resistant cattle. On the other hand, immediate hypersensitivity has been associated with susceptibility to tick infestation in tick susceptible cattle breeds (Marufu *et al*., 2013). The hypersensitivity reactions manifest in different forms and can be measured by an increase in thickness of the skin at the site of antigen inoculation (Bechara *et al*., 2000).

Therefore, in an endeavour to better understand the mechanisms used by different breeds to resist tick infestations, this study compares cutaneous hypersensitivity reactions between NA, Dorper and SAMM sheep.

7.2 Materials and Methods

7.2.1 Research venue and housing of the animals

This study was conducted at the Nortier Research Farm in the Western Cape province of South Africa. A total of 18 mature ewes (NA = 6, SAMM = 6 and Dorper = 6) was selected to be used in this study. However, 1 Dorper ewe became sick during the experiment. Therefore only 5 Dorpers were eventually used. The ewes were housed in a pen of 8m x 6m in the sheep handling facility at Nortier. Although the pen is open, it has a roofed area of 2 x 6 m at the western side to provide shade and shelter from rain to the animals. The pen has a solid brick wall on the western side that also ensured shelter against the predominantly north-westerly wind that bring rain to the area. The other three sides of the paddock were made up of a split-pole fence reinforced with meshed wire. The animals were thought to be better maintained in a relatively open area, since they are free-ranging animals used to an extensive production environment. They were fed oat and/or lucerne hay on an ad libitum basis for the duration of the experiment and had unrestricted access to potable drinking water.

7.2.2 Tick count

Ticks were counted on the ewes 2 weeks prior to the commencement of the hypersensitivity study. The ewes were exposed to natural tick challenge for at least three months. Then the ewes were treated with an accaricide containing 1% m/v Flumetrin (Drastic Deadline®) two weeks before the commencement of the study. Treatment was according to the stated guidelines for application of the product, namely to partition the required dose in four parts for application to the axillae and groin of experimental animal. However, additional care was taken to treat all

visible ticks on the animals using a small paintbrush. The animals were inspected for ticks on a weekly basis post-treatment and no ticks were found.

7.2.3 Larvae extract preparation

The unfed larvae which were used in this research were produced by the University of the Free State Entomology department. Fully engorged adult *Rhipicephalus evertsi evertsi* ticks were collected from sheep on the Nortier Research Farm and incubated at 29 ^{o}C and 75% humidity in the Entomology laboratory at the University of the Free State to produce eggs. These eggs were incubated at 29 ^{o}C and 75% humidity until they hatched. The unfed larvae extract (ULE) was prepared according to specifications reported by Marufu *et al*. (2013). The 2-months old larvae were ground in 200 µl Phosphate Buffered Saline (PBS) containing protease inhibitors cocktail in a tube. After grinding the larvae another 200 µl of PBS solution was added. The homogenate was sonicated at 30s for one minute, shaken for 10 minutes on a horizontal shaker at 70 shakes/minute. The crude larvae were centrifuged for 30 minutes at 6 000 rpm at 4 ^{o}C. The supernatant was removed and 600 µl of PBS solution added. Protein concentration of the extract was determined using Bradford method. The ULE was further diluted to 50µg/ml protein concentration and kept in a -80 ^{o}C refrigerator. The extracts were tested for *Theileria ovis*, *Anaplasma ovis* and *Borrelia theileria* at the University of Pretoria since these pathogens can potentially be transmitted by *Rhipicephalus evertsi evertsi*. The larvae extract tested negative for the screened pathogens.

7.2.4 Intradermal test procedure

Since all the ewes were kept in the area which has ticks it was presumed they were pre-sensitized, as was confirmed by individual tick count prior to the procedure. The thickness of the ear of each sheep at the sites described below was measured prior to ULE inoculation. Then 0.1 ml of ULE of *Rhipicephalus evertsi evertsi* was injected intradermally on the upper side of each ewe's right ear 1 cm away from the edge of the ear by a qualified veterinarian. At the same time, PBS was injected on the lower side of the right ear opposite the site where the ULE was

inoculated to serve as control. The ear thickness at the two sites of individual ewes was measured at 1, 6, 24, 48 and 72 hours post inoculation (PI) using electronic callipers. This study was approved by the University of the Free State Interfaculty Animal Ethics Committee (Approval number is AED2015-45).

7.2.5 Data analysis

The initial ear thickness was measured prior to treatment as described above. Ear thickness measurements differed between animals also even between the treatment site and the control site. Based on this, the data could not be analysed by using means of ear thickness as the pre-inoculation measurements would have an effect after treatment and give inaccurate results. Instead the mean percentage increase from the pre-inoculation measurements was analysed. The differences between the mean percentage increase of ear thickness between breeds, time PI as well as between status (treated and control) were tested using Mixed Model procedure in SAS (SAS Institute Inc., 2013). A 3 (breeds) x 2 (treated vs control) x 5 (times PI) factorial was used with animal fitted as a random effect to account for repeated sampling of the same subject. Also ANOVA in SAS was used to test for the mean differences between the two statuses (treated versus control) within breeds.

7.3 Results

7.3.1 Tick count

NA ewes had significantly ($P<0.05$) lower tick numbers in total compared to SAMM and Dorper ewes (Table 7.1). Tick numbers were similar for Dorper and SAMM ewes. The bulk of the ticks were on the hind region of the ewes in all the three breeds.

Table 7.1 Mean total tick count (± SE) on Dorper, NA and SAMM ewes

Breed	Mean of total tick count
Dorper	42 ± 7^{a}
NA	14 ± 4^{b}
SAMM	44 ± 9^{a}

[ab] Values with different superscripts were significantly different at $P<0.05$

7.3.2 Hypersensitivity response to unfed larvae extract

The results are presented as the mean percentage increase of ear thickness at various times PI relative to pre-inoculation measurements. The ULE prepared from *R. evertsi evertsi* induced hypersensitive reactions in all three breeds used. However, the sheep breeds also reacted to the PBS solution used as the control, but the reaction to the PBS was mild compared to the reaction to the tick larvae extract.

Breed tended ($P=0.062$) to have effect on the increase of ear thickness. Status and time PI had a significant effect ($P<0.05$) on the increase of ear thickness. Similarly, the interaction of breed and status, status and time PI as well as breed and time PI significantly affected the ear thickness increase ($P=0.018$, $P=0.033$ and $P=0.039$, respectively). In contrast the interaction of the three factors (breed*status*time PI) did not affect the increase of ear thickness. The inflammation, reflected by the thickness of the ear increased substantially by approximately 40-80 % an hour after ULE inoculation and was roughly maintained at this level up to 24 hours PI (Figure 7.1). Subsequently, ear thickness declined in NA and Dorper ewes after 48 and 72 PI, but remained at approximately the same level for SAMM ewes. NA ewes responded intensely during the first hour after treatment and 24 hours PI suggesting strong immediate and delayed reactions. Overall, NA ewes had the highest hypersensitivity response at treated sites after 1, 6 and 24 hours, followed by SAMM and Dorper ewes which did not differ. The highest percentage increases, 80.8, 54.1 and 72.1% were observed in NA ewes at 1, 6 and 24 hour PI. Corresponding values amounted to 55.5, 34.4 and 39.8% for SAMM ewes and 39.9, 32.0 and 39.5% for Dorper ewes.

Within breeds, ear thickness (as indicative of the hypersensitivity response) was significantly higher ($P<0.05$) at the treated site compared to control site at some time points PI, although the trends differed between breeds. The ear thickness of Dorper ewes at the ULE inoculated sites only differed from the control site at 24 hours PI (Figure 7.2A). In contrast, the ear thickness of NA ewes at the ULE treated sites exceeded ($P<0.05$) control site values at 1, 6 and 24 hours PI, with no subsequent differences (Figure 7.2B). Unlike the Dorper and NA, SAMM ear thickness values were significantly higher ($P<0.05$) at ULE inoculated sites than at control sites at all times of measurement PI (Figure 7.2C).

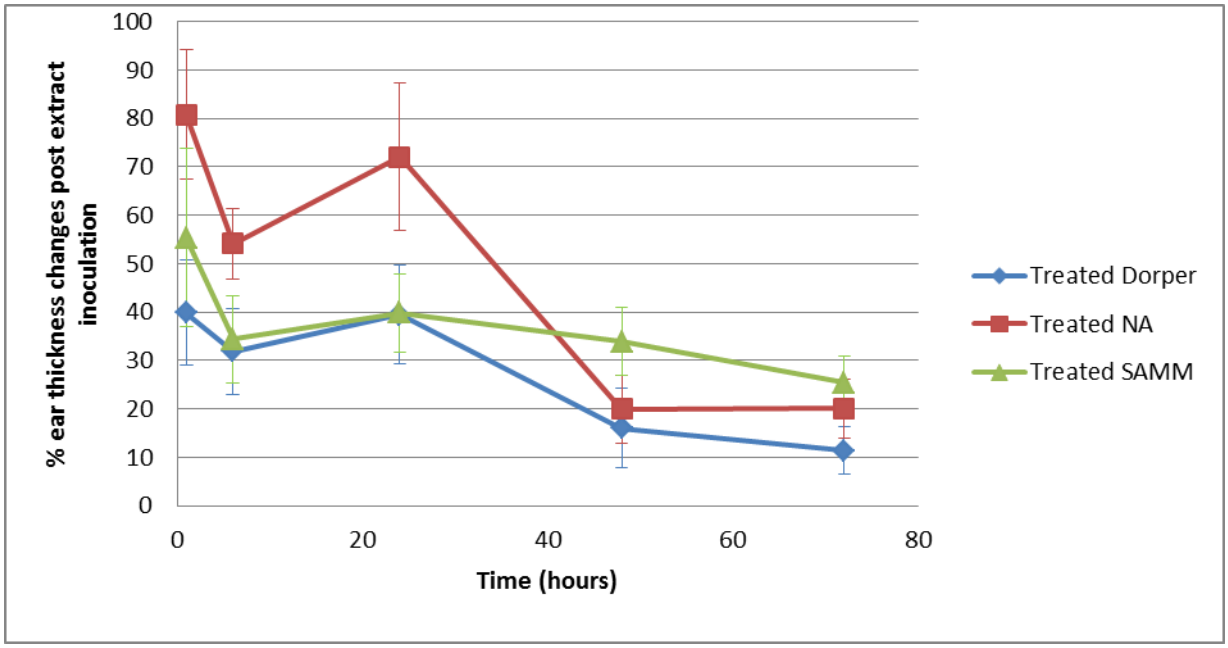

Figure 7.1 Least squares means depicting the interaction between breed and time post-inoculation for the mean percentage change of ear thickness induced by the inoculation with unfed larvae extract (ULE) of *R. evertsi evertsi* intradermally in the ears of ewes from three sheep breeds, namely the Dorper, Namaqua Afrikaner (NA) and SA Mutton Merino (SAMM). Vertical bars around means indicate standard errors.

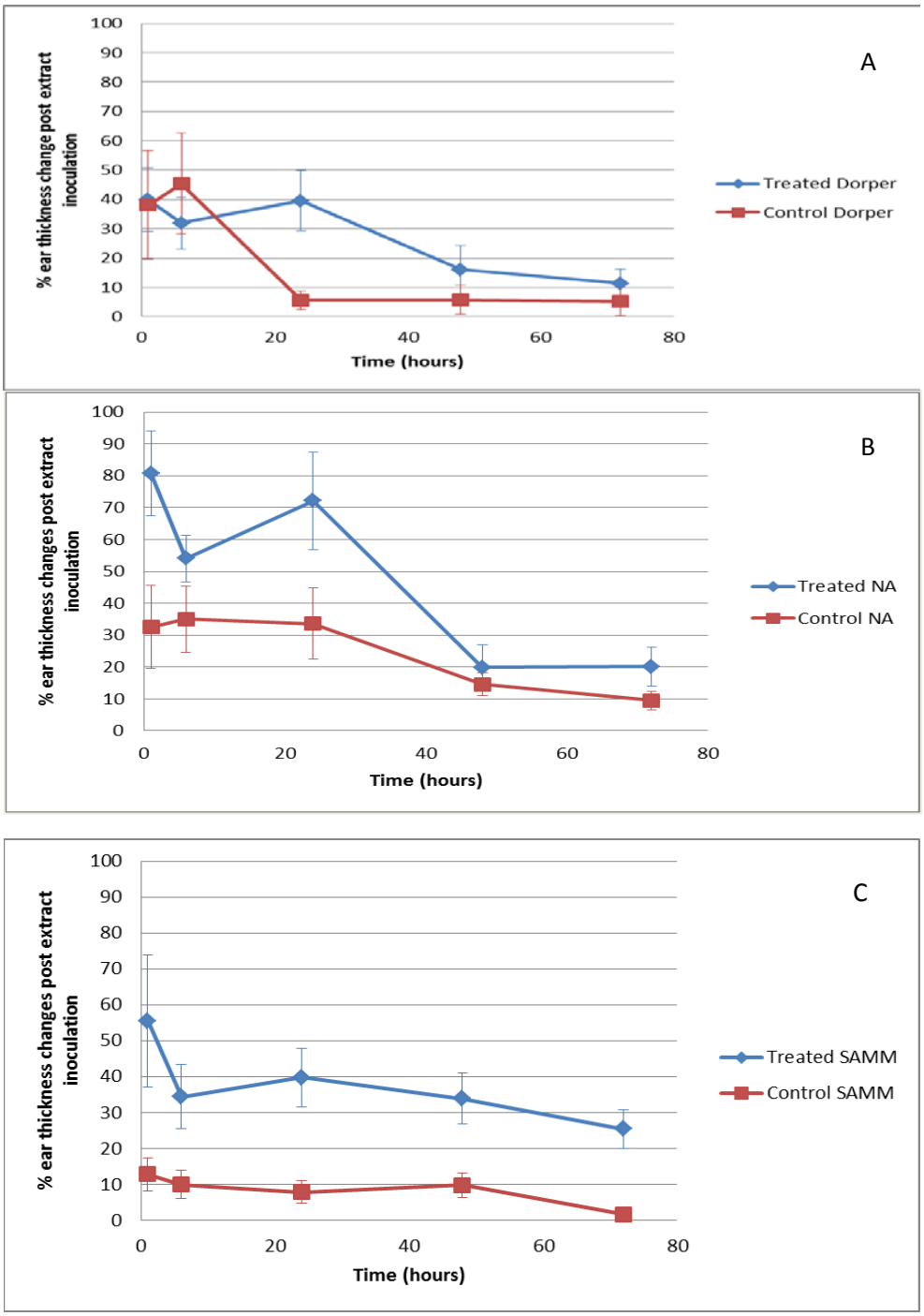

Figure 7.2 Least squares means depicting the mean percentage change of ear thickness induced by the inoculation with unfed larvae extract (ULE) of *R. evertsi evertsi* intradermally in the ears of ewes from three sheep breeds, namely the Dorper (A), Namaqua Afrikaner (NA; B) and SA Mutton Merino (SAMM; C) in comparison with control sites injected with phosphate buffered saline. Vertical bars around means indicate standard errors.

7.4 Discussion

The results of overall tick counts confirm the previous findings reporting higher total tick numbers in SAMM and Dorper ewes compared to the NA breed (Cloete *et al*., 2013). These results confirm that the NA ewes used in this study were more resistant to tick infestations compared to the SAMM and Dorper ewes used.

The results provide evidence that cell-mediated immune responses are involved in fight against tick infestation. The delayed hypersensitivity type has been indicated as an *in vivo* expression of cell-mediated immune response (Heriazon *et al*., 2013). *R. evertsi evertsi* ULE induced marked hypersensitive reactions at the inoculation site on the ears of all the experimental subjects, but PBS also caused mild increases in ear thickness where it was inoculated. All three breeds developed both immediate (1-6 hours PI) and delayed (24 hours PI and later) hypersensitivity reactions to ULE inoculation. However, the delayed responses dropped back to be similar to control sites in the Dorper and NA at 48 and 72 hours PI but were still noticeable in SAMM ewes. Corresponding immediate and delayed cutaneous hypersensitive reactions to tick ULE inoculation have been reported in previous studies in cattle, dogs, rabbits, mice and guinea pigs (Bechara *et al*., 1996; Szabo *et al*., 1995; Bechara *et al*., 2000; Ferreira *et al*., 2003; Hlatshwayo *et al*., 2004; Prudencio *et al*., 2011; Marufu *et al*., 2013).

The more intense immediate type reaction displayed by NA ewes with ear thickness increasing up to 80.8% 1 hour PI compared to the other breeds agree with previous studies in cattle. Bechara *et al*. (1996) reported an immediate hypersensitivity reaction of 72% ear thickness increase and Bechara *et al*. (2000) reported an immediate hypersensitivity reaction of 75% ear thickness increase in *Bos taurus* calves challenged with ULE of *Boophilus microplus*. Prudencio *et al*. (2011) similarly reported both immediate and delayed hypersensitive reaction to *R. microplus* tick extract in cattle. Marufu *et al*., 2013 also reported the immediate increase of ear thickness in Bonsmara cattle at 30 minutes to 1 hour PI. Contrary to the present study, the more intense immediate hypersensitivity reaction was observed in more susceptible cattle (Bechara *et al*., 1996; Prudencio *et al*., 2011; Marufu *et al*., 2013).

Szabo *et al*. (1995) reported a more intense immediate reaction and less intense delayed reaction in dogs intradermally inoculated with *R. sanguineus* tick extract and they suggested that it might be the reason why dogs lack resistance to this tick species. Accordingly, the intensity of skin reactions to tick extracts has been established to be negatively correlated with number of ticks on animals. Hypersensitive reactions are associated with an increased resistance to ticks (Lloyed & Walker, 1993, Allen, 1994). Based on this background the results of this study suggest that a fast and intense hypersensitivity reaction may be among the mechanisms used by NA sheep to resist tick infestation.

Although previous research in cattle and dogs associated immediate reaction with susceptibility, the fact that NA showed both pronounced immediate and delayed type reaction is evidence that hypersensitivity reaction play a great role in the NA's resistance to ticks. The less noticeable immediate and delayed reactions observed in the Dorper might explain why this breed is less resistant to tick challenge. The response of SAMM ewes to ULE inoculation was surprising, as it is a susceptible breed and it was not expected to have a delayed type reaction for up to 72 hours PI. This outcome may have been caused by a number of factors. Firstly, there is the possibility that hosts may immunologically respond to natural tick attachment differently compared to artificially inoculated tick antigens (Hlatshwayo *et al*., 2004). It could be hypothesized that tick saliva may suppress the onset of a delayed reaction in SAMM ewes during natural tick infestation, thus preventing this breed to resist natural tick infestation. The suppression of this reaction may not be achieved with ULE. On the other hand, it may be that there are other distinctive features (such as coat characteristics of SAMM as a dual-purpose breed) that create a conducive environment for ticks to attach to SAMM sheep regardless of the presence of cell-mediated immune responses.

It is not uncommon to have a secondary increase in hypersensitivity reaction after it has declined PI with tick ULE. The increase in the ear thickness observed at 24 hours PI after a slight decline at 6 hours PI primarily seen in NA ewes was also observed by Bechara *et al*. (1996) in resistant *Bos indicus* calves and by Marufu *et al*. (2013) in resistant Nguni cattle. These findings dispute those of Wang *et al*. (2007) suggesting that the down-regulation of pro-inflammatory molecules at 24 hours PI is an indication of the end of local inflammation reactions.

The intense immediate and delayed cutaneous hypersensitive reaction observed in NA suggests that inflammation may be part of mechanisms used by NA to protect itself against tick infestation. The fact that the reactions to tick ULE varied between breeds at various times PI also imply that inflammation may play a role in tick resistance. ULE was used in this study because tick resistance has been reported to mostly manifest against tick larvae attachment (Jonsson *et al*., 2014). This suggests that crude larvae extract may be used as an antigen to immunize sheep against tick infestation.

7.5 Conclusions

ULE of *R. evertsi evertsi* induced hypersensitive reactions in all three breeds of sheep. The indigenous NA, perceived as more resistant to tick infestation, displayed stronger reactions, both immediate and delayed, compared to the SAMM and Dorper regarded as more susceptible. The results of this study provide an insight into the role of immunology in host resistance to ticks. The results give evidence that cell-mediated immune responses are involved in fight against tick infestation. Together with other immunological aspects that contribute to host resistance to tick infestation, such as antibody-mediated immune responses to tick infestation or extracts, that might be studied in the future, the hypersensitivity reaction may be used as a phenotypic marker to select the animals or breeds that are more resistant to tick infestation. It is well-known that challenge-based research for promoting resistance to pathogens is under scrutiny from an ethical and welfare perspective. The methods employed in this study could be refined to enable routine evaluation of valuable animals (such as breeding rams) without resorting to more invasive strategies, such as allowing adequate natural challenge to accrue over time in selection candidates.

CHAPTER 8

General Conclusions and Recommendations

8.1 Conclusions

The presence of differences in tick burdens between sheep breeds which were exposed to similar tick challenges in the same flock has been reported. Furthermore it has been indicated that genetically influenced immunological responses are involved in resistance to parasite infestation. Hence, the current study investigated genetic parameters of tick resistance and immunological responses induced by tick attachment in Dorper, NA and SAMM breeds.

Firstly, tick burden differed between the three breeds studied, with NA having a significantly lower tick count than the other two breeds (Chapters 3 & 7). The findings of the current study concur with a previous study on the same resource flock which also indicated NA to be more resistant to tick infestations. Breed differences are often considered as indicative of genetic variation for a specific trait, whether it being additive or non-additive. These concepts were explored further in Chapter 3.

Variation in tick count between animals was associated with heterosis in the cross of indigenous, fat-tailed NA rams with Dorper ewes, but not in the reciprocal cross between the commercial SAMM and Dorper breeds. This result suggests that the ability of the hardy, indigenous NA breed to withstand tick challenge is transferred to progeny generated with Dorpers as representative of the commercial ovine genetic resource. An interesting feature of the results is that NA x Dorper progeny resembled the superior commercial breed for weaning weight, but resembled the more resistant indigenous breed for tick count. Further studies on crosses of indigenous sheep with commercial breeds as part of integrated tick management seem to be warranted.

The heritability (h^2) of tick count within breeds was relatively low at approximately 10%. The data structure was not optimal for comprehensive genetic analyses. However, with a CV of approximately 50% for transformed tick count, it is comprehensible that substantial genetic

progress may accrue, should directed selection be applied. The across-breed genetic analysis suggested substantial genetic variation, reflected by an h^2 estimate of approximately 30%. It thus seems as if the hardy, indigenous NA may have an adaptation advantage above the commercial breeds under severe tick challenge conditions. At this stage, it is unknown whether other indigenous breeds would exhibit the same advantage in terms of adaptation.

The current study thus suggests that resistance to tick infestation is mediated through non-additive and additive genetic components in the sheep breeds studied. This resistance to ticks may be divided into two main groups, non-specific mechanisms and specific mechanisms involving the immune response.

Cell-mediated immunity is involved in sheep resistance to ticks. Basophils are known to be regulators of Th2 cell responses. Cellular infiltration was significantly increased at tick attachment sites compared to control sites in all the three sheep breeds in this study (Chapter 4). NA ewes presented a generally higher infiltration of cells (basophils, eosinophils and mast cells) at tick attachment sites compared to other breeds. These cells have been reported to be associated with resistance to ticks. On the other hand, SAMM had significantly higher neutrophil numbers at attachment sites than other breeds. The infiltration of immune cells at tick attachment sites has also been suggested in cattle. Tick attachment induced skin reactions (crusts, acanthosis, hyperkeratosis, prominent granular layer and oedema) in most cases, but no significant differences were found between tick attachment and control sites in NA for spongiosis, apopthosis, necrosis and collagen degeneration. No significant difference was found on the effect of tick attachment on skin reactions between breeds. The absence of differences in skin reactions to tick attachment between resistant and susceptible breeds implies that there might be other mechanisms used by the more resistant NA breed to resist tick infestation.

Reference genes that could be used to normalize gene expression data in the breeds considered were validated for stability in Chapter 5. The commonly used reference genes were not all stable in the current study on NA, SAMM and Dorper sheep. The most suitable reference genes for the current study were *SDHA*, *YWHAZ* and *B2M*. These findings highlighted the need to validate reference genes for every gene expression study.

Cytokine expression was influenced by tick attachment and this varied between the sheep breeds in Chapter 6. The inflammatory genes seemed to be differentially highly expressed at the attachment sites compared to control sites in NA ewes. In the SAMM breed, the inflammatory cytokine genes also tended to be more expressed at the attachment site than at control sites. In contrast, no difference was observed in cytokine gene expression between tick attachment and control sites in Dorper ewes. Even with the chemokine genes, the NA breed had the up-regulation of these genes at tick attachment sites compared to control sites. In contrast, SAMM and Dorper ewes, in general, had the down-regulation of chemokines at tick attachment site. These results suggest that the more resistant NA breed was able to overcome the immunosuppression defensive mechanisms of ticks while the more susceptible commercial breeds were unable to do so. The results support the findings that was reported in cattle that immunology contributed to resistance to tick infestation. The presence of differences in the extent of immune responses to ticks between breeds might partly explain the differences in tick burden among breeds of sheep.

A hypersensitivity study was done on the three breeds of sheep at Nortier research farm (Chapter 7). This study suggested immediate as well as delayed type hypersensitivity reactions when ewes of the three breeds studied were inoculated with *R. evertsi evertsi* unfed larvae extract (ULE). The NA and SAMM breeds both displayed immediate and delayed type hypersensitivity reactions to *R. evertsi evertsi* ULE while the Dorper breed had an immediate mild delayed type skin reaction. Even though both NA and SAMM displayed both immediate and delayed type hypersensitivity reaction, the NA breed tended to have appreciably stronger reactions than either the Dorper or SAMM breeds. These findings lend further support to a contention that immunological responses are involved in resistance to tick infestation. The study suggests that, if other immunological responses such as antibody responses to tick infestation and tick extracts are also studied, such immunological responses may be adapted to serve as phenotypic markers to identify resistant or susceptible animals or breeds. The methods employed in this study could potentially be refined to enable routine evaluation of valuable animals (such as breeding rams) without resorting to more invasive strategies, such as allowing adequate natural challenge to accrue over time in selection candidates to gain access to phenotypic data.

8.2 Recommendations

This study was carried out in a natural environment, which was an advantage to investigate the responses to tick attachment in naturally challenged sheep. However, the shortcoming of investigating the naturally challenged sheep was that the time of tick attachment was unknown. In future, more controlled studies with artificial infestation needs to be carried out. Although the present study could not find significant effects of tick life stage on cell counts, future studies should consider looking at a different tick life stage at a time. This will give an idea of which tick life stage (larvae, nymph or adult) are more resisted by different sheep breeds and may thus help in making informed decisions regarding management of the flock or tick control measures in an integrated control strategy.

This study suggested the involvement of immunological responses in resistance to tick infestation. Before the results of this study can be used to decide on management regarding tick control, there is still other studies that need to be done. These include determining the relationship between tick resistance and traits of economic importance such as growth for mutton sheep and wool traits in wool and dual-purpose breeds, such as the SAMM. Selection for tick resistance would be less complicated if this trait is favorably correlated with other traits of economic importance.

Evidently, the breeds studied here are only part of those available in the South African ovine genetic resource. Studies on the additive and non-additive genetic components of other indigenous and commercial breeds contributing to this genetic resource seem to be warranted. Such studies should include those genetic resources under the care of small-scale farmers, producing meat and wool under challenging conditions.

Finally, the breeds studied differed appreciably in their ability to withstand tick challenge. They may be used as model breeds for further genomic studies on sheep, searching for single nucleotide polymorphisms (SNP) associated with resistance to ticks as well as exploring the possibilities of using genomic tools for selection of local breeds for their ability to withstand challenge by ticks. Such studies may be complicated by ascertainment bias against indigenous breeds during SNP-chip development.

ABSTRACT

The study investigated genetic parameters and immunological responses to tick infestation in three South African sheep breeds (Namaqua Afrikaner [NA], Dorper and SA Mutton Merino [SAMM]). The study aimed to estimate genetic and crossbreeding parameters for tick count (TC) and weaning weight (WW), to examine the histology of tick attachment and control sites, to select reference genes for normalizing gene expression data in this study, to compare cytokines gene expression at tick attachment and control sites and finally to compare cutaneous hypersensitivity reactions to unfed larvae extracts (ULE) of *Rhipicephalus evertsi evertsi* between NA, Dorper and SAMM sheep.

Genetic parameters for WW and TC were estimated using data of lambs maintained on the Nortier Research Farm from 2010 to 2015. Firstly, data of purebred commercial Dorper and SAMM lambs were combined with data of their reciprocal crosses to assess breed effects and the possible effect of non-additive genetic variation on WW and TC. In the second analysis, data of purebred commercial Dorper lambs were combined with data of the unimproved, indigenous NA and the NA x Dorper cross. In Analysis 1 the coefficient of variations (CV) were 24%, 95% and 50% for WW, untransformed total TC and square root transformed total TC, respectively. Genotype affected WW but not TC. A heterosis estimate of approximately 4% was derived for WW. A single-trait h^2 estimate for TC was 0.11±0.09. A model analysing across-genotype h^2 yielded a slightly lower h^2 estimate of 0.08 ± 0.07. In Analysis 2 the CV were 27% and 55% for WW and square root transformed total TC, respectively. WW and TC were affected by genotype. WW exhibited heterosis amounting to 8.5% while the corresponding value for TC amounted to -23%. The single-trait h^2 estimate for TC was 0.06±0.05. A model analysing across-genotype h^2 yielded a substantially higher h^2 estimate of 0.27±0.07. These results suggest that genetic variation in TC was primarily associated with differences among genetic groups while differences between individual animals within genetic groups were not as important. Heterosis estimates for WW were variable between two analyses, but within ranges reported in the literature. This study established significant variation in TC between sheep genotypes when the indigenous NA breed formed part of the analysis. The NA x Dorper cross resembled the improved Dorper breed for WW but the unimproved, resistant NA for TC and exhibited worthwhile levels of heterosis for both traits. Indigenous ovine genetic resources may be instrumental in providing genetic material for adaptive traits in environments susceptible to high levels of tick infestation. Further research is required to elucidate the role that adapted indigenous ovine genetic resources may play in an integrated tick management strategy under conditions characterised by high levels of tick challenge.

A histological study was conducted to assess histological features at tick attachment and control sites in pure breeds. Skin biopsies were examined using routine histological techniques for immunological cell infiltration and skin reactions. Marked variation in immunological responses to tick attachment within and between sheep breeds was observed. There were differences between the attachment and control sites in most of the skin changes (defects) except for four skin defects in the NA. However, all breeds had similar

frequencies of skin defects at tick attachment sites. Tick attachment sites were more likely to be infiltrated by cells within as well as across breeds. The NA and SAMM breeds tended to demonstrate greater cellular infiltrations of specific leukocytes at tick attachment sites compared to Dorpers. Basophils, mast cells and eosinophils were increasingly recruited at tick attachment site in NA ewes compared to the Dorper and, occasionally, the SAMM breeds. These results suggest the importance of these cells in sheep resistance to tick infestation. Tick genera influenced the recruitment of neutrophils to tick attachment sites. Tick gender, sampling site as well as tick engorgement level did not affect the number of immunological cells. Further studies should be done with one tick species at a time to better comprehend the species-specific impact of tick attachment to animals belonging to divergent sheep breeds.

Five genes (*18S*, *GAPDH*, *YWHAZ*, *B2M* and *SDHA*) were tested for their stability. *SDHA*, *YWHAZ* and *B2M* were the most suitable reference genes recommended by geNorm analysis for normalizing gene expression data in sheep skin. These findings will assist in normalizing data in gene expression studies at tick attachment and control sites of the NA, Dorper and SAMM breeds. This study suggested that no reference gene is stably expressed in different experimental conditions.

The expression of *IL-1β*, *IL-8*, *CCL2* and *CCL26* was quantified in real-time qPCR. *IL-1β and IL-8* were more highly expressed at tick attachment than at control sites. NA ewes expressed *IL-1β* more at tick attachment sites than Dorpers. The NA breed was also more likely to upregulate the expression of the *CCL2*, *CCL26* and *IL-8* genes at tick attachment sites compared to control sites than the other breeds. This indicates that *IL-1 β*, *CCL26* and *IL-8* may play a part in resistance or susceptibility of sheep to tick infestation. The differences in expression of the two chemokines between the resistant NA and more susceptible SAMM and Dorper imply that the NA breed could be able to overcome the anti-chemokine activity of tick saliva.

ULE of *R. evertsi evertsi* induced hypersensitivity reactions in all the breeds. The indigenous NA displayed stronger reactions, immediate and delayed, than the commercial breeds. The results suggest that cell-mediated immune responses are invoked to fight against tick infestation in the NA. The hypersensitivity reaction may be used as a phenotypic marker to select animals or breeds that are more resistant to tick infestation. It is well-known that challenge-based research for promoting resistance to pathogens is under scrutiny from an ethical and welfare perspective. The methods employed here could be refined to enable routine evaluation of valuable animals without resorting to more invasive strategies, such as allowing adequate natural challenge to accrue over time in selection candidates. Overall, the component studies reported in the thesis increased the present understanding of ovine tick-host interactions and factors contibuting to breed differences in tick loads.

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