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Expression of cytokine genes at tick attachment and control sites of Namaqua Afrikaner, Dorper and South African Mutton Merino sheep

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Highlights:

- This study investigated expression of four selected cytokine genes at tick attachment and control sites
- Animal resources included an indigenous sheep breed the Namaqua Afrikaner (NA) and the Dorper and SA Mutton Merino (SAMM)
- NA ewes expressed *interleukin 1 beta* more at tick attachment sites compared to Dorpers
- NA ewes were also more likely to upregulate the expression of genes at tick attachment sites than the other breeds
- The study laid the foundation for further studies using specific tick species

ABSTRACT

Cytokines are immune response components important in innate immunity and inflammatory response. They are harnessed as part of local immunological responses by animals to combat local infections and/or infestations. This study investigated expression of four selected cytokine genes, namely, *interleukin 1 beta (IL-1β)*, *chemokine C-C ligand 2 (CCL2)*, *chemokine C-C ligand 26 (CCL26)* and *interleukin 8 (IL-8)*, at tick attachment and control sites in a South African indigenous sheep breed the Namaqua Afrikaner (NA) and two commercial breeds, the Dorper and South African Mutton Merino (SAMM). The NA was previously shown to be more resistant to infestation by ticks than the two commercial 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. 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 under controlled challenge conditions.

Key words: Dorper, Immune response. Namaqua Afrikaner, South African Mutton Merino, Tick attachment

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 (Wikel, 2013). 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*; Cameron and Kelvin, 2013). 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).

No similar studies on sheep in South Africa or elsewhere could be sourced from the literature. Gene expression studies using the real-time qualitative polymerase chain reaction (RT-qPCR) technique are becoming popular in life sciences as scientists endeavor 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 and 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 a specific experiment need to be analyzed and the most stable ones selected. Several common reference genes are used to normalize gene expression data in the literature and most of them are used without validation. Some of the commonly used reference genes are *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH), 18S ribosomal RNA (18S rRNA), TATA box binding protein (TBP), Beta-2-microglobulin (B2M), Beta actin (ACTB), Succinate dehydrogenase complex, subunit A (SDHA)* and *Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ)* (Vandesompele et al., 2002; Jain et al., 2006). This is done in spite of reports that the reference genes may not be stable under different experimental conditions, different tissues, species, breeds and even individuals within breeds.

The dual aim of this study was therefore to firstly 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; the Namaqua Afrikaner (NA), Dorper and South African Mutton Merino (SAMM). Secondly, differences in expression of four selected cytokine genes, namely, *IL-1β*, *CCL2*, *CCL26* and *IL-8* were studied between tick attachment sites and nearby control sites of an indigenous fat-tail breed, the NA and ewes of two commercial breeds, the Dorper and SAMM. There is evidence that the NA is more resistant to infestation by ticks than the two commercial breeds (Cloete et al., 2016). The indigenous NA also displayed stronger immunological reactions, immediate and delayed, when injected with unfed tick larval extract than the commercial breeds (Thutwa et al., 2018). In the absence of detailed information on the

mechanisms that could be involved in these differences, it is important to know whether the reported breed differences can be related to the differential expression of genes in the respective breeds.

2. Material and methods

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'S; 18º 20'E, and is about 10 km north of Lambert's Bay in the Western Cape, South Africa. 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. All animals included in the study were maintained as a single flock under extensive production conditions and utilized indigenous shrub pastures typical of the Strandveld of the western seaboard as described by Cloete et al. (2013; 2016).

2.2 Skin biopsy sampling

The study involved two phases, namely to first identify suitable reference (housekeeping) genes for subsequent use (Phase 1) and second to study the expression of genes after tick infestation (Phase 2). Both phases were conducted on animals managed in a single flock that were naturally infested by ticks. During Phase 1, 1 ml of Lignocaine (lidocaine) local anesthetic was administered per sample site to the test animals before sampling. Whole skin biopsies were collected from 15 animals (NA = 4, Dorper = 5 and SAMM = 6). Two samples were taken per animal, one at a tick bite site (after the infesting tick was detached) and one at a nearby tick free control site, using 4 mm diameter 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 ((Ezetil Electric Cooler E 3000 R AC/DC; Type no: 7766 12100000) 240 V AC, 65W – 12V DC, 48W; Manufactured for IPV GmbH, Germany) 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.

During Phase 2, skin biopsies were similarly collected from 40 Dorper, 26 NA and 29 SAMM ewes (95 sheep in total) in April 2015 after the attached tick was removed as described for Phase 1. Tick attachment sites were identified with a permanent marker pen on each animal and a nearby control site (unaffected by tick activity) was also marked for each attachment site. Sampling progressed as described for Phase 1 in ewes that preferably had at least two ticks on appropriate attachment sites (see below) that were needed for the gene expression and a concurrently running histology study (see Thutwa, 2016). Rarely a single tick on an animal was randomly allocated to either one of the two studies. Nearby tick-free control sites we samples for each infested site, whether it be for the gene expression or histology study.

All samples were collected by a qualified veterinarian and animal health technicians under the supervision of the veterinarian. Ticks detached from the sites where biopsies were taken were also collected and submerged in 70% alcohol for identification. All the detached ticks were later individually weighed, using a digital scale. The engorgement level and the sheep location (sampling site) from which the ticks were detached were recorded. The ticks' attachment sites were primarily from areas that had limited fibre coverage, namely the perineum, tail, inside thigh and udder regions of ewes from all breeds. The engorgement of ticks was visually evaluated and ticks classified as fully engorged, moderately engorged, slightly engorged and un-engorged (flat). Tick species, tick life stage (nymph and adults) and tick sex were determined using the Tick Identification Guide (Madder et al., 2020). Skin samples were individually submerged in 5 ml RNAlater RNA stabilization Reagent (Qiagen Gmbh) immediately after collection and the tubes were tightly closed. The tubes containing the skin samples were treated and transported to the laboratory as described above.

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

2.3 RNA extraction

RNA for the identification of reference genes was extracted from the 30 skin samples obtained during Phase 1 (15 tick bite sites and 15 control sites) at the molecular laboratory in the Plant Sciences Department, University of the Free State. The skin tissue was removed from the RNAlater and put in a 2 ml Eppendorf tube containing 500 µl TRIzol™ Reagent (Manufactured by Thermo Fisher Scientific, Cat. No. 15596018; Supplied by Whitehead scientific Pty Ltd) and two 2 mm stainless steel beads (Qiagen). The tissue was disrupted using the tissue lyser (Qiagen®). Then the disrupted tissue was homogenized to reduce the viscosity of the cell lysates and then another 500 µl Trizol™ was added before incubation at room temperature for 10 min. 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 min prior to centrifuging the tubes at 12 000 x g for 15 min at 4 ºC. 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 color) with proteins. A volume of 500 µl of the clear supernatant was transferred to tubes containing 2-Propanol/Isopropanol (C_3H_8O; Sigma Aldrich) and mixed well before being incubated at room temperature for 10 min. The tubes were centrifuged for 10 min at 12 000 x g at 4 ºC to pellet the RNA before 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 x g for 10 min at 4 ºC and the supernatant was removed from the pellet using a water jet pump. The tubes were incubated on the bench for 5 min and 50 µl Diethylpyrocarbonate (DEPC) treated water added to each pellet. The tubes were incubated on ice for 1 h 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 min at 12 000 x g at 4 ºC to pellet any undissolved RNA. Lastly the supernatant was transferred to a newly labeled Eppendorf tube and stored at -20 ºC until usage. RNA of all

tick attachment and control samples obtained from the gene expression study during Phase 2 was treated similarly.

2.4 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 rRNA*, *GAPDH*, *ACTB*, *B2M*, *TBP*, *YWHAZ* and *SDHA* were selected. All the primers (Tables 2 and 3) used were based on results obtained from the literature and have been validated in other studies.

2.5 RNA concentration and quality determination

During the determination of the reference genes as well as the gene expression study, RNA concentrations were determined using the Nano-drop Spectrophotometer (Thermo Scientific), 1 µl of RNA was measured after measuring 1 µl of a blank solution (DEPC treated water) in the Nano-drop Spectrophotometer. The RNA quality was checked by running a 1.2% agarose gel at 100 V for 30 min.

2.6 Optimization of reference gene 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). For 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 (Kapa Biosystems, supplied by Whitehead Scientific Pty Ltd), 0.2 µl KAPA RT Mix (Kapa Biosystems, supplied by Whitehead Scientific Pty Ltd) and 1.8 µl of DEPC treated water

(Thermofisher Scientific, supplied by Whitehead Scientific). The RT-qPCR conditions were; initial incubation at 42 ^{o}C for 5 min, Incubation at 95 ^{o}C for 5 min, denaturation at 95 ^{o}C for 10 sec, annealing temperature gradient (temperature varied for each primer) for 30 sec , extension at 72 ^{o}C for 20 sec and melting curve to evaluate specific amplification. The RT-qPCR ran for 40 cycles.

2.7 Determination of primer's efficiency

The efficiencies of the primers were determined by running the standard curve reactions as reflected in Table 2. The serial dilution of 5-fold RNA concentrations was used for the standard curve. The reaction mixture preparations and RT-qPCR conditions were similar to those used for optimization except that the annealing temperatures varied for each primer as given in Table 1.

2.8 Design and optimization of primers for the gene expression study

The *IL-1β*, *CCL2*, *CCL26* and *IL-8* genes were analyzed in this study. Two primers (*CCL2* and *CCL26*) were designed using the IDTDNA program (http://www.idtdna.com) and synthesized by Inqaba Biotechnical Industries (Pty) Ltd, trading as Iqaba Biotec™, (Pretoria, South Africa). The sequence for these genes was obtained from the database: http://www.ncbi.nlm.nih.gov/nuccore/NC, Accession number for CCL2 was NC_019468.1 and for CCL26 was NC_019481.1. The other five primers (including three reference genes) were previously published and were also synthesized by Inqaba Biotec^{TM}. 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 5 ng/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 DEPC treated water, giving a total of 10 µl volume of the reaction mixture per tube. The RT-

qPCR conditions were; initial incubation at 42 ^{o}C for 5 min, incubation at 95 ^{o}C for 5 min, denaturation at 95 ^{o}C for 10 sec, annealing temperature gradient (temperature varied for each primer) for 30 sec, extension at 72 ^{o}C for 20 sec and melting curve to evaluate specific amplification. The RT-qPCR ran for 40 cycles.

2.9 Gene expression quantification

Gene expression was quantified in RT-qPCR using a C1000^{TM} Thermal Cycler CFX96^{TM} Real-Time System (BIO-RAD). The reaction mixture was the same as the one used for optimization of the primers. Each sample was analyzed in triplicate and three reference genes (*SDHA*, *YWHAZ* and *B2M*) were also quantified. These reference genes were validated in the present study and have been found to be suitable for normalizing gene expression data in the studied sheep. The standard curve was also included in the reaction plate in each gene analysis to give the efficiency of the primers per reaction. The RT-qPCR conditions were identical to those used during the optimization of primers.

2.10 Data analysis

The reference gene data were analysed using the geNorm program in qbasePLUS, version 2.6.1 (Biogazelle, Belgium, info@biogazelle.com). All the outliers pertaining to quantitation cycle (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. Furthermore all samples with only one technical replicate amplification 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 three reference genes (*SDHA*, *YWHAZ* and *B2M*) using the qbasePLUS program (Biogazelle). All the statistical analyses in the reference gene study were done by using SAS Enterprise Guide 6.1 (SAS

Institute Inc. 2013). The data were analysed using the GLM procedure in a 2 (sample site; attachment or control) x 3 (breed; SAMM, Dorper or NA) factorial analysis. The following factorial model was used for analyzing the data:

$$Y_{ijk} = \mu + \alpha_i + \beta j + (\alpha\beta)_{ij} + e_{ijk} \quad (1)$$

Where;

Y_{ijk} = data reflecting the expression of the *IL-1β* or *IL-8 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_{ijk} = randomly distributed error term. Additionally, one-way ANOVA procedures were 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.

3. Results

3.1 Reference gene selection (Phase 1)

When doing the standard curves to determine the efficiency of the primers, the *ACTB* and *TBP* primers did not have a good efficiency and were nonspecific, this was indicated by melt curves displaying more than one peak. These genes were excluded from the analysis. The remaining five genes (*18S rRNA*, *GAPDH*, *YWHAZ*, *B2M* and *SDHA*) had good efficiencies and had specific melt curves with only one peak. A melt curve for *YWHAZ* is provided in Fig. 1 as a representative graph of all five reference genes' This melt curve is presented together with a no template control where no change was observed with a change in temperature.

RNA showed clear 18s and 28s bands on the gel indicating that it was not degraded (Fig. 2). 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.

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 genes have high CT values. The average CT values for the reference genes were 14.58 for *18S rRNA*, 21.67 for *B2M*, 23.75 for *GAPDH*, 24.22 for *YWHAZ* and 25.92 for *SDHA*. The *18S rRNA* gene was the most variably expressed in samples with the range of 6 to 36 CT (close to a 20 CT difference) while *SDHA* was the least variably expressed with the range of 22 to 29 CTs (data not shown). The results in Fig. 3 show that the commonly used reference genes *18S rRNA* and *GAPDH* had high expression stability values (M), which suggests that they were the least stable. Two reference genes of the five studied had M-values below one, which is the threshold value for gene stability in heterogeneous samples. *SDHA* was the most stable with an M-value of 0.965 followed by *YWHAZ* and *B2M*. *GAPDH* and *18S rRNA* proved not to be the preferred normalizing genes. Fig. 4 shows the optimal number of reference genes used for normalization. A threshold value of 0.15 was set by geNorm software. 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 Fig. 4, V2/3 was 0.34, V3/4 was 0.33 and V4/5 was 0.47. These results suggest 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 were thus needed to normalize skin gene expression data at tick attachment and control sites under the experimental conditions of this study.

3.2 Gene expression study (Phase 2)

3.2.1 *Tick species and sampling sites*

Tick species detached from the ewes used in this phase were identified as *Hyalomma rufipes* (large, coarse bont-legged tick), *Hyalomma truncatum* (bont-legged tick) and *Rhipicephalus evertsi evertsi* (red-legged tick; Neumann, 1897). Previous results showed that ticks were more likely to be found on the tails of NA ewes (Cloete et al., 2016), complicating the choice of sampling areas in this breed. Samples in NA ewes were thus taken from the smooth areas of the perineum or the bare strip underneath the tail and not from the fibre-covered kink or tip of the tail to render results comparable with those of the Dorper and SAMM breeds. In these breeds, samples were exclusively taken from parts with limited fibre coverage such as the udder, inside thighs and perineum.

3.2.1 *IL-1β*

Overall, the expression of the two Interleukin genes (*IL-1β* and *IL-8*) analysed in this study was higher ($P < 0.05$) across breeds at tick attachment sites compared to control sites. Within breeds, the NA and SAMM breeds had *IL-1β* being upregulated at tick attachment sites compared to control sites (Fig. 5). 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 the Dorper ($P = 0.006$) but not compared to the SAMM ($P = 0.096$). The interaction of breed with sampling site (tick attachment or control site) was not statistically significant ($P = 0.052$).

3.2.2 *IL-8*

There was no significant difference ($P = 0.155$) in the expression of the *IL-8* gene at control sites between breeds (Fig. 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 = 0.0008) only in NA ewes but not in SAMM (P = 0.096) and Dorper (P > 0.10) ewes. Furthermore, the interaction of breed with sampling site did not affect the expression of *IL-8* in sheep skin (P > 0.10).

3.2.3 *CCL2* and *CCL26*

Breed and sampling site interacted (P < 0.05) for *CCL2* expression (Fig. 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 was independent of sampling site (P > 0.05). Breed and sampling site did not affect the expression of the *CCL26* gene (P > 0.05) while the interaction of the breed and sampling site was not significant (P = 0.099; Fig 8). Still, *CCL26* was more highly expressed at the tick attachment sites of NA ewes than at control sites (P < 0.05). There was no significant difference in expression of this gene at tick attachment sites and control sites in the other two breeds.

3.3 Effects 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, adults) affected (P < 0.05) the expression of *IL-1β* when tested across breeds. This gene was expressed more where nymphs attached compared to where adult ticks attached (respective expression values: 0.306 ± 0.16 for females, 0.170 ± 0.15 for males vs. 1.498 ± 0.29 for nymphs). The expression of *IL-8*, *CCL2* and *CCL26* was unaffected by tick life stage (P = 0.105, P = 0.083 and P = 0.176, respectively). The expression of all genes was independent of the body location from which the ticks were detached, the tick engorgement level and tick species (data not shown). 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 (respectively 0.199 and 0.200), and also not significant (respectively P = 0.115 and P = 0.096).

4. Discussion

The reference genes identified during Phase 1 to study tick-bite sites in sheep could be ranked according to their stability (from most stable to least stable); *SDHA*, *YWHAZ*, *B2M*, *GAPDH* and *18S rRNA*. *SDHA* proved to be the most stable and is thus a good reference gene in sheep skin gene expression studies. The stability of *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 mice 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 of their reference genes. Similarly to the current study, 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 in pig tissues, nasopharyngeal carcinoma and ovine skin, respectively. Interestingly, two commonly used reference genes (*GAPDH* and *18S rRNA*) 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 rRNA* 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 therefore 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 in this study 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.

Overall, *IL-1β* was up-regulated ($P < 0.05$) at the tick attachment sites during Phase 2, although site effects did not reach significance ($P = 0.267$) in Dorper ewes. The up-regulation of *IL-1β* at tick attachment sites relative to control sites in NA sheep in this study is consistent with previous studies on different species, including cattle, sheep and fish (Bridle et al., 2006; Piper et al., 2008; Burgess et al., 2010; Heinze et al., 2012; Brannan et al., 2014; Hermance and Thangamani, 2014). Piper et al. (2008) also reported a significantly higher expression of *IL-1β* at tick attachment sites than at control sites in cattle. The latter authors reported significantly higher expression at the tick attachment sites than at control sites in Holstein-Friesian cattle, a breed considered as more susceptible to tick infestation. The lower expression level of *IL-1β* at tick attachment sites in Dorpers compared to the NA may be related to the results of Ramachandra and 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 concurs

with findings of Piper et al. (2008) who 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 and 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. Even though the latter authors investigated gene expression in cattle infected with protozoan parasites, while the present study investigated the effect of tick infestation on gene expression, 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 compared to Dorper ewes indicates that this gene may play a role in the resistance to ticks in NA ewes as reported by Cloete et al. (2013; 2016). The relative expression of *IL-1β* varied greatly between individual ewes within breeds (results not shown). Piper et al. (2008) and Piper et al. (2010) also reported similar variation in cattle. This observation might be attributed to several factors, including that animals were naturally infested in this study, which means ticks attached at different times. It is therefore 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* differs 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 was low in mite (*Psoroptes ovis*) infected host sheep compared to controls at 1 h post-infestation, peaked up at 3 h post-infestation before lowering down by 6 to 24 h post-infestation (Burgess et al., 2010). Gene expression levels are inherently variable by nature (Piper et al., 2008). The results of this study confirmed there were differences ($P < 0.05$) in *IL-1β* expression between the NA (tick-resistant breed) and the Dorper (tick-susceptible breed). It was interesting to note that the susceptible SAMM breed exhibited upregulated *IL-1β* expression ($P = 0.09$) at tick attachment sites. The breed differences in the expression of *IL-1β* in this study indicate that *IL-1β* may account for breed differences in resistance or susceptibility to tick infestation in sheep.

The up-regulation of the *IL-8* gene at the tick infested sites of all three breeds accorded with findings of previous studies on sheep infested with the sheep blowfly, *Lucilia cuprina* (Egan et al., 1996) and *P. 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 tick-resistant cattle, the results of this study showed the up-regulation of *IL-8* in the perceived resistant breed at tick attachment sites compared to the control sites. There were no significant differences in *IL-8* expression at control sites between the breeds. However, NA ewes had the highest *IL-8* expression at the tick attachment sites compared to the other two breeds. 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 cytokine genes, tick saliva suppresses its expression so 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 infested sites in NA ewes naturally infested by ticks were not achieved. This result implied that NA ewes may have a mechanism of fighting against tick attachment which may contribute to their resistance to tick infestation.

CCL2 was significantly up-regulated at tick attachment sites in NA ewes, while sample site did not affect its expression in other breeds. The lack of significant differences in *CCL2* expression between breeds is contrary to the 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. Those 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 provoked 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) reported the down-regulation of the *CCL2* gene expression in cells treated with tick saliva. The up-regulation of this gene at tick attachment sites in the NA suggested that the immune response of NA ewes is capable of overcoming the tick's defense mechanisms.

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 breed only. There were no significant differences between tick attachment and control sites in SAMM and Dorper ewes. 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 as reported by Cloete et al. (2013; 2016). Wang et al. (2007) reported a down-regulation of this gene at tick attachment sites in Hereford x Shorthorn cattle compared to control sites. 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). The discrepancies in the expression of this gene between sheep and cattle might be due to different mechanisms used by these species to combat tick infestation and requires further research.

The lack of animal body sampling site effects on gene expression recorded in this study concur with findings of Piper et al. (2008). The results obtained in this study suggest that biopsies for gene expression can be taken from any region of the animals' body without seriously biasing the results as was also reported for cattle. Contrary to Vančová et al. (2007)'s findings, the present study suggests that gene expression is independent of tick sex. The tick life stage only affected the expression of *IL-1β*, where nymphs elicited higher expression than adult ticks (both males and females). Hajnická et al. (2005) reported that anti-chemokine activity varied between tick species. No information was found in the literature to support or refute the lack of significant ($P > 0.05$) effects of tick engorgement levels as well as the low correlations between tick weight and gene expression in the present study.

Conclusions

Concerning reference gene selection, the geNorm analysis results indicated that at least three reference genes with the lowest M-values should be used. *SDHA*, *YWHAZ* and *B2M* were the most suitable reference genes for normalizing gene expression data in sheep skin in this study. These findings assisted in normalizing data in gene expression studies at tick attachment sites 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. Overall, the pro-inflammatory cytokine genes (*IL-1β and IL-8*) were more highly expressed at tick attachment sites than at control sites. No significant differences in chemokines (*CCL2* and *CCL26*) were found between tick attachment and control sites when compared across breeds. 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 than the other breeds to upregulate the expression of the *CCL2*, *CCL26* and *IL-8* genes at tick attachment sites compared to control sites. These results indicate that *IL-1 β*, *CCL26* and *IL-8* may play a part in resistance or susceptibility of sheep to tick infestation. On absolute values, *CCL2* was down-regulated at tick attachment sites in 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 may 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 provide an indication that cytokines are involved in immune responses to tick challenge and lay a foundation for further studies on gene expression under more controlled conditions. Since this study was extensive in nature, control over aspects such as tick species, sex and maturity stage as well as attachment time were impossible to achieve.

Ethical Clearance

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.

Conflict of Interest and Author's Contribution Statement

The authors wish to state that there is no conflict of interest involved in the study from Dr Thutwa's PhD that was submitted to the Veterinary Parasitology Journal. None of us received any material benefit from our involvement in the study. KT, JBvW, KD and SWPC were responsible for the conceptualisation, planning and execution of the study. JBvW and SWPC sourced funding to allow the study. KT, JBvW, AJS and SWPC arranged, coordinated and executed the study and arranged for access to the facilities and support systems (including materials and manpower) used in the process. The draft version of the manuscript was drawn up by KT and SWPC. All authors contributed to the further development, preparation and finalisation of the manuscript for submission.

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Figure captions:

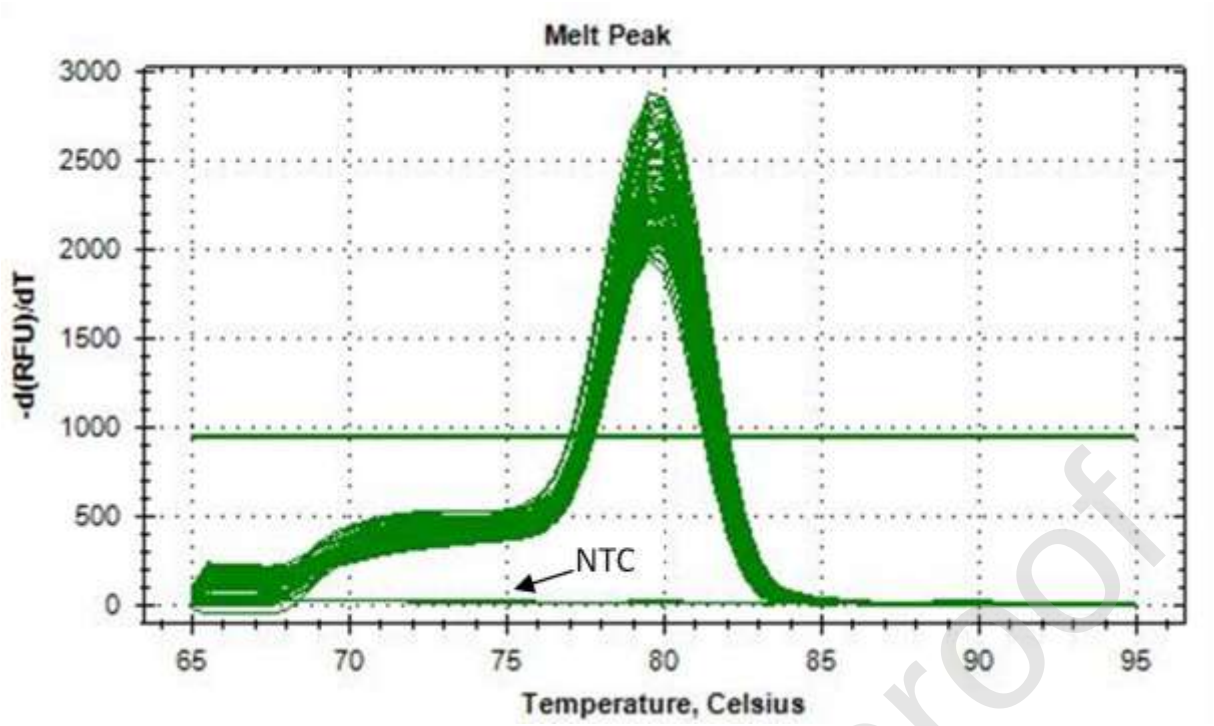

Fig. 1. Melt curve of *YWHAZ* with one peak and all the amplifications above the threshold line except for the no template control (NTC). A single peak indicates the specificity of the primers and that a single amplicon is amplified.

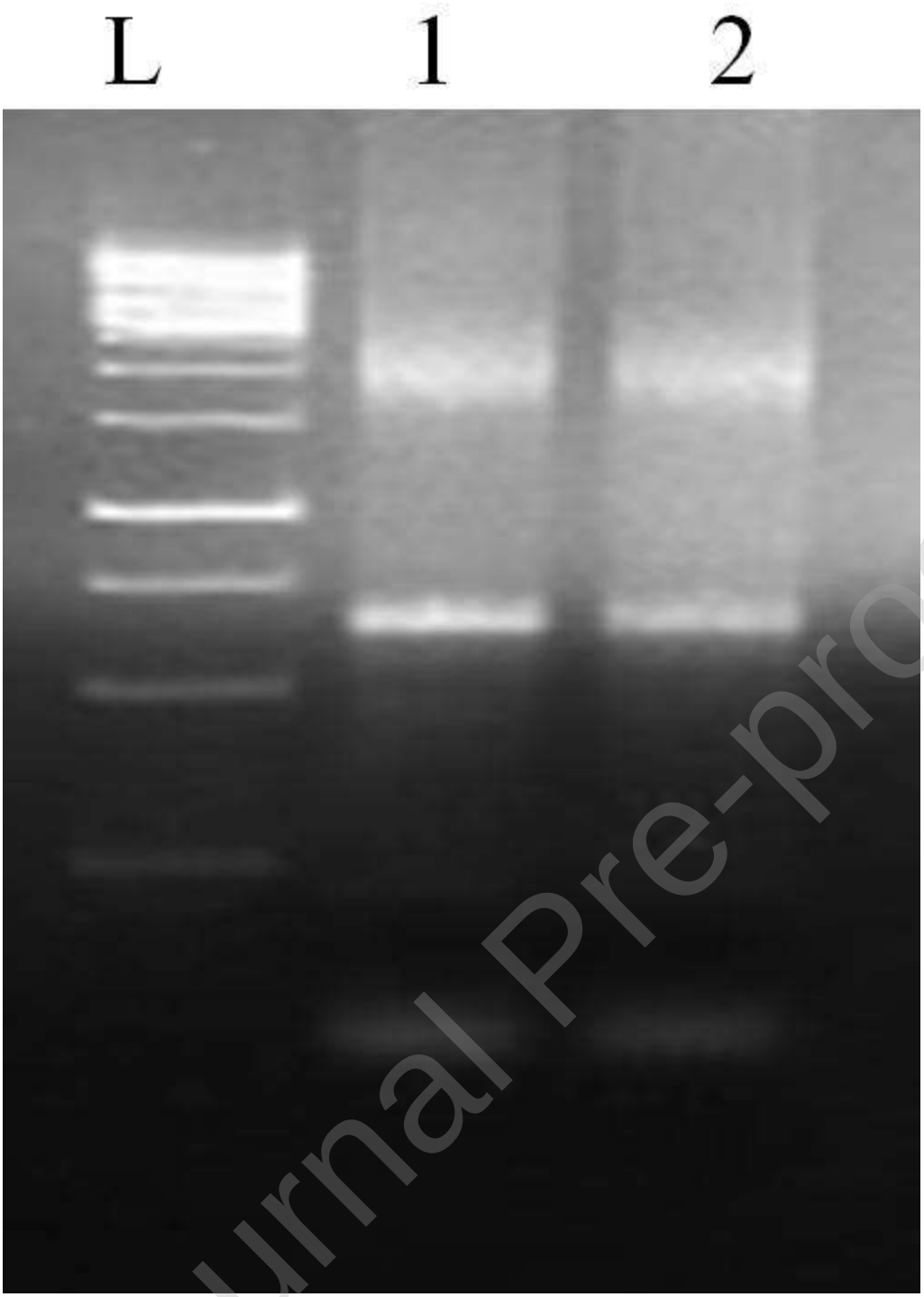

Fig. 2. An agarose gel showing representative RNA bands in the lanes 1 and 2, lane L is a 1 kb ladder.

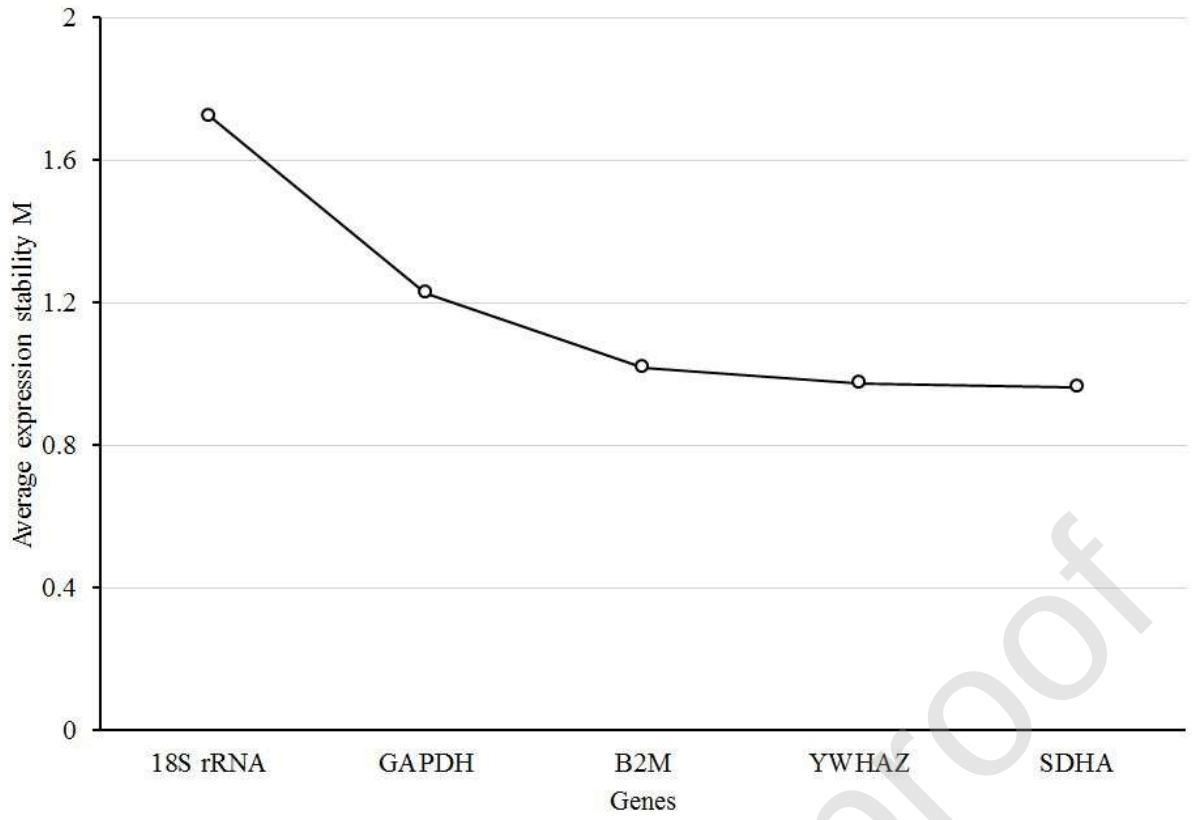

Fig. 3. The average expression stability (geNorm stability value) of the reference genes. The genes are listed from least stable to most stable (from left to right). Gene names: *18S rRNA* – 18S ribosomal RNA; *GAPDH* – Glyceraldehyde-3-phosphate dehydrogenase; *B2M* – Beta-2-microglobulin; *YWHAZ* – Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide*; SDHA* – Succinate dehydrogenase complex, subunit A*;*

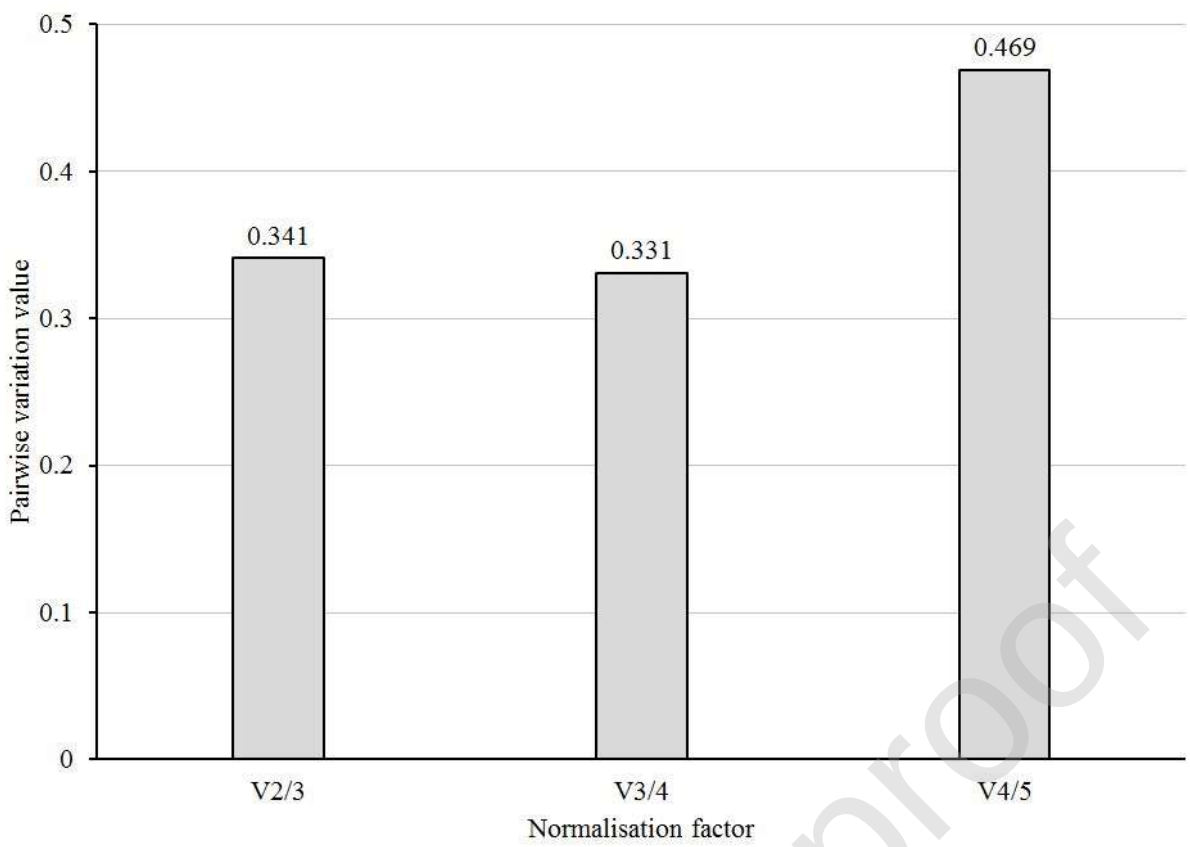

Fig. 4. The determination of the optimal number of reference genes for normalization. The normalization factor (NF) was calculated from at least two genes by the geNorm program. The mean pairwise variation (V) is determined between two sequential NFn/NFn+1, where n is the number of reference genes needed for normalization of the gene expression data.

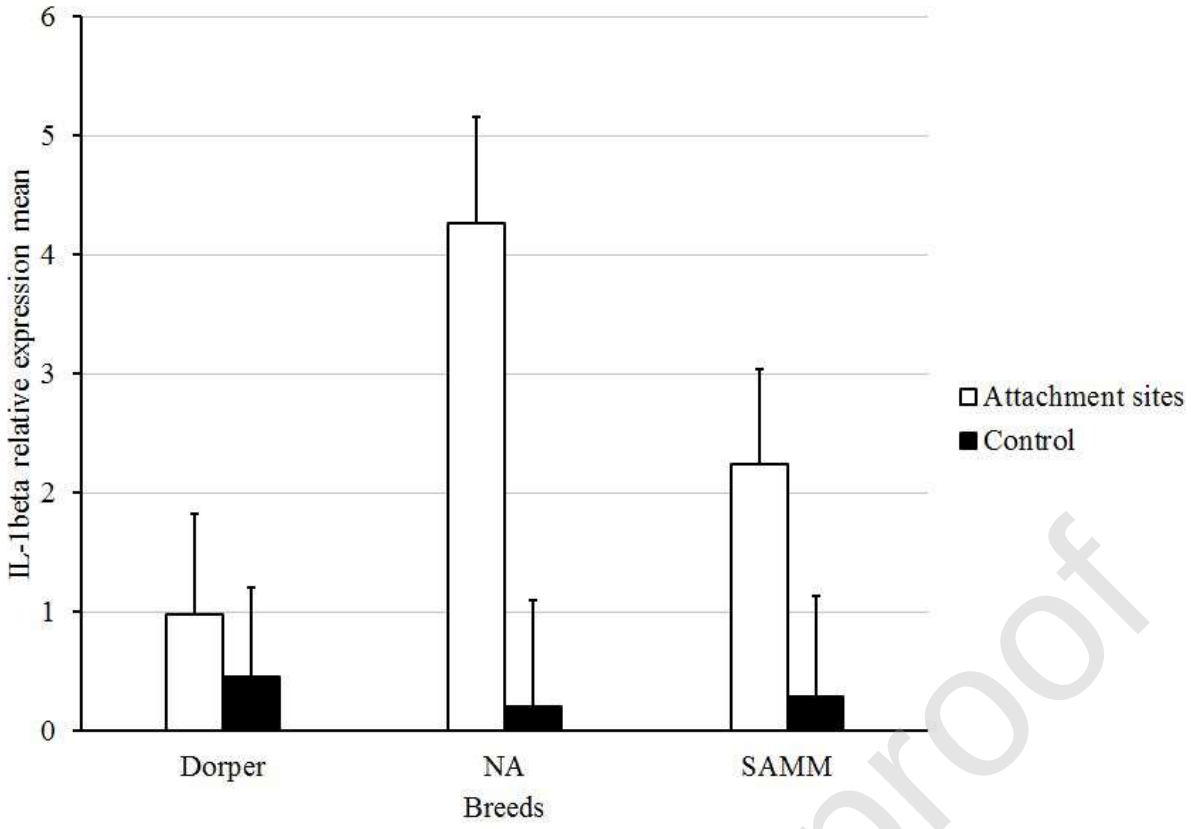

Fig. 5. Least-squares means depicting the effects of the breed x sampling site interaction on relative expression (normalized with three reference genes) of the *IL-1β* gene in Dorper, Namaqua Afrikaner (NA) and SA Mutton Merino (SAMM) ewes. Vertical lines about means represent SEs. Overall, *IL-1β* expression was increased at attachment sites compared to control sites across breeds. Within breeds, *IL-1β* expression at attachment sites exceeded that in control sites in the NA and SAMM breeds.

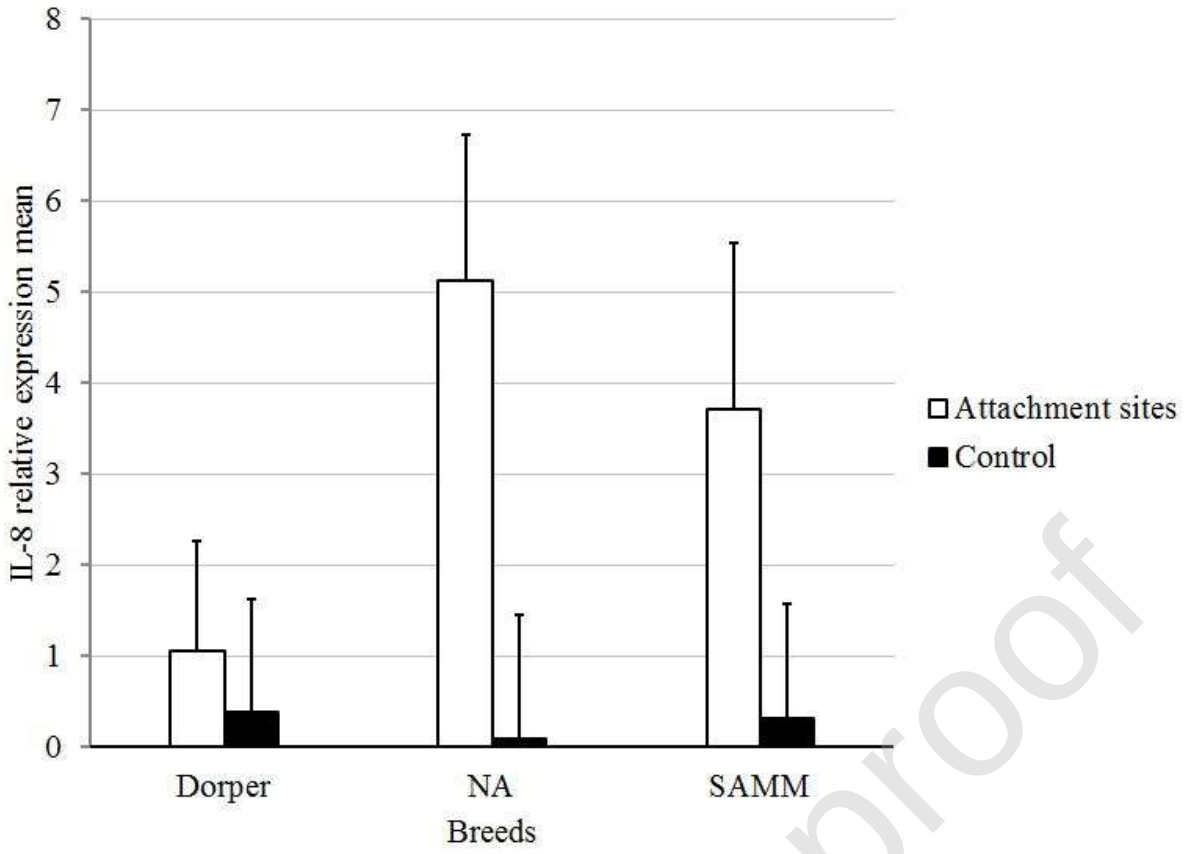

Fig. 6. Least-squares means depicting the effects of the breed x sampling site interaction on the relative expression (normalized with three reference genes) of the *IL-8* gene in Dorper, Namaqua Afrikaner (NA) and SA Mutton Merino (SAMM) ewes. Vertical lines about means represent SEs. Overall, *IL-8* expression was increased in attachment sites compared to control sites across breeds. Within breeds, *IL-8* expression at attachment sites exceeded that in control sites only in the NA breed.

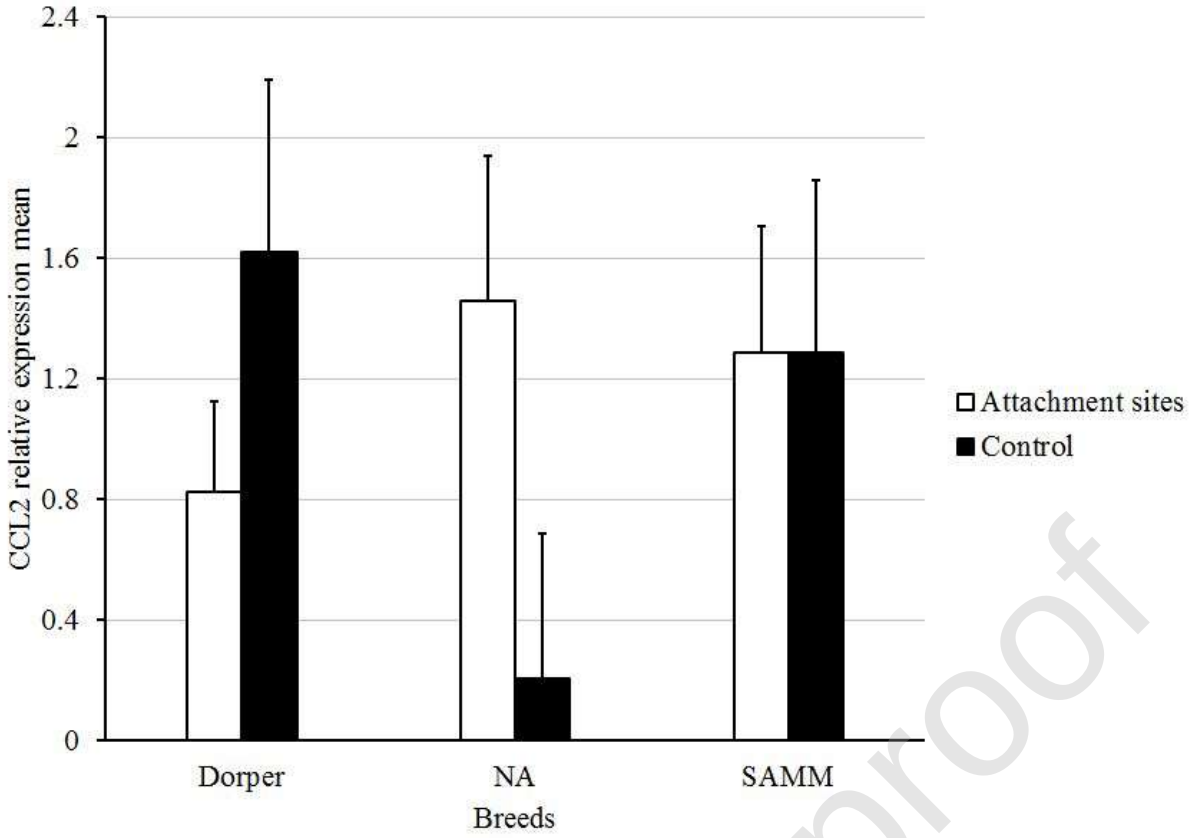

Fig. 7. Least-squares means depicting the effects of the breed x sampling site interaction on the relative expression (normalized with three reference genes) of the *CCL2* gene in Dorper, Namaqua Afrikaner (NA) and SA Mutton Merino (SAMM) ewes. Vertical lines about means represent SEs. Overall, *CCL2* expression was not affected by the main effects of breed or sampling site. However, *CCL2* expression at attachment sites exceeded that in control sites in the NA breed.

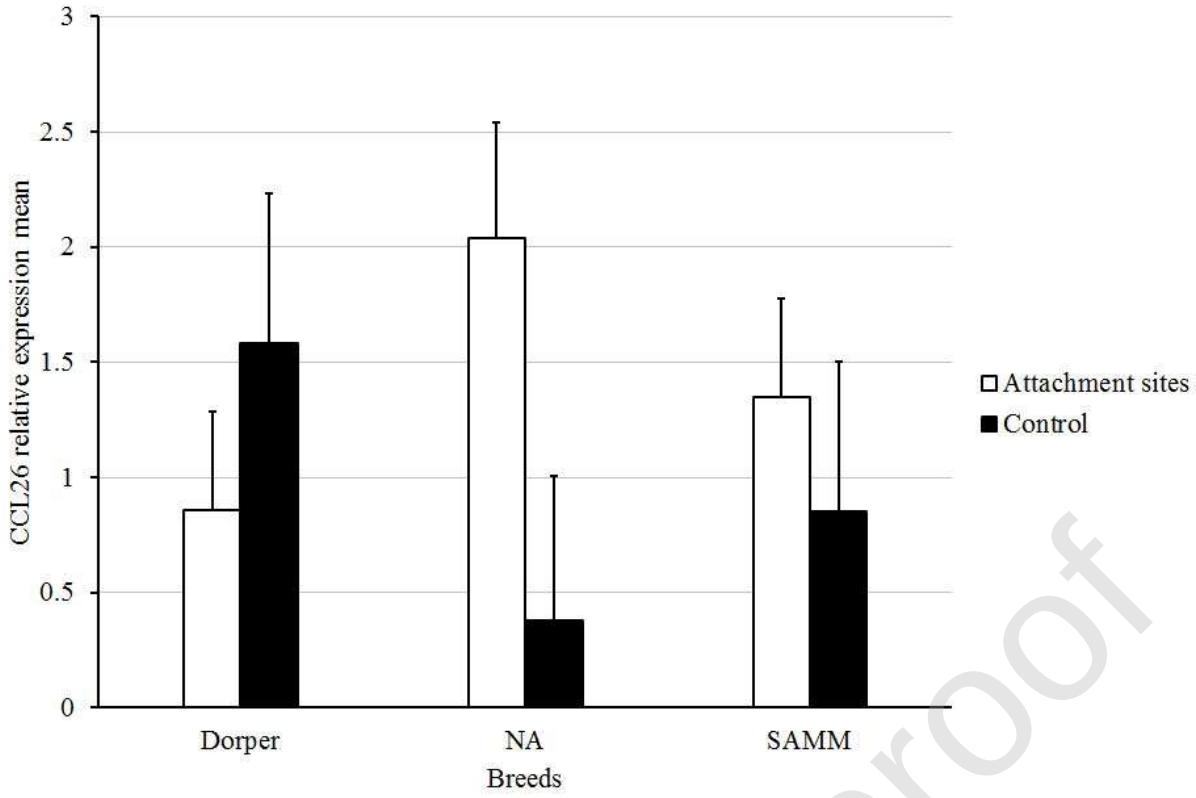

Fig. 8. Least-squares means depicting the effects of the breed x sampling site interaction on the relative expression (normalized with three reference genes) of the *CCL26* gene in Dorper, Namaqua Afrikaner (NA) and SA Mutton Merino (SAMM) ewes. Vertical lines about means represent SEs. Overall, *CCL26* expression was independent from breed and sampling site. Within breeds, *CCL26* expression at attachment sites exceeded that in control sites only in the NA breed.

Table 1

Sequences of the primers used for reference genes, product size and annealing temperature.

Gene*	Sequence	Product size (bp)	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 rRNA	Forward: GAGAAACGGCTACCACATC Reverse: GCTATTGGAGCTGGAATTAC	185	57
GAPDH	Forward: AAGTTCAACGGCACAGTCAA Reverse: ACCACATACTCAGCACCAGC	181	59

YWHAZ – Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide*; SDHA* – Succinate dehydrogenase complex, subunit A*; B2M* – Beta-2-microglobulin; *18S rRNA* – 18S ribosomal RNA; *GAPDH* – Glyceraldehyde-3-phosphate dehydrogenase

Table 2

Appropriate literature source and efficiency of the primers used for the reference genes.

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

**YWHAZ* – Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide*; B2M* – Beta-2-microglobulin; *SDHA* – Succinate dehydrogenase complex, subunit A*; 18S rRNA* – 18S ribosomal RNA; *GAPDH* – Glyceraldehyde-3-phosphate dehydrogenase

Table 3

A summary of the specific primers used for both studied genes and reference genes, literature source, primer sequences and annealing temperatures.

Gene*	Primer pairs	Annealing temp (°C)	Literature sourc
IL-8	Forward: ATGAGTACAGAACTTCGA Reverse: TCATGGATCTTGCTTCTC	55.0	Egan et al. (1996)
IL-1β	Forward: CAGCCGTGCAGTCAGTAAAA Reverse: GAAGCTCATGCAGAACACCA	57.0	Herman et a (2010)
CCL2	Forward: CCAGCAAGTGTCCCAAGA Reverse: AGATGGTTTATGGCGTCCTG	55.7	Present study
CCL26	Forward: TCCCTATGGCTTCCCTTCTT Reverse: TACTGGAAACAGCAGAACTTGG	55.7	Present study
YWHAZ	Forward: TGTAGGAGCCCGTAGGTCATCT Reverse: TTCTCTCTGTATTCTCGAGCCATCT	59	Garcia-Crespo al. (2005)
B2M	Forward: ATCCAGCGTATTCCAGAGGTC Reverse: AATCTTCTCCCCGTTCTTCAG	59	Tian et al. (2013)
SDHA	Forward: CATCCACTACATGACGGAGCA Reverse: ATCTTGCCATCTTCAGTTCTGCTA	64.5	Garcia-Crespo al. (2005)

**IL-1β* – Interleukin 1 beta, *IL-8* – Interleukin 8; *CCL2* – Chemokine C-C ligand 2; CCL26 Chemokine C-C ligand 26; *YWHAZ* – Tyrosine 3-monooxygenase/tryptophan 5-monooxygenas activation protein, zeta polypeptide; *B2M* – Beta-2-microglobulin; *SDHA* – Succinat dehydrogenase complex, subunit A