A genetic and immunological comparison of tick-resistance in beef cattle following artificial infestation with *Rhipicephalus* ticks

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Abstract

Host resistance to ticks can be explored as a possible approach of combating tick infestations to complement the existing unsustainable tick control methods. Thirty-six beef cattle animals were used, consisting of Angus, Brahman and Nguni breeds, with each breed contributing 12 animals. Half of the animals per breed were artificially challenged with *Rhipicephalus microplus* and the other half with *R. decoloratus* unfed larvae per animal. Skin biopsies and blood samples were collected pre-infestation and 12 h post-infestation from the feeding sites of visibly engorging ticks. The success rate of the ticks was high and had an influence even at the early time point. Increased lymphocytes and blood urea nitrogen levels as well as decreased levels of segmented neutrophils were observed in the Angus, which were the opposite of those in the Brahman and Nguni. The increase in cholesterol, which was highest in the Angus and lowest in the Nguni, may be due to altered protein metabolism. The expression profiles of genes *TRAF6*, *TBP*, *LUM* and *B2M* were significantly different among breeds. Five genes (*CCR1*, *TLR5*, *TRAF6*, *TBP*, *BDA20*) had increased or constant expression post-infestation, whereas the expression of *CXCL8*, *IL-10* and *TNF-α* decreased or remained the same after tick challenge. Genes that showed variation are involved in discouraging long-term supply of blood meal to the tick and those associated with immune responses. The gene *LUM* is a potential biomarker for tick resistance in cattle. The response to infestation by the breeds was consistent across the tick species.

Keywords: Tick-resistance, gene expression, serum biochemistry, haematology.

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INTRODUCTION

The severe economic and environmental effects of tick infestations on cattle production are the driving force behind developing effective strategies to combat tick infestations. Although acaricides and vaccines have been the dominant tick control methods (Jongejan and Uilenberg 1994), they are not considered sustainable because of various economic, environmental and social concerns (Jonsson 2006; Regitano et al. 2008; Machado et al. 2010). The host's natural resistance to ticks offers an opportunity that can be exploited to develop alternative tick control methods that can complement the existing strategies. Being a low-cost, permanent solution requiring no extra resources and incurring no additional costs to generate a given amount of product, host resistance is the single most important factor affecting the economics of tick control (Frisch 1999). In this regard, host resistance to ticks can be exploited to develop alternative tick control measures by utilizing the variation in resistance to ticks that exists between and among breeds. The Nguni are more resistant to ticks than Bonsmara and Angus cattle (Jonsson, 2006; Muchenje et al. 2008; Marufu et al. 2011a). On the other hand, the Brahman breed displayed superior resistance to ticks compared to its *Bos taurus* counterparts (Seifert 1971; Utecha et al. 1978; Piper et al. 2009). Several factors are responsible for the variation in the tick-resistance phenotype in cattle; thus, understanding these factors may form the basis of developing effective tick control strategies.

The variation in resistance to ticks that exists among cattle breeds has been attributed to differences in immunological and cellular responses. Strong evidence suggests that cattle rely on innate and acquired immunological mechanisms (Marufu et al. 2011). The animal's immunological response to infestation includes a range of components amongst which leukocytes, complement cytokines and antigen presenting cells have been listed. Studies on tick infestation site histology found that eosinophils, basophils, mast cells and lymphocytes are associated with the degree of resistance to ticks (Marufu et al. 2014). Changes in

parameters like haematocrit, white cell counts, plasma proteins, cholesterol and lactate dehydrogenase were directly associated with the effects of tick infestation in cattle (O'kelly 1968; O'Kelly and Kennedy 1981; Piper et al. 2009). The histology of the tick attachment sites has been studied to understand the cellular responses when cattle are exposed to different tick species. Mast cells, basophils, eosinophils and lymphocytes play a role in the animal's cellular response and degree of resistance when artificially infested with ticks (Veríssimo et al. 2008; Carvalho et al. 2010; Marufu et al. 2014). The involvement of and variation in proteins and cells involved in immunological responses to tick infestation within and among breeds may suggest an underlying genetic control of resistance to ticks.

Given that resistance to ticks is a polygenic trait, which is fairly heritable (Mapholi et al. 2014), there is an opportunity to improve resistance to ticks through genetic selection. Selection of animals should be based on higher accuracy of genetic evaluations so that significant genetic improvement is achieved; hence, molecular techniques should be utilized. Use of genomic technologies may help improve the accuracy of selection by identifying markers or genes associated with resistance to ticks. Wang et al. (2007) observed variation in the expressions of extracellular matrix genes in Hereford Shorthorn cattle. In separate studies, Piper et al. (2008, 2009) reported significant between-breed differences for toll-like receptors (*TLR5, TLR7, TLR9, NFKBp50, MyD88, Traf-6, CD14* and *IL-1β*), chemokines (CCL2 and CCL26) and chemokine receptor (CCR-1) on Brahman and Holstein-Friesian cattle. The majority of these studies were gene expression studies primarily focused on the genes of the major histocompatibility complex (MHC) rather than transcriptomic studies following challenge with one tick species (Turner et al. 2011). Transcriptome analyses provide a global picture of the cell function following infestation by profiling coding and non-coding transcriptional activity and gene expression following infestations. On the other hand, immune responses vary depending on the degree to which an animal's immune system has evolved in its ability to generate vigorous responses in defence against a biting tick species (Marufu et al. 2014). This may be attributed to the variations that exist in the characteristics of the tick species, such as, mouthparts, bioactive molecules in the saliva and other physiological properties (Francischetti et al. 2009). The objective of the study was therefore to compare gene expression and immunological responses of the Nguni, Brahman and Angus cattle artificially infested with *Rhipicephalus microplus* and *R. decoloratus*.

MATERIALS AND METHODS

Study site

The trial was conducted at the South African Agricultural Research Council - Animal Production (ARC-AP) in Irene, which is located 25º53'59.6"S and 28º12'51.6"E. Cattle were housed individually in 36 large stock feeding pens. The pens consisted of concrete floors which were cleaned daily and were each equipped with a feeding trough and an animal-operated water tap.

Tick species

Unfed larvae of the *R. microplus* and *R. decoloratus* species were obtained from aseptic colonies. The colonies were made up of engorged females fed in healthy animals free of tick-borne diseases and maintained in laboratory conditions at ClinVet International Laboratories, Bloemfontein, South Africa. The larvae were kept unfed and allowed to mature for 8 weeks in humidity chambers (75% RH) at 20°C prior to infestation. Larvae were prepared in a sterile environment and they were considered aseptic. This minimizes the risks involved surrounding the transmission of any tick-borne diseases.

Experimental cattle

In total 36 cattle – 12 Nguni bulls, 12 Brahman bulls, six Angus heifers and six bulls – were sourced from a selection of extensively managed farms. The cattle were aged between 12 and 15 months with similar body conditions and body weights, ranging from 250 to 300 kg. The Nguni and Brahman cattle came from the Mpumalanga Province, whereas the Angus cattle were sourced from the Free State Province of South Africa. Both areas have the *R. microplus* and *R. decoloratus* tick species prevalent. As a result, all animals were known to have been previously exposed to *R. microplus* and *R. decoloratus* tick challenge ahead of the trial. The cattle were all treated with amitraz (Decatix®, Cooper Veterinary Products, South Africa), short acting acaricide, upon arrival at the ARC-AP feedlot. They were housed in individual pens for the duration of the trial and allowed *ad libitum* access to a standard commercial feedlot diet and *ad libitum* supply of fresh, clean water.

Artificial infestation

The unfed tick larvae (UFL) were counted and organised into groups of approximately 100 before being placed into tick-safe vials. Each breed group of the experimental animals was randomly split in half, with six animals per breed undergoing artificial infestation with *R. microplus* larvae, whereas the remaining six were infested with *R. decoloratus* larvae. Subsequently, the Angus groups were further divided in terms of sex, with three animals per

sex undergoing infestation with *R. microplus* and the other three being infested with *R. decoloratus* larvae. A circular part of each animal's upper back was shaved using an industrial cattle clipper (Lister Legend Clipper, Wahl Clipper, UK). Shaved areas were adjacent to the cervico-thoracic humps of Brahman and Nguni cattle while maintaining a similar position on the Angus breed. One calico bag was attached to the clean-shaven area using a contact adhesive (Alcolin, South Africa) applied to the outer ring followed by a 24-h drying period. Once the bags were completely dry, one tick-containing vial was placed inside the bag of each animal, opened and left there to liberate the larvae for feeding. The open sock end of the bag was then shut and secured with one rubber castration ring.

Twelve hours post-infestation, the bags were opened to remove the vials and the tick bite sites were visually inspected for any actively feeding ticks, areas from which skin biopsy samples were collected. At the end of the experiment, 18 days post-infestation, the bags were carefully removed and the animals were all treated with amitraz (Decatix). The area of the skin where the outer ring of the calico bag was attached was disinfected with chlorfenvinphos 0.48% (Supona Aerosol Spray, Zoetis, South Africa) to prevent infection and wound myasis from any possible skin trauma.

Blood collection

Each animal was restrained in a crush pen and blood was collected by coccygeal venipuncture into 9-ml vacuum tubes (Vacuette, Lasec, South Africa). Two blood samples were taken per animal pre-infestation and another two at 12-h post infestation. One sample was collected into a 9-ml vacuum tube containing EDTA (Vacuette K2EDTA), whereas the other was collected into a plain 9-ml vacuum tube (Vacuette Z Serum Clot Activator). The blood samples were inverted gently several times and stored at room temperature for 1-2 h, followed by refrigeration at 4°C until sampling was complete and all samples were collected for processing.

Blood biochemistry

All blood biochemistry analyses were performed by IDEXX Laboratories (Johannesburg, South Africa) shortly after collection into the plain vacuum tubes. Blood serum was analysed for total serum protein, albumin, globulin, alanine amino-transferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), uric acid, bilirubin, cholesterol, creatine kinase (CK), blood urea nitrogen (BUN), creatinine, lactate dehydrogenase (LDH) and fibrinogen. All serum biochemistry analyses were performed using a Vitros® 350 Dry

Slide Chemistry Analyzer (The Scientific Group, South Africa). Analysis proceeded with the use of the parameter specific Vitros sides, the appropriate Vitros products calibrator kit and the Vitros 5600 Integrated System.

Haematology

All haematology analyses were performed by IDEXX Laboratories, on samples collected in EDTA-containing tubes within 4 h of collection. Whole blood was analysed for red blood cell count (RBCC), haemoglobin, haematocrit (packed cell volume), mean cell volume (MCV), red cell distribution width (RCDW), mean cell haemoglobin concentration (MCHC), white cell counts (WCC) and platelets. Differential analysis was also performed for segmented neutrophils, band neutrophils, lymphocytes, monocytes, eosinophils and basophils (all % and abs). All haematological analyses were performed using a Sysmex XT2000i Automated Haematology Analyzer (Sysmex, South Africa).

Skin biopsy collection

The animals were lightly sedated with 0.2 ml/50 kg body weight xylazine (Rompun, 20 mg/ml; Bayer, South Africa) administered intramuscularly in the rump. The Brahman breed has increased sensitivity to the sedative; thus, these animals were closely monitored thereafter. A local anaesthetic injection, at a dose of 0.1 ml/site of 2% lignocaine hydrochloride injection (Bayer), was administered subcutaneously around the punch biopsy collection site

Using a disposable 5 mm biopsy punch (Stel+Medcc, South Africa), three skin biopsies were taken, each with a 5 mm diameter and 10 mm deep. Two skin biopsies were taken from non-parasitized skin prior to infestation and one from parasitized skin from identifiable tick feeding sites 12-h post-infestation. The biopsies were for genetic analyses and were preserved individually in 5 ml RNAlater® RNA stabilization Reagent (Qiagen) and stored at-80°C. The biopsy collection sites were treated with chlorfenvinphos 0.48% (Supona Aerosol Spray) and oxytetracycline (Terramycin Wound Powder, Fivet, South Africa) to prevent bacterial infection and wound myiasis.

RNA extraction

Between 50 and 100 mg of each tissue sample was used for RNA extraction using the TRIzol® Reagent protocol (Ambion, Life technologies). Each biopsy sample was placed in a specialized 2-ml screw-cap tube containing two ceramic beads and 1000 µl of TRIzol reagent

and the samples were macerated, disrupted and homogenized using the Geno/Grinder 2010 machine (SPEX SamplePrep, Vacutec). The Qiagen RNeasy Mini Kit Quick-Start Protocol was followed to conduct total RNA clean-up and removal of genomic DNA (gDNA) contamination. The RNA concentrations were quantified using the Qubit 2.0 Fluorometer. However, the NanoDrop spectrophotometer (NanoDrop DN-100, Thermo Fisher Scientific) was used to quantify concentrations higher than 600 ng/µl. The quality or integrity of the RNA was further verified using a 1% agarose gel electrophoresis.

Complementary DNA (cDNA) synthesis

Complementary DNA (cDNA) was synthesised using equal amounts of total RNA (Huggett et al. 2005). The RT^2 First Strand Kit from Qiagen was used according to manufacturer's protocol. To obtain optimal results, 400 ng of total RNA per sample was used to obtain a total cDNA volume of 30 µl.

Primer design and optimization

The primers for each of the genes of interest were custom designed by Qiagen (Whitehead Scientific, South Africa) using forward and reverse primer sequences associated with the GenBank and UniGene reference sequence numbers listed in Table 1.

Quantitative real-time PCR (qPCR) analysis

Custom 96-well RT^2 Profiler PCR arrays (Qiagen) were used for the real-time PCR analyses and facilitated high-throughput focused expression analysis on the genes of interest. The arrays came pre-treated with the primers in each well for each of the genes of interest. Each plate enabled the analyses of four samples at a time to generate amplification data for 17 genes of interest and four reference genes per sample.

Using an ABI 7500 real-time PCR thermocycler, the gene expression profiles of the panel of genes listed in Table 1 were examined. A PCR components mix was prepared in a 5-ml tube for each sample according to manufacturer's protocol (Qiagen 2015). The mix comprised of 12.5 µl 2x RT^2 SYBR Green Mastermix, 11.5 µl high-quality RNase-free water and 1 µl cDNA synthesis reaction to make the required total volume of 25 µl per well. Each well received 25 µl of the components mix and was then tightly sealed with an Optical Thin-Walled 8-Cap Strips.

The arrays were also fitted with primers designed to amplify three Qiagen recommended quality control parameters, namely Bovine Genomic DNA Control (BGDC),

Reverse Transcription Control (RTC) and Positive PCR Control (PPC). The BGDC is a very sensitive assay that detects the unique non-coding region that is far removed from any transcriptional start site within the bovine genome. Any sample that produced C_T values below 35 was analysed carefully gene for gene, whereas those with C_T values lower than 30 had their RNA re-purified with genomic DNA removal, using DNase I and a spin column. BGDC values <30 indicated that genomic DNA was likely to have been contributing signal to most if not all the genes of interest for that specific sample (Qiagen 2015). The PPC matrix measured the PCR array reproducibility by measuring the technical variability of the PPC wells across all samples. The recommended difference between any two samples was not to be more than 2 from one another for suitable data to be produced for further data analysis (Qiagen 2015). The RTC measured the efficiency of the reverse transcription across samples by detecting the artificial mRNA with a poly-A tail not homologous to any mammalian or bacterial sequence that is preloaded into the primer buffer of the RT^2 First Strand cDNA synthesis kit (Qiagen 2015). The artificial mRNA is reverse transcribed with the messages in the samples and upon detection of this sequence; it was possible to determine whether the data from all the samples could be used for comparison. The RT efficiency was determined by calculating the ΔC_T (RTC-PPC) for each sample. The preferred difference between the C_T values was ≤ 5 above which the RNA was to be re-purified.

The threshold was set to 1 for all the arrays. This point lies slightly above the middle of the geometric phase of the amplification curve, where all the curves were straight and parallel to each other (Wong and Medrano 2005). The baseline was set to range from 2-15 cycles since the earliest amplification was visible between cycles 12 and 18.

Statistical analysis

Haematology and biochemistry

The data were analysed using Statistical Analysis System Enterprise guide software (SAS 2012). The linear-models procedure was used to perform ANOCOVA (Type III) analysis of the effects of treatments breed and tick species on the respective biochemical and haematological parameters. Mean effects of treatments were determined using LSMEANS option and compared using Bonferroni t-tests. The pre-infestation values for each parameter were used as the covariate in all ANOCOVA models. The statistical model for all biochemical and haematological parameters can be summarized as follows:

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

where Y_{ijkl} = biochemical or haematological parameter; X_i = effect of the i-th animal; β = regression coefficient for the effect of the animal on the dependent variable; A_j = effect of the j-th breed; B_k = effect of the k-th tick species; $A_i B_k$ = the effect of the breed by tick species interaction; and ε_{ijkl} = residual error.

Genetic analysis

The threshold cycle (C_T) values generated were used to calculate the expression level of each gene using the RT^2 Profiler PCR Array Data Analysis Webportal (SABioscience, Qiagen). The fold change value of each gene, normalised against the reference genes Ribosomal protein, large, P0 (*RPLP0*), 18S ribosomal RNA (*RN18S1*), Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and Beta-actin-like (*LOC616410*), was calculated using the $\Delta\Delta C_T$ method explained below (Livak and Schmittgen 2001; Wong and Medrano 2005):

$$\Delta C_T = C_{T(\text{Gene of interest})} - C_{T(\text{Reference gene})}$$

$$\Delta\Delta C_T = \Delta C_T(\text{Test group n}) - \Delta C_T(\text{Control group n})$$

$$\text{Fold change} = 2^{(-\Delta\Delta C_T)}$$

$$\text{Fold regulation} = \text{Fold change for values} \geq 1, \text{and Fold regulation} = -\frac{1}{\text{fold change}} \text{for values} < 1.$$

Mean fold regulation > 0 indicates upregulation, < 0 indicates down-regulation, ≥ 2 indicates over-expression, and < -2 indicates under-expression.

Instead of fold change values, fold regulation values were used to facilitate ease of interpretation of the data for all relative quantitation statistical analyses (Wong and Medrano 2005). Employing XLSTAT 2016 and SAS Enterprise Guide 9.4 (SAS 2016), an ANOVA for two-way factorial designs was used to generate the p-values for the interaction between the main effects, breed and tick species, for each of the genes ($\alpha = 0.05$). All genes which produced non-significant interaction values were tested for the significance of each of the main effects. Genes that exhibited significant values for either one of the main effects were further analysed using the Bonferroni pairwise test for Least Square (LS) means to determine

which treatments differed from each other. A coefficient of determination (R^2) was generated for each gene to provide an indication of the amount of variation that was explained by the formulated model, as well as the contribution of the interaction and each of the main effects to the observed variation. The mean and standard errors for each gene were generated in every treatment combination group, namely Angus-*R. microplus*, Angus-*R. decoloratus*, Brahman-*R. microplus*, Brahman-*R. decoloratus*, Nguni-*R. microplus* and Nguni-*R. decoloratus.*

Ethical clearance

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

RESULTS

Breed differences in haematological changes

Values for haematological changes presented in Table 2 are the differences between observations taken at the second (post-infestation, P2) and first (pre-infestation, P1) time points and calculated as follows: $\Delta Parameter = P2 - P1$. The Angus cattle had the largest increase in red blood cell counts (RBC) and haemoglobin levels of $0.77 \pm 0.46 \times 10^{12}$/l and 1.14 ± 0.56 g/dl, respectively, which differed significantly from that of the Brahman cattle ($0.19 \pm 0.39 \times 10^{12}$/l and 0.17 ± 0.47 g/dl). The smallest increase in packed cell volume levels of 0.61 ± 1.60% was observed in the Brahman, whereas the Angus and Nguni cattle had increases of 3.53 ± 1.90 and 2.78 ± 2.40%, respectively. There was a decrease of 0.06 ± 0.25 fl in mean cell volume in the Brahman that was significantly different from the increase in the Nguni cattle (0.54 ± 0.43 fl). The Angus cattle were the only group to display an increase in mean cell haemoglobin concentration of 0.10 ± 0.45 g/dl, which differed from the 0.72 ± 0.46 g/dl decrease observed in the Nguni group.

Breed differences in differential leukocyte changes

The Least Square means for all changes in leukocyte percentages and absolute numbers are contained in Table 3. There was an increase of 8 ± 11.77% in segmented neutrophils in the

Brahman, which differed significantly from the 3.67 ± 4.86% decrease in the Angus cattle. In addition, there was an increase of 2.67 ± 5.12% in lymphocytes in the Angus, whereas a decrease of 5.175 ± 12.42 and 9.08 ± 17.53% was recorded for the Brahman and Nguni, respectively. An increase of 2.08 ± 2.47% in monocytes was observed in Angus cattle and it differed significantly ($p<0.01$) from the 1.875 ± 2.62% decrease and 1.55 ± 3.29% increase displayed by the Brahman and Nguni cattle, respectively. Similar changes were also noted in the absolute number of leukocytes.

Breed differences in serum biochemistry changes

Table 4 presents changes in serum biochemistry parameters and shows that the Brahman cattle displayed a 0.65 ± 1.81 g/l decrease in albumin levels, which is significantly lower ($p<0.01$) than the 1.67 ± 1.23 g/l decrease observed in the Angus cattle. An increase of 3.28 ± 2.22 g/l in serum globulin was observed in the Brahman group, which was significantly higher ($p<0.01$) than the 0.98 ± 2.19 and 0.77 ± 1.78 g/l increases for the Nguni and Angus cattle, respectively. There was an increase in alanine amino-transferase levels in the Brahman (12.47 ± 9.94 U/l), whereas a lower increase was observed in the Nguni (0.98 ± 2.19 U/l) and a decrease was recorded in the Angus (-3.75 ± 5.99 U/l). The Angus cattle displayed the largest decrease in alkaline phosphatase levels of 40.08 ± 15.27 U/l compared to the 0.67 ± 36.98 and 24.08 ± 44.3 U/l decrease observed in the Nguni and Brahman, respectively. The Nguni cattle had the largest change in uric acid levels with a 12.63 ± 10.05 µmol/l decrease, whereas the Brahman and Angus uric acid changes were -1.53 ± 9.47 and -3.12 ± 6.30 µmol/l, respectively. Blood urea nitrogen only increased in the Angus (0.15 ± 0.95 mmol/l) as opposed to the decrease observed in the Brahman (1.11 ± 0.39 mmol/l) and Nguni (0.16 ± 1.20 mmol/l). There was an increase in cholesterol levels in the Angus cattle of 0.53 ± 0.45 mmol/l that was significantly different from the 0.10 ± 0.19 mmol/l increase displayed by the Nguni.

Tick type differences in immunological parameter changes

No significant differences between tick types were observed for changes in haematological and leukocyte percentages and absolute amounts following infestation. There were differences between the two tick species observed in the changes in some biochemistry parameters. Cattle infested with *R. microplus* had a significantly higher increase in serum globulin levels of 2.77 ± 2.28 g/l than their *R. decoloratus*-infested counterparts (0.58 ± 1.82 g/l). Animals infested with *R. microplus* ticks also showed a significantly larger increase in cholesterol levels at 0.49

± 0.38 mmol/l as compared to the *R. decoloratus*-infested cattle which displayed an increase of 0.21 ± 0.33 mmol/l.

Data normalisation through selection of suitable reference genes

Four reference genes were chosen: *RPLP0, RN18S1, GAPDH* and *LOC616410*. The average C_T values for the reference genes were 24.153, 15.717 and 25.399 for *RPLP0, RN18S1* and *GAPDH*, respectively. The least variably expressed gene was *RN18S1* with a C_T value range of 7.75 and an average pre- and post-infestation C_T value difference of 1.575. On the other hand, reference gene *GAPDH* was the most variably expressed, where the C_T value range and average pre- and post-infestation C_T values difference were 10.089 and 3.881, respectively. As a result of the other reference genes yielding values significantly above the RT-qPCR assay manufacturer's recommendation of 1.5 in the average difference of the control and test C_T values, *RN18S1* was selected as the most stable and only reference gene.

Expression levels

No significant interaction between the main effects, breed and tick species, was observed for any of the genes. However, a Bonferroni pairwise comparison for the interaction between breed and tick species revealed a significant difference in the Least Square (LS) means of treatment Nguni-*R. decoloratus* (LS = 9.372) and Angus-*R. microplus* (LS = -0.405). Although the expression of the majority of the genes did not differ significantly according to breed, the expression profiles of genes *TRAF6*, *TBP*, *LUM* and *B2M* were significantly different according to breed. A Bonferroni pairwise comparison combined with a one-way ANOVA of the breed types (Fig. 1) revealed significant differences between the Nguni and Angus for *TBP* ($P = 0.008$) and *TRAF6* ($P = 0.016$), as well as between the Brahman and Angus for *LUM* ($P = 0.003$) and *B2M* ($P = 0.007$). None of the genes differed significantly for the main effect tick species as indicated in the pairwise test in Fig. 2.

Table 5 presents the patterns of gene expression following challenge with ticks. There were increases in the expression levels of six genes (*CCL2*, *CCL26*, *CD14*, *OGN*, *LUM*, and *B2M*) post-infestation for all breed × tick species treatment groups. Five genes (*CCR1*, *TLR5*, *TRAF6*, *TBP*, *BDA20*) increased expression or remained approximately equal after infestation with ticks for all groups. Conversely, completely mixed results were obtained in the breed × tick species groups for expression levels for the genes *IL-1β*, *TLR7*, and *TLR9*, whereas the expression levels of three genes (*CXCL8*, *IL-10*, *TNF-α*) decreased or remained the same after tick challenge in all breed × tick species groups. In the treatment group Angus-*R. decoloratus*,

genes *IL-1β, IL-10, CCL2, CCL26, CCR1, TLR5, TLR7, CD14, TRAF6, OGN, TBP, LUM* and *B2M* were upregulated following infestation. Treatment groups Brahman-*R. decoloratus*, Nguni-*R. decoloratus* and Nguni-*R. microplus* mostly exhibited similar expression patterns to the Angus-*R. decoloratus* group. The exception was that genes *IL-10* and *TLR7* were downregulated in the Brahman-*R. decoloratus* group, whereas *B2M* was downregulated in the Nguni-*R. decoloratus* and Nguni-*R. microplus* groups. Treatment group Angus-*R. microplus* had genes *CCL2, CCL26, CCR1, TLR5, CD14, OGN, LUM, B2M* and *BDA20* upregulated. An expression pattern similar to that of Angus-*R. microplus* group was observed in the Brahman-*R. microplus* group with the only difference being that gene *CCR1* was downregulated, whereas *TRAF6* and *TBP* were upregulated in the latter group. The Angus-*R. microplus* group produced the minimum expression values for most of the genes of interest, whereas the Nguni groups produced maximum values for most of the genes with no specification of the tick species.

DISCUSSION

Haematology

Blood parameters are known to differ among breeds (Claxton and Ortiz 1996). The Angus cattle had the greatest increase in RBC, PCV and Hb in response to tick infestation compared to the Brahman and Nguni. Increases in red blood cell count and haemoglobin can be a response to infection (O'Kelly and Seifert 1970; Kocatürk 2010), whereas a decrease can be due to anaemia as a result of blood loss (Kim et al. 2017). Certain studies have, however, shown the ticks' influence on growth rate to be beyond the removal of blood (Rechav et al. 1980), which suggests a level of toxicity that accompanies the parasitic excretions during infestation. In the current study, the tick larvae were pathogen-free and post-infestation samples were collected at an early time point, when anaemia can be ruled out. Thus, the increases may be attributed to the animals' immune systems conditioned to respond to tick bites rather than the actual infection by disease-causing agents in ticks. The Angus had the higher increase in MCV than the Nguni breed. Both breeds had values in the lower end of the 40 to 60 fl range considered normal for cattle (Schalm et al. 1975), whereas it decreased in the Brahman that had lower MCV values. These observations are consistent with Piper et al. (2009), where MCV values for Brahman cattle were lower compared to British breeds. The breeds studied may be considered having microcytic hypochromic anaemia associated with an iron deficiency (Schalm et al. 1975) as they had similar levels of mean corpuscular haemoglobin concentrations.

The Angus generally had different trends from the other breeds for segmented neutrophils, lymphocytes, monocytes and eosinophils. Previous studies reported differences in leucocyte levels among breeds, where periods of infestation were longer with a higher infestation intensity (Piper et al. 2009; Rechav et al. 1990). Post infestation, both the Brahman and the Nguni displayed circulating neutrophil levels that could be classified as neutrophilia (>4.6 × 10^9/L) (Schalm et al. 1975), whereas the Angus was the only breed to display high levels of lymphocytes coupled with a decrease in neutrophil levels post-infestation. Leucocyte levels observed within circulation is directly proportional to its demand in tissues (Young et al. 2006). However, the neutrophilic response is initially masked as both the currently circulating and newly formed neutrophils are attracted to the formation of an acute inflammatory lesion (Schalm et al. 1975). With a sampling time point as early as 12 h post infestation with pathogen-free larvae, a higher level of circulating leukocyte values would be regarded as most likely reflecting an increased demand in tissue. Results suggesting that susceptible British breeds undergo a larger exodus of neutrophils from circulation agree with infestation site histology studies that have identified greater amounts of the leukocytes in susceptible cattle (Wada et al. 2010; Marufu et al. 2014).

The levels of circulatory eosinophils observed suggest that they play a role in the infestation response as they are associated with parasitic infestations (Francischetti et al. 2009). It should be noted, however, that the cellular composition within the circulation might not be a direct reflection of the local parasitic response, especially at low intensities of infestation. The differences in neutrophil levels were observed in breeds that proved susceptible in this trial and the contribution of eosinophils to the manifestation of resistance is still uncertain. There were significant differences in the lymphocytes, although clear conclusions may be difficult as differentiation among the cell subsets was not possible. Earlier studies found positive T-cell involvement regarding responses to infestation in *Bos indicus* cattle (Piper et al. 2009; Constantinoiu et al. 2010). Simultaneously, high antibody titres have been reported in tick-susceptible animals (Fivaz et al. 1991; Schorderet and Brossard 1993; Piper et al. 2016), which is a B-cell mediated response. This corresponds with the results of the present study, where the susceptible Angus displayed an increase in lymphocyte following infestation in comparison to the Nguni and Brahman breeds. The observations within the differential leukocyte values allow for distinction between the breeds regarding parameters that relate to their immunological responses. Assessment of local tick-host interfaces may be needed to enlighten the role of individual leukocytes. The individual involvement of leukocytes at infestation sites requires confirmation through histology site assessments.

Serum biochemistry

There were general increases in levels of globulin, gamma glutamyl-transferase, cholesterol, creatine kinase and lactate dehydrogenase across all the breeds. On the other hand, decreases were observed in alkaline phosphatase and uric acid. The Angus had different trends from other breeds for alanine amino transferase, bilirubin total and BUN. Albumin decreases are common in infected animals and are associated with a compensatory mechanism for globulin increases to maintain osmotic pressure (Dimopoullus 1963; Rechav et al. 1991, 1992). O'Kelly et al. (1970) reported albumin decreases due to *R. microplus* infestation on Hereford steers and suggested that the ticks have a specific toxic effect on the liver's ability to produce albumin. Differences in the current study, may be due to decreased levels of protein consumption in the susceptible animals as the anorectic effect and direct effect of infestation could not be separated. Given that the change in globulin and albumin profiles is a non-specific reaction to parasitic infestation (Herlich and Merkal 1963; Banerjee et al. 1990), it is interesting to note that *R. microplus*-infested cattle produced higher globulin levels than *R. decoloratus*-infested cattle. It can be concluded that success rate of the tick is much higher and would have had an influence despite the early time point. The difference might thus be attributed to a higher level of infesting ticks and their associated secretions (Francischetti et al. 2009) rather than a more severe reaction to an individual tick. If this were the case, however, it could have been expected that more of the parameters would differ significantly between tick species.

The increased level of blood urea nitrogen (BUN) in the Angus group post-infestation suggests some extent of altered protein metabolism compared to the Brahman group. Increased levels of BUN could be related to the increased catabolism of serum proteins, which is supported by the lower albumin level in the Angus (Khan et al. 2011). Similar results were reported for British Hereford cattle after experimental infection with *Trypanosoma congolense* (Wellde et al. 1974). Severe kidney or liver malfunction is unlikely as such conditions would have very likely been reflected in the serum creatinine or alkaline phosphatase levels. Although clear differences between the Brahman and Angus breeds in circulating ALT levels were observed, these may indicate muscle damage (Boyd 1988; Khan et al. 2011; Shahnawaz et al. 2011), as ALT is not a good indication of liver damage (Stogdale 1981). This might not be applicable to this study, however, as Brahman, which displayed the highest levels of ALT, was not subjected to significant tissue trauma given the low infestation intensity. The functional relationship between ALT and the animals' response

to infestation is unclear.

The greatest increase in cholesterol was observed in the Angus and was lowest in the Nguni. This is different from observations by O'Kelly et al. (1988), where there were decreases in cholesterol levels of Afrikaner and British breeds infested with *R. microplus*. Parasitic infection influences lipid levels; hence, it is expected to affect blood cholesterol (Bansal et al. 2005; Khan et al. 2011). Thus, O'Kelly (1968) suggested cholesterol levels as an index for resistance after reporting a significant inverse correlation with tick counts. The current results also suggest a functional relationship between cholesterol levels and tick resistance. The factors that determine the relationship between an animal's total cholesterol level and its susceptibility to ticks, however, remain unknown. Hypothyroidism is often associated with increases in cholesterol levels and decreased levels of thyroid activity is associated with parasitism (Ogwu et al. 1992; Shahnawaz et al. 2011). In the current study, however, the different levels may be a consequence of infestation due to ticks' influence on diet and liver function. Furthermore, blood cholesterol may play a role in an animals' ability to respond to an external/foreign threat. One of the functional roles of cholesterol includes the maintenance of specific cell membrane 'lipid-rafts' responsible for enabling cell transduction cascades (Simons and Toomre 2000), allowing for the processing of certain stimuli and cellular function. Further investigation into the role of cholesterol during infestation is necessary.

Tick species differences

Host-tick interactions in cattle, following *Rhipicephalus* (*Boophilus*) tick challenge, have resulted in an array of complex differential gene expression profiles. No significant tick-species related differences were observed in the immunological responses and gene expression patterns of all the genes of interest in the cattle infested with either the *R. microplus* or the *R. decoloratus* ticks. These two tick species are both from the subgenus *Boophilus* (The Center for Food Security and Public Health 2007) and have been shown to share numerous morphological characteristics (Jongejan and Uilengberg 2004). These might explain the lack of differences in their feeding signatures which subsequently resulted in gene expression profiles in the hosts that were indistinguishable from each other between these two species. Although there is a lack of literature on the comparative genetics of the two species, a lot of work has been undertaken towards the sequencing of the genomes of the *Rhipicephalus* ticks (Willadsen 2006). Studies have indicated that the wide variety of bioactive molecules contained in the tick's saliva may contain partially characterised immune-active proteins and

lipids that induce vasodilatory, anti-haemostatic and immunomodulatory activities, to facilitate successful feeding (Wikel 1996, 1999; Francischetti et al. 2010; Oliveira et al. 2010). Consequently, numerous candidate genes are differentially expressed and pathways are activated in the host animal in an attempt to re-establish homeostasis. Therefore, the feeding signatures of different tick species, as characterised by the differential gene expression profiles in the host, would be expected to differ even among species with shared morphological characteristics.

Breed differences (gene expression)

Piper et al. (2008) and Wang et al. (2007) support the data observed in the current study which showed significant differences in the expression profiles among breeds. All the significantly higher fold regulation values were observed within the high and medium resistance breed groups, the Brahman and Nguni, respectively. Apart from genes *TLR7*, *CXCL8* and *TNF*, none of the genes of interest were downregulated within the Nguni treatment combination groups. Conversely, all but two (*CCR1* and *CD14*) of the genes of interest produced their lowest expression values and were often downregulated within the Angus-associated treatment groups, predominantly the Angus-*R. microplus* group. Piper et al. (2008) reported contrasting results, where the low-resistance animals had indications of high levels of inflammation. It was apparent in the current study that lower-resistance cattle breeds had reduced inflammatory responses. This contradicts the perception that the increased level of tick resistance observed in tick-resistant animals is characterised by unique gene expression profiles rather than inflammatory responses.

Expression levels

The results of this study were broadly consistent with previous work (Piper et al. 2008, 2010; Wang et al. 2007). The genes encoding the extracellular matrix constituents, most importantly *LUM* and *B2M*, were upregulated at much higher levels in the high (Brahman) and intermediate (Nguni) resistance breeds than the genes involved in immune system regulation and inflammatory responses. This was consistent with the results by Piper et al. (2010), where a microarray study showed upregulation of genes encoding constituents of the extracellular matrix in the tick-resistant Brahman cattle in comparison to the susceptible Holstein-Friesian cattle. Furthermore, Kongsuwan et al. (2010) highlighted the importance of the epidermal permeability barrier of the skin as an important component of resistance in cattle against ticks. This explains the heightened expression of these genes in the tick-resistant Brahman cattle. Of

the four genes that displayed significant between-breed differential expression patterns, *LUM*, *B2M* and *TBP* induced tick resistance not by initiating host immune responses, but rather by promoting continued cellular regeneration, tissue repair and detoxification of the tick bite site. This activated the mechanism required to discourage long term supply of blood meal to the tick. These genes, except *TBP*, were upregulated within most treatment combination groups.

The highest upregulation values were detected for *LUM* within treatment groups Brahman-*R. microplus*, Brahman-*R. decoloratus* and Nguni-*R. microplus.* As a gene that encodes a member of the small leucine-rich proteoglycan (Weizmann Institute of Science 2016b), *LUM* serves in conjunction with *OGN* to induce immune responses. Gene *OGN* similarly presented higher upregulation values than the rest of the genes of interest. Both *LUM* and *OGN* are capable of regulating fibril organisation and circumferential growth as well as epithelial cell migration in the process of tissue repair at the tick bite site (Weizmann Institute of Science 2016b). The significantly high expression level of *LUM* in the Brahman animals more than the Angus suggested that the Brahman had a stronger capacity to prevent tick feeding through continuous tissue repair than Angus animals did. The gene *LUM* therefore shows potential as a biomarker for superior host resistance to both *R. microplus* and *R. decoloratus* tick species in these cattle breeds.

Unlike *LUM,* the significant differences in the expression levels of *TBP* between the Nguni and Angus treatment group were unexpected. *TBP* is a component of the RNA polymerase III and is expected to be a housekeeping gene exhibiting stable expression levels in all treatment combinations to facilitate continued cell growth; hence tissue repair regardless of the biological or environmental conditions (Vannini and Cramer 2012). Although *TBP* was upregulated in all treatment groups, it displayed a downregulated but stable expression level in group Angus-*R. microplus.* It is, however, evident that under stressful conditions, such as those inflicted by tick infestations, the regulatory protein *Maf1* may repress RNA polymerase III activity (Vannini et al. 2010). This may explain the downregulation of *TBP* in treatment group Angus-*R. microplus,* which in turn resulted in significant differences in expression levels between the Nguni-*R. decoloratus* and Angus-*R. microplus* treatment combinations.

Although *B2M* has often been identified among housekeeping genes, it was differentially expressed at significantly different levels between the Brahman and Angus treatment groups. This gene was upregulated in all the treatment groups, with much higher expression levels in the Brahman treatment groups. It is a component of the MHC class I that is responsible for presenting peptide antigens (including tick antigens) to the immune system while simultaneously forming amyloid fibrils in pathological challenges (Weizmann Institute of

Science 2016a). Therefore, the significantly low *B2M* expression levels produced by the Angus animals may suggest that this breed's nucleated cells had a poor capacity to detect the tick antigens to prompt host immune responses. Toll-like receptors have been implicated as key role-players in a myriad of immune functions correlated to their ability to differentially express and initiate appropriate immune responses to various pathogenic invasions at the earliest stage of immune development (Kopp and Medzhitov 1999; Menzies and Ingham 2006). It is widely understood that toll-like receptors vary in abundance in response to the host's altered immune responsiveness upon detection pathogen-associated molecular patterns (PAMP) (Menzies and Ingham 2006). Evidently, in the current study *TRAF6* showed significantly different expression patterns in Nguni in comparison to the Angus treatment groups. *TRAF6* is the only one within the *TRAF* family of proteins known to participate in signalling via *Toll/IL-1* receptors. *TRAF6* is activated by *IL-1β* mediated stimuli (Kopp and Medzhitov 1999), which explains the recognisable shared expression patterns observed between the two genes of interest. *TRAF6* was under-expressed, producing significantly lower fold regulation values in the Angus-*R. microplus* group.

Conclusions

This study showed that there is variation among the three cattle breeds in resistance to ticks and no differences in resistance to the two tick species as they share many morphological characteristics. There were, however, no tick species × breed interactions observed, which suggests resistance to the two tick species was consistent in all three breeds. The biochemistry parameters provided insights into the metabolic function of the animals' different systemic responses despite a very early time point, when blood loss could be ruled out. The functional differences can thus only be responses to the components of early infestation, such as the immunomodulatory excretions found in tick saliva. There was no indication of severe hepatic or renal malfunction, but analysis suggested altered protein metabolism, possibly decreased production or increased breakdown. Biochemical results also contributed to evidence of a functional relationship of tick infestation with blood cholesterol. Both on a systemic and cutaneous level, the study showed that the effect of ticks, as well as the hosts' responses to it, is already visible at very early time points. The Brahman had a stronger capacity to prevent continued tick feeding through continuous tissue repair unlike the Angus, in which the gene *LUM* showed diminished expression levels. Therefore, *LUM* shows potential as a biomarker for tick resistance in cattle. Genes that showed variation are involved in discouraging long-term supply of blood meal to the tick and those associated with immune responses.

The identification of biomarkers that correlate to tick resistance could improve the sensitivity of prediction tools, subsequently impacting the rational approach to developing novel, chemical-free tick control strategies. Additional biomarkers are necessary to improve the accuracy of selection programmes and predictive tests for tick-resistant cattle.

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Figure captions

Fig 1 Least Square means, using fold regulation, between day 0 pre-infestation and 12-h post-infestation, as a measure of the expression levels of 17 genes of interest in Angus, Brahman and Nguni cattle following tick infestations.

Fig 2 Comparison of Least Square means of genes expressed in Angus, Brahman and Nguni cattle infested with *Rhipicephalus microplus* and *R. decoloratus.*

Table 1 Description of the 17 genes of interest and their gene product functions

Gene Symbol	Gene name	RefSeq Number		Function of gene product
		UniGene	GenBank	
IL-1β	Interleukin 1, beta	Bt. 4856	NM_174093	Pleiotropic; pro-inflammatory
CXCL8	Interleukin 8	Bt.49470	NM_173925	Chemo-attractant for effector blood cells
IL-10	Interleukin 10	Bt.4723	NM_174088	Anti-inflammatory
CCL2	Chemokine (C-C motif) ligand 2	Bt.2408	NM_147006	Recruitment and activation of immune effector cells; inflammatory response
CCL26	Chemokine (C-C motif) ligand 26	Bt.23451	NM_001205635	Recruitment and activation of immune effector cells; inflammatory response
CCR1	Chemokine (C-C motif) receptor 1	Bt.62596	NM_00107739	Recruitment of immune effector cells to site of inflammation
TLR5	Toll-like receptor 5	Bt.66307	NM_001040501	Pathogen recognition and activation of innate immunity
TLR7	Toll-like receptor 7	Bt.111931	NM_001033761	Pathogen recognition and activation of innate immunity
TLR9	Toll-like receptor 9	Bt.12810	NM_183081	Pathogen recognition and activation of innate immunity
CD14	Cluster of differentiation 14	Bt.4285	NM_174008	Confers lipopolysaccharide sensitivity to neutrophils, monocyte & macrophages
TRAF6	TNF receptor-associated factor 6	Bt.9201	NM_001034661	Mediates signal transduction from the TNF receptor family
TNF-α	Tumor necrosis factor – alpha	Bt.12756	NM_173966	Cell signalling protein (cytokine) involved in systemic inflammation
OGN	Osteoglycin	Bt.5341	NM_173946	Corneal keratan sulfate proteoglycan; regulates collagen fibrillogenesis in skin
TBP	TATA box binding protein	Bt.22662	NM_001075742	General transcription factor
LUM	Lumican	Bt.2452	NM_173934	Collagen fibril organization; epithelial cell migration; tissue repair
B2M	Beta-2-micropglobulin	Bt.64557	NM_173893	Formation of amyloid fibrils in some pathological conditions; presentation of peptide antigens to the immune system
BDA20	Bovine dander allergen 20	Bt.550	NM_174761	Weak inducer of both humoral and cellular responses

Table 2 Change in haematological parameters calculated by the difference between sampling time points (post-infestation – pre-infestation).

Parameter	Brahman	Nguni	Angus	P
Δ Red blood cell count (× 10^{12}/l)	0.19 ± 0.39	0.57 ± 0.60	0.77 ± 0.46	0.034
Δ Haemoglobin (g/dl)	0.17 ± 0.47	0.7 ± 0.79	1.14 ± 0.56	0.006
Δ Packed cell volume (%)	0.61 ± 1.60	2.78 ± 2.40	3.53 ± 1.90	0.007
Δ Mean cell volume (fl)	-0.06 ± 0.25	0.54 ± 0.43	0.17 ± 0.44	0.012
Δ Red cell distribution width (%)	0.28 ± 0.52	0.34 ± 0.74	0.45 ± 1.13	0.914
Δ Mean cell haemoglobin cons. (g/dl)	-0.13 ± 0.89	-0.72 ± 0.46	0.10 ± 0.45	0.026
Δ White cell count (× 10^9/dl)	-0.07 ± 1.23	0.55 ± 4.34	0.99 ± 1.38	0.688
Δ Platelets (× 10^9/dl)	143.03 ± 147.57	-78.83 ± 135.67	98.79 ± 179.52	0.009

P-values are based on Bonferroni pairwise test for Least Square (LS) means

Table 3 Change in differential leukocyte percentages calculated by the difference between sampling time points (post-infestation – pre-infestation).

Leukocyte	Brahman	Nguni	Angus	P
Δ Segmented neutrophils (%)	8 ± 11.77	1.45 ± 9.27	-3.67 ± 4.68	0.036
Δ Lymphocytes (%)	-5.175 ± 12.42	-9.08 ± 17.53	2.67 ± 5.12	0.14
Δ Monocytes (%)	-1.875 ± 2.62	1.55 ± 3.29	2.08 ± 2.47	0.007
Δ Eosinophils (%)	-0.7 ± 1.32	0.48 ± 2.73	0.79 ± 1.14	0.254
Δ Segmented neutrophils (abs × 10^9/l)	0.84 ± 1.71	0.68 ± 2.68	-0.27 ± 0.71	0.386
Δ Lymphocytes (abs × 10^9/l)	-0.66 ± 2.28	-1.80 ± 2.71	0.98 ± 1.15	0.035
Δ Monocytes (abs × 10^9/l)	-0.25 ± 0.39	0.33 ± 0.70	0.45 ± 0.34	0.023
Δ Eosinophils (abs × 10^9/l)	-0.11 ± 0.19	0.11 ± 0.38	0.14 ± 0.15	0.129

P-values are based on Bonferroni pairwise test for Least Square (LS) means

Table 4 Breed-specific changes in serum biochemistry parameters calculated by the difference between sampling time points (post infestation – pre-infestation).

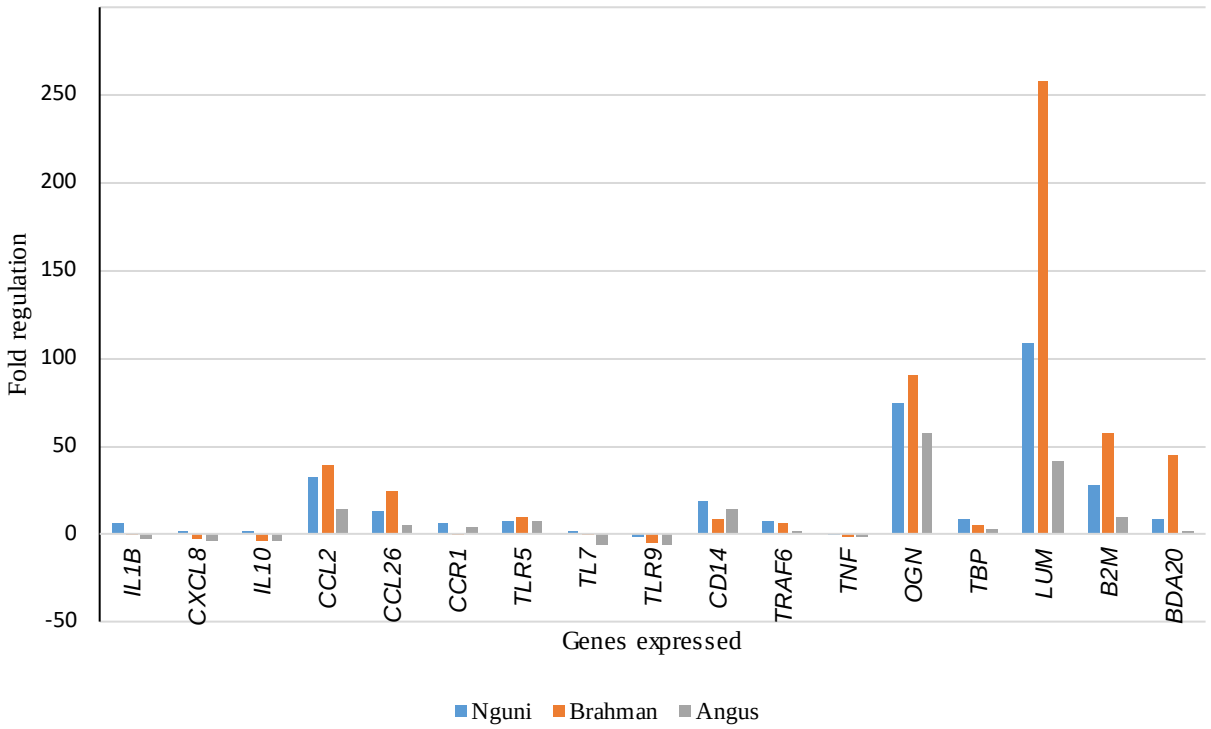
Parameter	Brahman	Nguni	Angus	P
Δ Albumin (g/l)	0.65 ± 1.81	-0.33 ± 1.44	-1.67 ± 1.23	0.0025
Δ Globulin (g/l)	3.28 ± 2.22	0.98 ± 2.19	0.77 ± 1.78	0.0033
Δ Alanine Amino-Transferase (U/l)	12.47 ± 9.94	0.58 ± 4.81	-3.75 ± 5.99	<0.0001
Δ Alkaline Phosphatase (U/l)	-24.08 ± 44.31	-0.67 ± 36.98	-40.08 ± 15.27	0.036
Δ Gamma Glutamyl-Transferase (U/l)	6.22 ± 8.64	0 ± 12.075	8.08 ± 17.01	0.280
Δ Uric Acid (µmol/l)	-1.53 ± 9.47	-12.63 ± 10.05	-3.12 ± 6.30	0.0069
Δ Bilirubin Total (µmol/l)	-1.92 ± 5.67	-0.58 ± 8.51	0.25 ± 7.42	0.788
Δ Cholesterol (mmol/l)	0.45 ± 0.32	0.10 ± 0.19	0.53 ± 0.45	0.006
Δ Creatine Kinase (U/l)	1037.1 ± 2165.19	512.92 ± 1185.58	299.08 ± 1191.65	0.540
Δ BUN (mmol/l)	-1.11 ± 0.39	-0.16 ± 1.20	0.15 ± 0.95	0.0069
Δ Creatinine (µmol/l)	-4.8 ± 14.22	4.92 ± 7.17	0.17 ± 5.79	0.067
Δ Lactate Dehydrogenase (U/l)	1117.83 ± 1404.97	977.83 ± 1358.66	1034.83 ± 673.258	0.963
Δ Fibrinogen (g/l)	-2.08 ± 2.37	1.35 ± 2.03	0.52 ± 2.91	0.0085

P-values are based on Bonferroni pairwise test for Least Square (LS) means

Table 5 Relative change in expression for 17 genes of interest in each of the six treatment groups. Data are presented as arrows according to the magnitude of the normalised fold regulation values as follows: ≥2, ↑; ≥10, ↑↑; ≥100, ↑↑↑; ≤–2, ↓; ≤–10, ↓↓; ≤–100, ↓↓↓; |fold regulation| <2, ↔. Rows are coloured according to whether all groups showed an increase or equivalence of expression (light green) or a decrease or equivalence of expression (red/yellow) or were inconsistent (grey).

	Angus-*R. decoloratus*	**Angus-*R. microplus***	**Brahman-*R. decoloratus***	**Brahman-*R. microplus***	**Nguni-*R. decoloratus***	**Nguni-*R. microplus***
IL1β	↔	↓	↔	↔	↑	↑
CXCL8	↓	↔	↓	↓	↔	↔
IL10	↔	↓	↔	↓	↔	↑
CCL2	↑	↑↑	↑↑	↑↑	↑↑	↑↑
CCL26	↑	↑	↑↑	↑↑	↑	↑↑
CCR1	↑	↑	↔	↔	↑	↑
TLR5	↑↑	↔	↑↑	↑	↑	↑
TLR7	↔	↓↓	↔	↔	↔	↑
TLR9	↓	↓↓	↓	↓	↓	↔
CD14	↑↑	↑↑	↑	↑	↑↑	↑↑
TRAF6	↑	↔	↑	↑	↑	↑
TNFA	↔	↓	↔	↓	↔	↔
OGN	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑
TBP	↑	↔	↑	↑	↑	↑
LUM	↑↑	↑↑	↑↑↑	↑↑↑	↑↑	↑↑↑
B2M	↑	↑↑	↑↑	↑↑	↑↑	↑↑
BDA20	↔	↑	↑↑	↑↑	↑	↑

Key	All increased	All decreased	All increased or no change	All decreased or no change	Mixed Inconsistent	Increased relative to other breeds	Decreased relative to other breeds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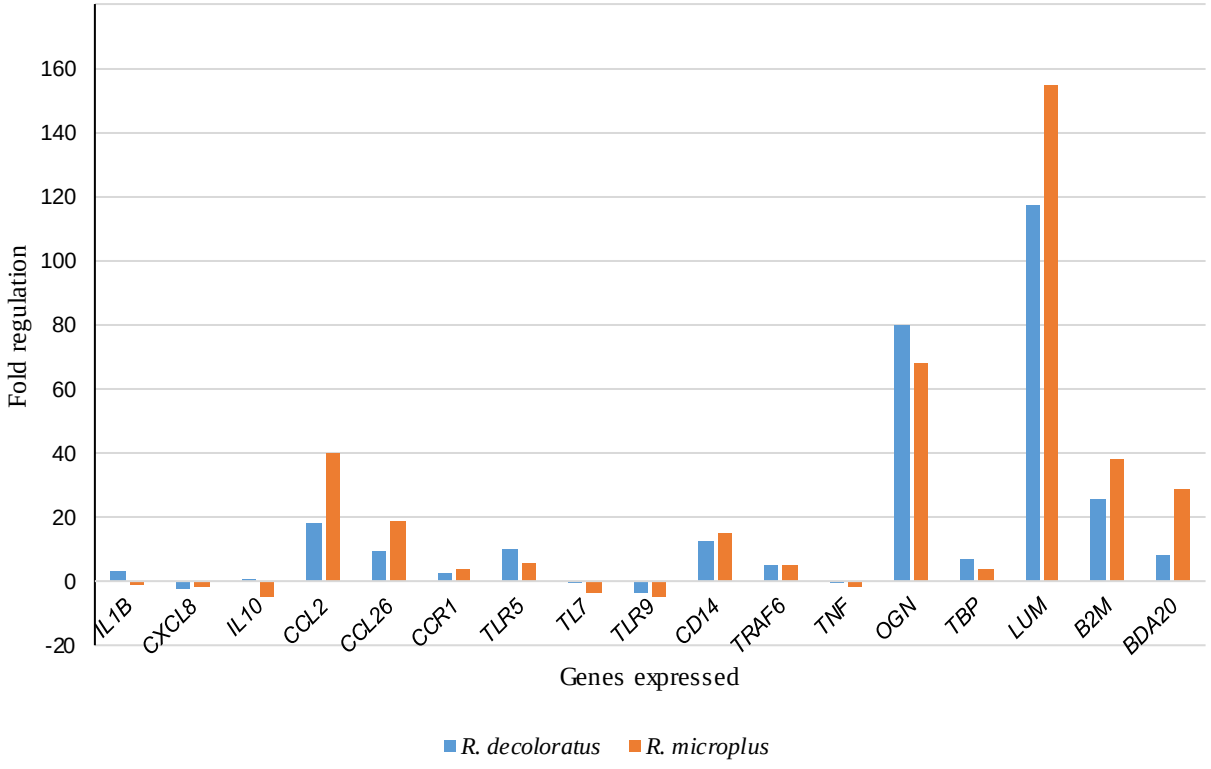
200
150
100
50
0
-50
IL1B
CXCL8
IL10
CCL2
CCL26
CCR1
TLR5
TL7
TLR9
CD14
TRAF6
TNF
OGN
TBP
LUM
B2M
BDA20
Genes expressed
Nguni
Brahman
Angus

Fig 1


Fold regulation
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140
120
100
80
60
40
20
0
-20
IL1B
CXCL8
IL10
CCL2
CCL26
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TLR5
TL7
TLR9
CD14
TRAF6
TNF
OGN
TBP
LUM
B2M
BDA20
Genes expressed
R. decoloratus
R. microplus

Fig 2