TITLE: Differences in leukocyte profile, gene expression, and metabolite status of dairy cows with or without sole ulcers

AUTHORS: Keelin O'Driscoll,Matthew McCabe, Bernadette Earley

1

Interpretive summary

Sole ulcers affect dairy cow immune status *by O'Driscoll et al*

Dairy cows afflicted with sole ulcers are often clinically lame, and experience pain and impaired welfare. However little is known of the cows' stress and immune response to sole ulcers. This study demonstrated that cows that are affected with sole ulcers, and clinically lame, had a different leukocyte gene expression profile to healthy cows. They also had higher concentrations of the stress hormone, cortisol, the endogenous precursor steroid hormone – DHEA, and the various metabolites that are suggestive of systemic inflammation and stress.

Differences in leukocyte profile, gene expression and metabolite status of dairy cows with or without sole ulcers

Keelin O'Driscoll, Matthew McCabe and Bernadette Earley

Animal and Bioscience Research Department, Animal and Grassland Research and Innovation Centre, Teagasc, Dunsany, Co. Meath, Ireland

Corresponding author: Keelin O'Driscoll, Tel: 353-25-42290; Fax: 353 25 42340; email: keelin.odriscoll@teagasc.ie

ABSTRACT

Sole ulcers are one of the most severe lameness causing pathologies for dairy cows and are associated with abnormal behaviour and impaired production performance. However, little is known about how or whether lameness caused by sole ulcers affects the cow systemically. This study compared haematology profile, leukocyte gene expression, and physiological responses (metabolite, cortisol, the endogenous steroid hormone – DHEA, and haptoglobin concentrations) of cows with sole ulcers and healthy cows. Twelve clinically lame cows (LAME) were identified as having at least one sole ulcer, and no other disorder and matched with a cow that had good locomotion and no disorders (SOUND), using days in milk, liveweight, body condition score and diet. Blood samples were taken from all 24 cows within 24h of sole ulcer diagnosis. Leukocyte counts were obtained using an automated cell counter, cortisol and DHEA concentration by ELISA, and plasma haptoglobin (Hb), urea, total protein, creatine kinase and glucose were analysed on an Olympus analyser. Expression of 16 genes associated with lameness or stress were estimated using qRT-PCR. Data were analysed using the Mixed procedure in SAS (v 9.3). LAME cows had a higher neutrophil %, a numerically lower lymphocyte %, and tended to have a higher neutrophil:lymphocyte ratio than SOUND. Serum cortisol and DHEA concentrations were higher in LAME than in SOUND cows. LAME cows also tended to have higher haptoglobin and glucose levels than SOUND, as well as higher protein yet lower urea levels. SOUND cows tended to have higher relative expression of the gene coding for colony stimulating factor 2 than LAME, but in all other cases where differences were detected in cytokine gene expression (IL-1α, IL-1β, CXCL8 and IL-10), relative gene expression in SOUND cows tended to be, or was, lower than in LAME. Relative expression of MMP-13, GR-α, Fas, haptoglobin and CD62L were, or tended to be, higher in LAME than SOUND cows. A high neutrophil:lymphocyte ratio in combination with higher cortisol levels in cows with ulcers is indicative of physiological stress. Moreover, increased DHEA and a higher cortisol:DHEA ratio, as well as a tendency for higher haptoglobin levels and increased

haptoglobin mRNA expression are indicative of systemic inflammation. Increased cytokine mRNA expression indicates activation of the immune system compared with healthy cows. Increased expression of MMP13 mRNA has been found in cows with impaired locomotion and thus could be implicated in development of claw horn disorders.

Keywords: lameness, sole ulcer, immune, gene expression, stress

INTRODUCTION

Lameness is the most serious welfare problem facing both the dairy cow, and the European dairy industry (EFSA, 2009). The disorder causes pain and distress for the cow (Whay et al., 2003) and substantial economic losses (Kossaibati and Esslemont, 1997). Sole ulcers, which are one of the most severe lameness causing pathologies, are non-infectious in nature, and occur when claw horn formation is disrupted. The underlying tissue then becomes inflamed and sometimes exposed (Murray et al., 1986). Cows are particularly vulnerable around the time of parturition; changes in hormone levels cause increased vascular permeability, increasing the risk of oedema and ischaemia in the hoof, (Tarlton et al., 2002; Nocek, 1997; Knott et al., 2007), while concurrent weakening of connective tissue causes the pedal bone to drop and compress the corium, further disrupting claw horn formation (Thoefner et al., 2005).

Lameness is associated with behavioural and physiological changes indicative of a sickness response (Whay et al., 1997; Almeida et al., 2008), which facilitates host survival during illness and tissue injury (Hart, 1988). The sickness response is regulated by immune-to-brain communication initiated by pro-inflammatory cytokines released by activated immune cells (Watkins and Maier, 1999). Thus lameness likely causes systemic activation of the immune system.

Using a microarray-based gene expression profiling of peripheral blood mononuclear cells, Almeida et al. (2007) identified a small number of genes that the authors hypothesised could be differentially expressed specifically as a response to lameness. However, use of a whole leukocyte population approach, rather than focusing on particular cell types, could provide a broader picture of immunological interactions (Bleavins et al., 2011). Immune system genes are often expressed by more than one leukocyte cell type, and thus their overall expression level in response to lameness could be more accurately represented by examining combined leukocyte populations.

As well as differences in gene expression, there are several other physiological responses that could be expected as a response to lameness. For instance, Almeida et al. (2008) found that

lame cows had a tendency for higher serum cortisol levels, but lower DHEA levels, compared with healthy cows. They also had a higher cortisol:DHEA ratio, which is often used as an indicator of inflammation. Moreover the acute phase protein, haptoglobin, is elevated in lame cows (Jawor et al., 2008; Tadich et al., 2013), which in turn could drive up total serum protein levels (Jawor et al., 2008). Contrarily, serum urea is lower in sheep and beef cattle that have been exposed to acute stressors (Galyean et al., 1981; Apple et al., 1993). Other variables that could be possibly be influenced by the presence of lameness include creatine kinase and glucose. Creatine kinase is associated with tissue breakdown, whereas glucose levels can be raised in response to a tissue injury (Blebuyck, 1990).

Neutrophils are phagocytic granulocytes of the innate immune system that respond to glucocorticoids (Weber et al., 2006); as lame cows have higher cortisol levels than sound, it is possible that lame animals could experience neutrophilia, as well as altered expression of genes associated with apoptosis and inflammation (Burton et al., 2005).

As yet, the majority of studies that have investigated physiological and immune parameters in relation to lameness have simply compared lame and healthy cows, without considering the cause of the lameness. The objective of this study was to characterise the leukocyte profile, leukocyte gene expression, cortisol and DHEA (dehydroepiandrosterone) response, and aspects of the metabolite profile of cows that have a single lameness causing pathology, sole ulcers, and compare these with healthy cows. We hypothesised that cows with sole ulcers would show differences from healthy cows in all variables. The information generated will provide more insight into the systemic effects of lameness caused by sole ulcers.

MATERIALS AND METHODS

The study animals were located at the 'Moorepark' research farms, part of the Animal and Grassland Research and Innovation Centre, Teagasc, Moorepark, Fermoy, Co. Cork, Ireland. Blood samples were collected between November 2010 and October 2011, and analysis carried out at the

Animal and Bioscience Research Department, Animal and Grassland Research and Innovation Centre, Teagasc, Grange, Dunsany, Co. Meath, Ireland.

Animals and Treatments

All animal procedures performed in this study were conducted under experimental licence (B100/4319) from the Irish Department of Health and Children in accordance with the Cruelty to Animals Act 1876 and the European Communities (Amendment of Cruelty to Animals Act 1876) Regulation 2002 and 2005. The study subjects consisted of 24 lactating Holstein-Friesian cows. Twelve cows were clinically lame due to the presence of one or more sole ulcers, but otherwise healthy with no other foot disorders. Each lame animal was paired with a healthy cow.

Locomotion and hoof scoring

All cows from the herd were observed individually walking from the milking parlour once per week, on a clean grooved concrete floor. Cows that were clinically lame (obvious impairment of one or more limbs when moving forward) were selected for locomotion and hoof scoring the following morning. Locomotion and hoof scoring was carried out by the same person throughout the trial.

Cow locomotory ability was determined after morning milking as cows walked along a clean concrete track away from the milking parlour. They were observed from the side as they walked past, and then from behind. Five aspects of locomotion were scored (spine curvature, tracking up, ab/aduction, speed and head bob) using the system adopted by O'Driscoll et al. (2010). Each of the five aspects were scored from 1 (perfect) to 5 (extremely impaired). These five aspects were summed to give one overall locomotion score per cow. Thus the minimum score was 5 (perfect locomotion), and the maximum was 25 (extreme clinical lameness, cow virtually immobile).

Cows were restrained in a metal crate, their hind feet lifted, and the claws individually examined. The hind claws were cleaned and a sliver of horn was trimmed using a quittor knife

(Hauptner Quittor Hoof Knife, Channelle Vet Products, Loughrea, Ireland) from the entire area of the weight-bearing surface to expose fresh horn. Hooves were examined for the presence of sole lesions, heel erosion, digital dermatitis and white line disease (as described by O'Driscoll et al., 2008), as well as sole ulcers. Only cows that had no other disorder besides sole ulcers were included in the LAME cohort.

Once a LAME cow was identified for inclusion in the study, a SOUND partner was selected from the herd. Using herd list details, a shortlist of possible partner cows that were of a similar lactation number, days in milk, live-weight and body condition score (BCS) was drawn up. The day after identification of the LAME cow, these potential partners were locomotion scored, then had their hooves examined, as described previously. Only cows with good locomotion and no hoof pathologies were selected for inclusion as a SOUND cow. Table 1 shows details of the 12 LAME and SOUND pairs.

Blood sampling

Blood samples were collected from each pair of cows by jugular venepuncture before treatment for sole ulcers the day after hoof examination of the SOUND cow. Blood samples were transported to the laboratory, stored at ambient temperature, and processed within 3.5 h for haematological analysis, or centrifuged, separated, and stored at −20°C until assays were performed.

Cortisol and DHEA

Blood collected into a 9-mL serum separator tube (Vacutainer, Unitech Ltd., Dublin, Ireland) was used for measurement of cortisol and DHEA concentrations. Tubes were stored horizontally overnight at 4°C, then centrifuged at $1,000 \times g$ for 15 min at 4°C. Serum was aspirated using a pipette, then stored at −20°C until analysis. Cortisol and DHEA concentrations were measured using an ELISA (Enzo Life Sciences, Sigford Road, Exeter, UK; Cortisol = Catalog no. ADI-901-071; DHEA = Catalog no. ADI-900-093). The sensitivity of the cortisol assay was

9

56.72 pg/mL, and the intra- and interassay CV based on controls were < 10.5 and < 13.4%, respectively. The sensitivity of the DHEA assay was 2.90 pg/mL, and the intra- and interassay CV based on controls were < 6.4 and < 8.8%, respectively.

Physiological variables

Plasma haptoglobin (Hb), urea, total protein, creatine kinase and glucose were analyzed on an Olympus analyzer (Olympus AU 400, Japan) using reagents supplied by Olympus.

Leukocyte isolation from whole blood

Thirty-six mL of blood collected into ACD tubes from a subset of 8 animals in each cohort was processed within 30 min of collection according to the method of O'Loughlin et al. (2011). Briefly, red blood cells were lysed in a hypotonic solution followed by restoration in 12 mL of a hypertonic solution. The tubes were then centrifuged to collect the leukocyte pellet which was washed twice re-suspended in 1 mL of TRI Reagent (Sigma-Aldrich Ireland Ltd., Dublin, Ireland), pooled by animal and stored in a sterile tube at -80°C until RNA extractions were performed.

RNA Extraction and cDNA Synthesis

A modified TRI Reagent extraction method was used to extract total RNA from leukocytes via homogenization of the pellet in TRI Reagent and the subsequent
addition of chloroform followed by precipitation using isopropanol and ethanol. RNA was quantified using a Nanodrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA quality was assessed on an Agilent 2100 Bioanalyser (Agilent Technologies Ireland Ltd., Dublin, Ireland) and only RNA samples with a
RNA Integrity Number (RIN) of greater than 9.0 were used. Samples were DNase treated and purified using an RNeasy mini kit (Qiagen Ltd., Crawley, UK). One µg of total RNA per animal was reverse transcribed into cDNA using random hexamers and the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Ireland) in a 20 µl reaction and stored at -20°C.

Real-Time (RT)-qPCR

Primers for candidate genes (Table 2) were designed based on bovine sequences obtained from the NCBI database using Primer3 software and were commercially synthesised (Sigma-Aldrich Ireland Ltd., Dublin, Ireland). Amplified PCR products were sequenced (Cambridge, UK) and verified with BLAST http://blast.ncbi.nlm.nih.gov/ to be identical to their respective bovine sequence. Amplification efficiencies were determined for all genes using serial dilutions of pooled cDNA samples. The formula $E = -1 + 10(-1/\text{slope})$ was used where slope refers to the slope of the linear curve of cycle threshold (Cq) values plotted against log dilution. Only primers with PCR efficiencies between 90 and 130% were used in the current study.

Reference genes used in this study were selected based on previous experience within the research group (O'Loughlin et al., 2011). Three genes were used: beta-actin (ACTB), succinate dehydrogenase complex subunit A (SDHA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). These were found to have an average stability value of $M = 0.51$. A normalisation factor, calculated based on the geometric mean of the three reference genes, was used to normalise the expression of each gene of interest.

RT-qPCR was performed using the Applied Biosystems 7500 FAST RT-PCR equipment v2.0.1 (Applied Biosystems, Ireland). One μL of cDNA was added to a 19 μL master mix which included 10 μL of Fast SYBR Green I master mix (Applied Biosystems, Ireland), 8 μL of nuclease-free water and 0.5 μL each of forward and reverse primers at concentrations individually optimised for each primer set. The following RTqPCR cycle conditions were applied: 95°C for 20 s followed by 40 cycles of 95°C for 3 s and 60°C for 30 s, finishing with amplicon dissociation at 95°C for 15 s, 60°C for 1 min increasing 0.5°C per cycle until 95°C was reached for 15 s followed by 60°C for 15 s. In accordance with the MIQE guidelines, raw Cq values were imported to GenEx Software v.5.2.2.8 (2010) (MultiD Analyses AB, Göteborg, Sweden). Outliers were removed from replicate wells using a modified Grubbs test at a $P < 0.05$ confidence interval for any replicate differing from the replicate mean by a standard deviation of more than 0.25 cycles. The Cq values were adjusted

for calculated efficiencies before averaging of replicates. These values were then normalised to the reference genes followed by calculation of relative quantities to the highest Cq value.

Statistical analysis

Statistical analyses were carried out using SAS V9.3 (2002; SAS Institute Inc., Cary, NC), and all data were tested for normality before analysis by examination of box and normal distribution plots, then transformed where necessary. When transformed data were analysed, LSmeans were calculated using raw data to ease clarity of interpretation. Outliers that were outside the 95th percentile were removed.

All data, including locomotion score, were analyzed using the Mixed procedure. The model included fixed effects of cohort (LAME or SOUND), lactation number (1 or greater than 1) and date (n = 8). Weight (kg), days in milk (DIM), and body condition score were included as covariates. Pair was included as a random effect.

When significant effects were found, the Tukey test was used to establish pair-wise differences. Differences were considered significant at $P \leq 0.05$. Tendencies towards significance ($0.05 < P \leq 0.10$) are also presented. Data are presented as LSmeans $\pm$ s.e.

RESULTS

Locomotion scoring

Cows in the LAME group had higher locomotion scores (13.5 ± 0.5) than SOUND cows (6.7 ± 0.5; $P \leq 0.001$).

Leukocyte population and haematological analysis

We found no difference between lame and sound cows with regard to total leukocyte number (Table 3). However, LAME cows had a higher neutrophil % than SOUND and had a numerically lower lymphocyte %. LAME cows also tended to have a higher neutrophil lymphocyte ratio than SOUND. There were no other differences in leukocyte differential. There was no

difference between LAME and SOUND cows with regard to platelet number, red blood cell count, haemoglobin concentration, haematocrit percentage, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration.

Cortisol and DHEA

Serum cortisol concentration was higher in LAME (9.84 ± 0.95 ng/mL) than in SOUND cows (6.80 ± 1.17ng/mL; $P \leq 0.05$; Figure 1). LAME cows also had higher serum DHEA levels (2.35 ± 0.41 ng/mL) than SOUND (1.52 ± 0.43 ng/mL; $P \leq 0.001$). The cortisol:DHEA ratio was numerically higher in LAME (6.49 ± 1.43) than in SOUND (3.68 ± 1.56) cows, but there was no significant difference.

Haptoglobin, glucose, protein, urea and creatine kinase

LAME cows tended to have higher haptoglobin levels than SOUND cows (2.3 ± 0.2 mg/mL v's 1.8 ± 0.2 mg/mL; $P \leq 0.1$) as well as tending to have higher glucose levels (3.13 ± 0.09 mmol/L v's 2.89 ± 0.12 mmol/L; $P \leq 0.1$). Protein levels were also higher in LAME than SOUND cows (89.6 ± 1.2 g/L *v's* 84.8 ± 1.2 g/L; $P \leq 0.01$). Contrarily, LAME cows had lower urea levels than SOUND (4.35 ± 0.27 μmol/L *v's* 5.17 ± 0.26 μmol/L; $P \leq 0.05$), and we observed no difference in creatine kinase levels between LAME and SOUND cows (875.91 ± 161.07 μmol/L *v's* 850.03 ± 159.31 μmol/L).

Real time RT-PCR

Cytokine gene expression

There was no difference in expression of IL2, IL4, IFN-λ or TNF-α between LAME and SOUND cows. However SOUND cows tended to have higher relative expression of the gene coding for colony stimulating factor 2 than LAME (1.52 ± 0.25 *vs* 0.97 ± 0.25; $P \leq 0.1$). In all other cases where differences were detected (IL-1α = P = 0.05; IL-1β = P = 0.1; CXCL8 = $P \leq 0.1$; IL-10 = P = 0.05), relative gene expression in SOUND cows was, or tended to be, lower than in LAME (Figure 2).

13

General immunological gene expression

There was no difference in expression of Matrix metalloproteinase (MMP)-9 or chemokine receptor 5 (CCR5). Relative expression of MMP-13 (P ≤ 0.01), GR-α (P ≤ 0.1), Fas (P ≤ 0.05), haptoglobin (P ≤ 0.1) and CD62L (P ≤ 0.01) was, or tended to be, higher in LAME than SOUND cows (Figure 3).

DISCUSSION

To the authors' knowledge, this study is the first study to describe in detail physiological and immune characteristics of cows that are lame due to presence of sole ulcers but no other pathology compared with otherwise healthy cows. We hypothesised that the pain and stress associated with lameness (Whay et al., 2003) has a negative effect on the systemic immune and inflammatory status of the cow, and thus selected genes for comparison that are differentially expressed in cows that undergo a stressful event, such as parturition or transport (Buckham-Sporer et al., 2008), as well as lame and sound cows (Almeida et al., 2007; O'Driscoll et al., 2009). Thus this study provides novel information about the physiological reaction of the cow to a non-infectious inflammatory health disorder.

Cows with ulcers had higher levels of circulating cortisol than sound cows, and it was outside the normal range for dairy cattle (4.7 – 7.6 ng/mL; Radostits et al., 2007). Consideration of leukocyte differential in addition to cortisol level can provide an increasingly reliable insight when considering the state of the animal; the leukocyte response to a stressor occurs over a longer time span than the hormonal response and is hypothesised to be more enduring, and thus indicative not only of immediate, but also of longer term stress (Davis et al., 2008). Although leukocyte numbers were within the normal range for cattle in both cohorts (Kulberg et al., 2002), cows with ulcers had a higher neutrophil percentage, numerically lower lymphocyte percentage, and a higher neutrophil:lymphocyte ratio than sound cows. This type of leukogram is seen in all vertebrate taxa

in response to either natural stressors, or administration of stress hormones (Davis et al., 2008). A high N:L ratio has also been described in animals in stressful circumstances (Fell et al., 1999), in cows that are genetically more susceptible to disease (Kulberg et al., 2002), and in cows with poor locomotion (O'Driscoll et al., 2009). Thus this leukocyte profile is in concordance with the elevated cortisol levels we detected in cows with ulcers.

Contrary to our results, Almeida et al. (2008) reported no significant difference in cortisol levels between lame and sound cows. Yet, in that study, lame cows had cortisol levels that were 49% higher than sound; the smaller sample size than in our study may have precluded detection of a significant difference. Although in that study lame cows had lower DHEA levels than sound, and we found the opposite, both studies found that cortisol:DHEA ratio was higher in lame than sound cows (although not significant in ours). Thus cortisol:DHEA ratio could yet prove a useful indicator of inflammatory hoof disorders. Further work could investigate the usefulness of this indicator in cows with other foot disorders, and at different stages of inflammation.

Haptoglobin is an acute phase protein. In cattle, serum haptoglobin concentration increases in cases of acute, sub-acute and chronic inflammation (Alsemgeest et al., 1994). It is elevated in cows with all levels of lameness, from mild to severe, compared with sound cows (Jawor et al., 2008; Smith et al., 2010; Tadich et al., 2013). Thus it is unsurprising that lame cows tended to have higher levels of both circulating haptoglobin and haptoglobin mRNA expression, and our study confirms that elevated haptoglobin levels appear to be a reliable measure of lameness associated inflammation. To our knowledge this is the first study to investigate both haptoglobin levels and associated mRNA expression in lame cows. Cooray et al. (2007) reported that bovine granulocytes contain abundant amounts of haptoglobin in healthy cattle. Thus it is likely that the increased percentage of neutrophils in lame cows in this study could be related to the tendency for higher levels of both serum haptoglobin and haptoglobin mRNA expression in these animals.

15

Jawor et al. (2008) reported that clinically lame cows had numerically higher total serum protein levels than healthy cows, and that the protein decreased as lesions healed. This was mainly due to high levels of α- and γ-globulins, and low levels of albumin, compared with healthy cows. Haptoglobin is an α-globulin, and thus the tendency for higher serum haptoglobin levels in lame cows than sound in this study likely contributed to the higher total serum protein. It could be useful in future studies to investigate in more detail the makeup of the total serum protein in lame animals to understand further the pathophysiology of the disorder.

Although there is little information regarding glucose levels in lame cows, Lischer et al. (2001) found elevated levels of glucose in one third of cows affected by sole ulcers. One of the metabolic responses to stress is hyperglycemia, due to the release of liver glycogen as a result of injury or sepsis (Blebuyck, 1990). Moreover, an increase in serum glucose concentration is often attributed to glycogenolysis, which is associated with increased catecholamines and glucocorticoid secretion at the onset of a stressor (McDowell, 1983). Thus an elevated serum cortisol concentration, as well as the presence of an ulcer, could have caused the higher serum glucose levels. Likewise, although we could not find any data pertaining to serum urea concentrations in lame dairy cows, serum urea levels have been observed to be lower in sheep and beef cattle that are submitted to stressors such as isolation, restraint, and transportation (Galyean et al., 1981; Apple et al., 1993).

The primary cause of sole ulcers is displacement of the third phalanx (pedal bone) within the claw capsule, and the resultant concussive forces (Bicalho and Oikonomou, 2013; Lischer et al. 2002). This usually occurs as a result of loosening of the collagen in the hoof around the time of parturition, aggravated by management factors (e.g. concrete flooring) (Bicalho and Oikonomou, 2013). The digital cushion provides a protective layer between the pedal bone and the horn producing cells (Bicalho and Oikonomou, 2013), and a reduction in its thickness is also implicated in sole ulcer development (Bicalho et al., 2009). Thus sole ulcers develop under sterile conditions.

16

Even in sterile conditions, a tissue injury can cause cellular necrosis. This can stimulate an innate immune response (Rider et al., 2011), including recruitment of neutrophils and macrophages and production of cytokines and chemokines (Chen and Nunez, 2010).

We selected our references genes for this study on the basis of a previously executed candidate reference gene validation study from our group (O'Loughlin et al., 2011). In that study the same tissue was used (bovine leukocytes) as in the current one, and a comparison was also being made between healthy control animals, and animals which underwent a sterile stress/inflammatory response (i.e. expected activation of the immune system without being exposed to an infectious agent), which is why we considered the genes to be appropriate. Under normal circumstances a reference gene validation study should include 8 – 10 genes, to ensure accurate and reliable results, and thus the fact that we only investigated 3 can be considered a limitation to our study. Nevertheless, the M-values for our selected reference genes were well within an acceptable range (average M -values in the 0.5–1 range are expected when evaluating candidate reference targets on a heterogeneous set of samples; Hellemans and Vandesompele, 2014), and our use of the geometric mean of the three reference genes improves the reliability of the normalization of the data (Derveaux., 2010).

Cytokines are small proteins made and released by cells of the immune system that affect the behaviour of other cells (Janeway et al., 2005). They can be either pro- or anti-inflammatory. This study investigated expression of several genes coding for cytokines, as well as genes associated with a more generalised immune and stress response. Nevertheless, we detected differences in mRNA expression between lame and sound cows for both pro- and anti-inflammatory cytokines; lame cows had greater expression of the pro-inflammatory cytokines IL-1α, and tended to have greater expression of IL-1β and CXCL8 mRNA, as well as greater expression mRNA expression of the anti-inflammatory cytokine IL-10, and tended to have less expression of the pro-

17

inflammatory cytokine CSF-2. This indicates probable regulation of an innate immune response to a sole ulcer (Opal and DePalo, 2000).

Both IL-1α and IL-1β, which were more highly expressed in leukocytes of lame cows, are involved in the initiation of a sterile inflammatory response (Rider et al., 2011). Thus these cytokines could be implicated in a specific response to sole ulcers. In turn, IL-1β can increase expression of CXCL8 mRNA in leukocytes (Fujishima et al., 1993), and both IL-1β and CXCL8 are implicated in activating haptoglobin secretion (Godson et al., 1995; Wigmore et al., 1997). This is in agreement with the tendency for higher serum haptoglobin, CXCL8 mRNA, and haptoglobin mRNA expression in the lame cows in this study. In stressful situations CXCL8 mRNA is up-regulated in bovine leukocytes, and is hypothesised to contribute to neutrophilia (O'Loughlin et al., 2011; Buckham Sporer et al., 2008). Thus stress associated with sole ulcers could have contributed to the tendency for higher CXCL8 expression, and the higher neutrophil percentage in lame cows. It is worth noting however, that several of the differences we detected in gene expression were tendencies, and that gene expression may not correlate to expression of the actual protein. Future work could investigate presence of the gene product.

Lame cows also had greater expression of the anti-inflammatory cytokine IL-10, and tended to have less expression of CSF-2, which is pro-inflammatory. Likewise, Almeida et al. (2007) reported that IL-10 mRNA was up-regulated in the leukocytes of lame cows compared with sound, and concluded that this could be indicative of an anti-inflammatory response that has a role in reducing tissue damage and pain. Interleukin-10 is also involved in regulation of keratinocytes (Moore et al., 2001), which could be significant in development of sole ulcers, as hoof horn is produced through keratinisation of epidermal cells (Tomlinson et al., 2004).

In the few studies that have investigated differences in leukocyte gene expression in lame and sound cows MMP-13 is consistently identified as being differentially expressed. Not only has MMP-13 mRNA been observed to be up-regulated in the leukocytes of clinically lame cows, when

using both a micro-array and targeted gene expression approach (Almeida et al., 2007), but O'Driscoll et al. (2009) found that even in cows that weren't clinically lame, but with impaired locomotion, leukocytes MMP-13 mRNA was up-regulated. Matrix metalloproteinases (MMPs) are involved in extracellular protein degradation, with MMP-13 also known as collagenase 3, due to its role in collagen degradation (Entrezgene, 2013). It is possible that MMP-13 plays a role in degradation of the collagen fibres of the suspensory apparatus within the hoof, which could lead to displacement and sinking of the pedal bone, and as a consequence the tissue necrosis that ultimately results in sole ulcers (Lischer et al., 2002). MMP-13 also has a role in activation of other MMPs such as MMP-2 and MMP-9, which could cause further damage (Leeman et al., 2002). A major cytokine that is associated with MMP-13 secretion is IL-1, so the increased expression of IL-1α and IL-1β in cows with ulcers could be linked to its production. Thus any increase in expression of MMP-13 may not have been the root cause of solar ulceration, but rather a result of the inflammatory process.

GRα mRNA is abundantly expressed in neutrophils, yet down-regulated by glucocorticoids (Preisler et al., 2000; Burton et al., 2005). However, we found that even though lame cows had elevated cortisol levels compared with sound, they did not have lower expression levels of GRα mRNA. In fact, O'Loughlin et al. (2011) and Buckham-Sporer et al. (2007) found increased expression of GRα mRNA along with a surge in cortisol concentration after exposure to stress. It is possible that the higher neutrophil percentage in lame than sound cows offset any drop in expression by individual cells.

Similar to the work by O'Loughlin et al. (2011), we found a significant increase in expression of CD62L (L-selectin) in cows that were in the 'stressed' (lame) cohort. L-selectin is a cell adhesion molecule, and involved in migration of neutrophils to sites of inflammation (Kansas, 1996). However, CD62L mRNA expression was down-regulated in neutrophils when cattle were exposed to a glucocorticoid challenge test, and this down-regulation correlates with peak

neutrophilia (Weber et al., 2001). The neutrophilia was hypothesised to occur because CD62L is necessary for cells to migrate to peripheral tissues, and without its expression neutrophils accumulated in circulation. On the other hand, O'Loughlin et al. (2011) found that CD62L expression was higher in leukocytes of stressed animals, and Buckham-Sporer et al. (2007) found that an initial drop in neutrophil CD62L expression after transportation was followed by a surge in expression after 9h. These authors hypothesised that after the initial drop in expression there is a rebound increase as a result of young neutrophils being released from bone marrow. Chao et al. (1997) also identified a tri-phasic pattern to CD62L mRNA after activation of T-cells, with an initial decrease, followed by an increase due to increased message stability, and a final loss of expression. Thus it is possible that our sampling time coincided with a surge in expression of CD62L after the initial inflammation.

Fas codes for a trans-membrane receptor protein which is an apoptosis promoter (Nagata and Golstein, 1995). The finding that Fas mRNA expression levels was higher in lame cows than sound was surprising, as it has been suggested that depressed Fas expression could be a common scenario that contributes to neutrophilia and neutrophil longevity (Burton et al., 2005; Buckham-Sporer et al., 2007). However, O'Loughlin et al. (2011) found that leukocyte Fas mRNA expression was increased in animals that were exposed to weaning stress, and suggested that its expression could be an immunological attempt to restore homeostasis. It is possible that contrary to being associated with neutrophil percentage, in chronically stressful situations, Fas expression could be related to the reduction of lymphocyte numbers, and contributes to the immunosuppression associated with chronic stress (Shi et al., 2003). Indeed increased Fas expression is also associated with oxidative stress, and several chronic autoimmune disorders (e.g. rheumatoid arthritis, Aggarwal et al., 2013; diabetes, Arya et al., 2012). Thus it is possible that rather than acting upon neutrophils, in the case of a sterile inflammatory condition such as solar ulceration, elevated Fas

mRNA expression is related to lymphocyte apoptosis, and thus a contributing factor to the high neutrophil lymphocyte ratio.

CONCLUSION

This study provides novel information on the systemic effects of sole ulcers on the dairy cow. The data indicate activation of the immune system, as well as a generalised stress response. Further work to investigate expression of the protein products of differentially expressed genes will provide more insight into pathogenesis of solar ulceration. In particular, the identification of increased expression of MMP13 mRNA in cows with sole ulcers adds to a growing body of evidence implicating increased expression of this gene in cows with impaired locomotion.

References

Aggarwal, A., Sharma, A. and Bhatnagar, A. 2013. Bi(o)communications among peripheral blood fractions: A focus on NK and NKT cell biology in rheumatoid arthritis. Autoimmun. 46:238-250.

Almeida, P.E., Weber, P.S.D., Burton, J.L., Tempelman, R.J., Steiber, J.P. and Zanella, A.J. 2007. Gene expression profiling of peripheral mononuclear cells in lame dairy cows with foot lesions. Vet Immunol Immunop. 120:234-245.

Almeida, P.E., Weber, P.S.D., Burton, J.L. and Zanella, A.J. 2008. Depressed DHEA and increased sickness response behaviors in lame dairy cows with inflammatory foot lesions. Domest. Anim. Endocrin. 34:89-99.

Alsemgeest, S.P.M., Kalsbeek, H.C., Wensing, Th., Koeman, J.P., van Ederen, A.M. and Gruys, E. 1994. Concentrations of serum amyloid-A (SAA) and haptoglobin (Hp) as parameters of inflammatory diseases in cattle. Vet. Quart. 16:21-23.

Apple, J.K., Minton, J.E., Parsons, K.M and Unruh, J.A. 1993. Influence of repeated restraint and isolation stress and electrolyte administration on pituitary-adrenal secretions, electrolytes, and other blood constituents of sheep. J. Anim. Sci. 71:71-77.

Arya, A.A., Deepa, P. and Kamlakar, T. 2012. Relationship between oxidative stress and apoptotic markers in lymphocytes of diabetic patients with chronic non-healing wound. Diabetes Res. Clin. Pr. 94:377-384

Bicalho, R.C., Machado, V.S. and Caixeta, L.S. 2009. Lameness in dairy cattle: a debilitating disease or a disease of debilitated cattle? A cross-sectional study of lameness prevalence and thickness of the digital cushion. J. Dairy Sci. 92:3175-3184.

Bicalho, R.C. and Oikonomou, G. 2013. Control and prevention of lameness associated with claw lesions in dairy cows. Livest. Sci. 156:96-105.

Bleavins, M.R, Carini, C., Jurima-Romet, M. and Rahbari, R. (eds). 2011.Biomarkers in Drug Development: A Handbook of Practice, Application, and Strategy. John Wiley and Sons, Hoboken, NJ, USA.edited by Michael R. Bleavins, Claudio Carini, Mallé Jurima-Romet, Ramin Rahbari

Blebuyck, J.F. 1990. Review article: The metabolic response to stress: an overview and update. Anesthesiology 73:308-327.

Buckham Sporer, K.R., Burton, J.L., Earley, B. and Crowe, M.A. 2007. Transportation stress in young bulls alters expression of neutrophil genes important for the regulation of apoptosis, tissue remodeling, margination, and antibacterial function. Vet. Immunol. Immunopathol. 118:19-29.

Buckham-Sporer, K., Xaio, L., Tempelman, R.J., Burton, J.L., Earley, B. and Crowe, M.A. 2008. Transportation stress alters the circulating steroid environment and neutrophil gene expression in beef bulls. Vet. Immunol. Immunopathol. 121:300-320.

Burton, J.L., Madsen, S.A., Chang, L.-C., Weber, P.S.D., Buckham, K.R., van Dorp, R., Hickey, M.-C. and Earley, B., 2005. Gene expression signatures in neutrophils exposed to glucocorticoids: a new paradigm to help explain ''neutrophil dysfunction'' in parturient dairy cows. Vet. Immunol. Immunopathol. 105:197– 219.

Chao, C.C., Jensen, R. and O'Dailey, M. 1997. Mechanisms of L-selectin regulation by activated T-cells. J. Immunol. 159:1686-1694.

Chen, G.Y. and Nunez, G. 2010. Sterile inflammation: sensing and reacting to damage. Nature Reviews 10:826-837.

Cooray, R. Waller, K.P. and Venge, P. 2007. Haptoglobin comprises about 10% of granule protein extracted from bovine granulocytes isolated from healthy cattle. Vet. Immunol. Immunopathol. 119:310-315.

Davis, A.K., Maney, D.L. and Maerz, J.C. 2008. The use of leukocyte profiles to measure stress in vertebrates: A review for ecologists. Funct. Ecol. 22:760-772.

Derveaux, S., Vandesompele, J. and Hellemans, J. 2010. How to do successful gene expression analysis using real-time PCR. Methods 50:227-230.

EFSA (European Food Safety Authority). 2009. Scientific report of EFSA prepared by the Animal Health and Animal Welfare Unit on the effects of farming systems on dairy cow welfare and disease. Annex to the EFSA Journal 1143:1-7.

Entrez Gene. 2013.MMP13 matrix metallopeptidase 13 (collagenase 3).

http://www.ncbi.nlm.nih.gov/gene?Db=gene&Cmd=ShowDetailView&TermToSearch=4322 Accessed 7 Jan 2013.

Fell, L.R., Colditz, I.G., Walker, K.H. and Watson, D.L. 1999. Associations between temperament, performance and immune function in cattle entering a commercial feedlot. Aust. J. Exp. Agric. 39:795–802.

Fujishima, S., Hoffman, A.R., Vu, T., Kim, K.J., Zheng, H., Daniel, D., Kim, Y., Wallace, E.F., Larrick, J.W. and Raffin, T.A. 1993. Regulation of neutrophil interleukin 8 gene expression and protein secretion by LPS, TNF-α, and IL-1β. J. Cell. Physiol. 154: 478–485.

Galyean, M.L., Lee, R.W. and Hubbert, M.E. 1981. Influence of fasting and transit on ruminal and blood metabolites in beef steers. J. Anim. Sci. 53:7-18.

Godson, D.L., Baca-Estrada, M.E., Van Kessel, A.G., Hughes, H.P.A., Morsy, M.A., Van Donkersgoed, J., Harland, R.J., Shuster, D.E., Daley, M.J. and Babiuk, L.A. 1995. Regulation of bovine acute phase responses by recombinant interleukin-1β. Can. J. Vet. Res. 59:249-255.

Hart BL.1988. Biological basis of the behavior of sick animals. Neurosci. Biobehav. R. 12:123–37.

Hellemans, J. and Vandesompele, J. 2014. Selection of reliable reference genes for RT-qPCR analysis. Methods Mol. Biol. 1160:19-26..

Janeway, C.R., Travers, P., Walport, M. and Shlomchik, M.J. 2005. Immunobiology. The immune system in health and disease. 6th Edition. Garland Science Publishing, New york, USA.

Jawor, P., Steiner, S., Stefaniak, T., Baumgartner, W. and Rzasa1, A. 2008. Determination of selected acute phase proteins during the treatment of limb diseases in dairy cows. Vet Med-Czech. 53:173-183.

Kansas GS. 1996. Selectins and their ligands: current concepts and controversies. Blood 88:3259-3287.

Knott, L., Tarlton, J. Craft, H. and Webster, A.J.F. 2007. Effects of housing, parturition and diet change on the biochemistry and biomechanics of the support structures of the hoof of dairy heifers. Vet. J. 174:277-287.

Kossaibati, M. and Esslemont, R. 1997. The costs of production diseases in dairy herds in England. Vet. J.154:41–51.

Kulberg, S., Storset, A.K., Heringstad, B. and Larsen, H.J.S. 2002. Reduced levels of total leukocytes and neutrophils in Norwegian cattle selected for decreased mastitis incidence. J. Dairy Sci. 85:3470–3475.

Leeman, M.F., Curran, S. and Murray, G., 2002. The structure, regulation, and function of human matrix metalloproteinase-13. Crit. Rev. Biochem. Mol. Biol. 37, 149–166.

Lischer, C.J., Dietrich-Hunkeler, A., Geyer, H., Schulze, J. and Ossent, P. 2001. Healing process of uncomplicated sole ulcers in dairy cows kept in tie stalls: clinical description and blood chemical investigations. Schweiz. Arch. Tierheilkd. 143:125-33.

Lischer, C.J., Ossent, P., Raber, M. and Geyer, H. 2002. Suspensory structures and supporting tissues of the third phalanx of cows and their relevance to the development of typical sole ulcers (Rusterholz ulcers). Vet. Rec. 151:694-698.

McDowell, G.H. 1983. Hormonal control of glucose homeostasis in ruminants. Proc. Nutr. Soc. 42:149-167.

Moore, K.W., De Waal Malefyt, R., Coffman, R.L. and O'Garra, A. 2001. Interleukin-10 and the interleukin-10 receptor. Annu. Rev. Immunol. 19:683–765.

Murray, R.D., Downham, D.Y., Clarkson, M.J., Faull, W.B., Hughes, J.W., Manson, F.J., Merrit, J.B., Russel, W.B., Sutherst, J.E. and Ward, W.R. 1996. Epidemiology of lameness in dairy cattle: description and analysis of foot lesions. Vet. Rec.138:586-591.

Nagata, S. and Golstein, P. 1995. The Fas death factor. Science 267:1449-1456.

Nocek, J. E. 1997. Bovine acidosis: Implications on laminitis. J. Dairy Sci. 80:1005-1028.

O'Driscoll, K., Boyle, L., French, P. and Hanlon, A. 2008. The effect of out-wintering pad design on hoof health and locomotion score of dairy cows. J. Dairy Sci. 91:544-553.

O'Driscoll, K.K.M., Lossie, A.C., Schutz, M.M. and Eicher, S.D. 2009. The effect of floor surface on dairy cow immune function and locomotion score. J. Dairy Sci. 92:4249-4261.

O'Driscoll, K., O'Brien, B., Gleeson, D. and Boyle, L. 2010. Effect of milking frequency and nutritional level on hoof health, locomotion score and lying behaviour of dairy cows Livest. Sci.. 127:248–256

O'Loughlin, A., McGee, M., Waters, S.M., Doyle, S. and Earley, B. 2011. Examination of the bovine leukocyte environment using immunogenic biomarkers to assess immunocompetence following exposure to weaning stress. BMC Vet. Res. 7:45

Opal, S.M. and DePalo, V.A. 2000. Anti-Inflammatory Cytokines. Chest 117:1162-72.

M.T. Preisler, Weber, P.S.D., Tempelman, R.J., Erskine, R.J., Hunt, H. and Burton, J.L. 2000. Glucocorticoid receptor down-regulation in neutrophils of periparturient cows. Am. J.Vet. Res. 61:14-19.

Radostits, O.M., Gay, C.C., Hinchcliff, K.W. and Constable, P.D. 2007 Veterinary Medicine. Saunders- Elsevier: Philadelphia, USA.

Rider, P., Carmi, Y., Guttman, O., Braiman, A., Cohen, I., Voronov, E., White, M.R., Dinarello, C.A. and Apte, R.N. 2011. IL-1α and IL-1β recruit different myeloid cells and promote different stages of sterile inflammation. J. Immunol. 187:4835-4843.

Shi, Y., Devadas, S., Greeneltch, K.M., Yin, D., Mufson, R.A. and Zhou, J. 2003. Stressed to death: Implication of lymphocyte apoptosis for psychoneuroimmunology. Brain Behav. Immun.17:18–26

Smith, B.L., Kauffold, J. and Sherman, L. 2010. Serum haptoglobin concentrations in dairy cattle with lameness due to claw disorders. Vet. J. 186:162-165.

Tadich, C., Bastias, S., Rosenfeld, C. and Green, L.E. 2013. Nociceptive threshold, blood constituents and physiological values in 213 cows with locomotion scores ranging from normal to severely lame. Vet. J. 197:401-405.

Tarlton, J.F., Holah, D.E., Evans, K.M., Jones, S., Pearson, G.R. and Webster, A.J.F. 2002. Biomechanical and histopathological changes in the support structures of bovine hooves around the time of first calving. Vet. J.163:196-204.

Thoefner, M.B., Wattle, O., Pollitt, C.C., French, K.R. and Nielsen, S.S. 2005. Histopathology of oligofructose –induced acute laminitis in heifers. J. Dairy Sci. 88:2774-2782.

Tomlinson, D.J., Mulling, C.H. and Fakler, T.M. 2004. Invited review: formation of keratins in the bovine claw: roles of hormones, minerals, and vitamins in functional claw integrity. J. Dairy Sci. 87:797-809.

Watkins, L.R. and Maier, S.F. 1999. Implications of immune-to-brain communications for sickness and pain. Proc. Natl. Acad. Sci. USA. 96:7710-7713.

Weber, P.S.D., Madsen, S.A., Smith, G.W., Ireland, J.J. and Burton, J.L. 2001. Pre-translational regulation of neutrophil L-selectin in glucocorticoid-challenged cattle. Vet. Immunol. Immunopathol. 83:213-240.

Weber, P.S.D., Madsen-Bouterse, S.A., Rosa, G.J.M., Sipkovsky, S.S., Ren, X., Almeida, P.E., Kruska, R., Halgren, R.G., Barrick, J.L., and Burton, J.L. 2006. Analysis of the bovine neutrophil transcriptome during glucocorticoid treatment. Physiol. Genomics 28:97-112.

Werner, S. and Grose, R. 2003. Regulation of wound healing by growth factors and cytokines. Physiol. Rev. 83:835-870.

Whay, H.R., Watermann, A.E. and Webster, A.J. 1997. Association between locomotion, claw lesions and nociceptive threshold in dairy heifers during the prepartum-period. Vet. J. 154:155–161.

Whay, H.R., Main, D.C.J., Green, L.E. and Webster, A.J.F., 2003. Assessment of the Welfare of Dairy Cattle using animal-based measurements: direct observations and investigation of farm records. . Vet. Rec. 153:197-202.

Wigmore, S.J., Faron, K.C., Maingay, J.P., Lai, P.B. and Ross, J.A. 1997. Interleukin-8 can mediate acute-phase protein production by isolated human hepatocytes. Am. J. of Physiol. 273:E720-726.

27

1 **Table 1.** Details of cows that were clinically lame had an ulcer (Lame) and their partner cow that was healthy (Sound). NA = not available

Cow pair	Lactation Number.		DIM[1]		Liveweight (kg)		BCS[2]	
	Lame	Sound	Lame	Sound	Lame	Sound	Lame	Sound
1	4	5	265	268	538	616	2.75	3.25
2	6	6	208	204	590	604	2.75	2.75
3	6	5	269	277	626	558	2.75	3.00
4	3	3	152	152	624	648	2.75	2.75
5	1	1	120	119	576	548	3.00	3.00
6	5	4	12	14	628	642	3.00	3.25
7	2	2	22	24	590	552	3.25	3.00
8	6	5	14	30	636	NA	NA	NA
9	3	3	39	41	666	620	3.25	3.25
10	4	3	43	36	524	534	3.00	3.00
11	5	4	75	76	453	499	2.50	2.75
12	3	3	343	353	586	744	2.75	2.75

2 [1] Days in milk (DIM); [2]Body condition score (1 (too thin) to 5 (too fat))

28

Table 2. Gene, sequence, amplicon size and National Center for Biotechnology Information (NCBI) accession number of primers used to

analyze gene expression by quantitative PCR. The primers for RT-qPCR candidate genes were obtained using Primer3 software and based on

bovine sequences obtained from the NCBI database

Gene[1]		Sequence 5' → 3'	Amplicon size	Primer efficiency	NCBI Accession
		Cytokine gene expression			
IL-1α	F	TACCCATGACAGCAGTTGGA	132bp	92%	NM_174092
	R	CAGCAGCAGCAAACTGAGAC			
IL-1β	F	CAGTGCCTACGCACATGTCT	167bp	90%	NM_174093
	R	CCAGGGATTTTTGCTCTCTG			
IL-2	F	AGAACCTCAAGCTCTCCAGGATGC	150bp	121%	NM_180997.2
	R	TGGGGTTCAGGTTTTTGCTTGGAGC			
IL-4	F	AGCAGACGTCTTTGCTGCCCC	163bp	98%	NM_173921.2
	R	CACAGAACAGGTCTTGCTTGCCA			

CXCL8	F	TGGGCCACACTGTGAAAATTC	92 bp	102%	O'Loughlin et al. 2011
	R	CCTTCTGCACCCACTTTTCC			
IL-10	F	ACAGGCTGAGAACCACGGGC	175bp	105%	NM_174088.1
	R	GACACCCCTCTCTTGGAGCTCACT			
CSF-2	F	CTCCGCACCTACTCGCCCAC	164bp	109%	NM_174027.2
	R	GGCACGTTGGTTCCTGGGAGTC			
IFN-λ	F	TTCAGAGCCAAATTGTCTCC	205bp	108%	O'Loughlin et al. 2011
	R	AGTTCATTTATGGCTTTGCGC			
TNF-α	F	TGGAGGGAGAAGGGATTCTT	140bp	105%	AF011926
	R	CCAGGAACTCGCTGAAACTC			

General gene expression

MMP-9	F	CCCGACCCGAGTCGATGCAA	213bp	109%	NM_174744.2
	R	GCGGCCACAAGGAACAGGCT			
MMP-13	F	CCTCTGGTCTGTTGGCTCACGC	172bp	101%	NM_174389.2

30

	R	GCTCCTGGGTCCTTGGAGTGGT			
CCR-5	F	CCCCTACGAGAATCTCTCTCGAGTC	150bp	93%	NM_001011672.2
	R	CCAGCGAGTAGAGTGGGGGCA			
GR-α	F	CCATTTCTGTTCACGGTGTG	132bp	105%	AY238475
	R	CTGAACCGACAGGAATTGGT			
Fas	F	AGTTGGGGAGATGAATGCTG	171bp	104%	NM_174662
	R	CCTGTGGATAGGCATGTGTG			
Haptoglobin	F	TGGTCTCCCAGCATAACCTC	185bp	92%	BC109668
	R	AGGGTGGAGAACCACCTTCT			
CD62L	F	CCGATTGCTGGACTTACCAT	194bp	93%	NM_174182
	R	CCAAGTCCACACCCCTTCTA			

Reference genes

ACTB	F	ACTTGCGCAGAAAACGAGAT	123bp	103%	BT030480
	R	CACCTTCACCGTTCCAGTTT			
SDHA	F	AACTGCGACTCAACATGCAG	132bp	94%	NM_001034034

31

	R	TGTCGAACGTCTTCAGATGC			
GAPDH	F	GGGTCATCATCTCTGCACCT	176bp	102%	DQ402990
	R	GGTCATAAGTCCCTCCACGA			

6   [1] *IL-1α* = Interleukin-1 alpha, *IL-1β* = Interleukin-1 beta, *IL-2* = Interleukin-2, *IL-4* = Interleukin-4, *CXCL8* = Interleukin-8, *IL-10* =

7   Interleukin-10, *CSF-2* = Colony stimulating factor-2, *IFN-λ* = Interferon gamma, *TNF-α* = Tumour necrosis factor alpha, *MMP-9* = Matrix

8   metallopeptidase-9, *MMP-13* = Matrix metallopeptidase-13, *CCR-5* = C C chemokine receptor-5, *GR-α* = glucocorticoid receptor alpha, *CD62L*

9   − L-selectin, *ACTB* = Beta atctin, *SDHA* = Succinate dehydrogenase complex subunit A, *GAPDH* = glyceraldehyde-3 phosphate dehydrogenase

10   **Table 3.** Comparison of total leukocyte, neutrophil and lymphocyte number, neutrophil: lymphocyte (N:L) ratio, eosinophil and monocyte

11   number in cows diagnosed with sole ulcers and no other pathology, and sound cows

	Count ($\times 10^3$ cells/µL)			Percentage		
Variable	**LAME**	**SOUND**	**P-value**	**LAME**	**SOUND**	**P-value**
Total leukocytes	7.47 ± 0.55	7.16 ± 0.55	NS			
Neutrophils	3.10 ± 0.29	2.56 ± 0.29	NS	42.9 ± 2.8	35.9 ± 2.8	0.03
Lymphocytes	5.71 ± 1.72	5.93 ± 1.72	NS	42.5 ± 2.8	48.7 ± 2.8	0.12
N:L (ratio)	1.04 ± 0.10	0.76 ± 0.10	0.05			
Eosinophils	0.41 ± 0.04	0.41 ± 0.04	NS	8.7 ± 1.8	9.2 ± 1.8	NS
Monocytes	0.68 ± 0.15	0.68 ± 0.15	NS	5.9 ± 0.5	5.9 ± 0.5	NS

12

33

13   **Figure 1.** Serum cortisol and DHEA concentrations in cows that either had an ulcer

14   (Lame) or were otherwise healthy (Sound). ** indicates means differ by $P \leq 0.05$, ***

15   by $P \leq 0.01$.

16   **Figure 2.** Differences in cytokine relative gene expression in leukocytes of cows that

17   either had an ulcer (Lame) or were otherwise healthy (Sound). * indicates means

18   differ by $P \leq 0.1$, ** by $P \leq 0.05$. Values were normalised to reference genes followed

19   by calculation of relative quantities to the highest Cq value.

20   **Figure 3.** Differences in gene expression in leukocytes of cows that either had an

21   ulcer (Lame) or were otherwise healthy (Sound). * indicates means differ by $P \leq 0.1$,

22   ** by $P \leq 0.05$, *** by $P \leq 0.01$. Values were normalised to reference genes followed

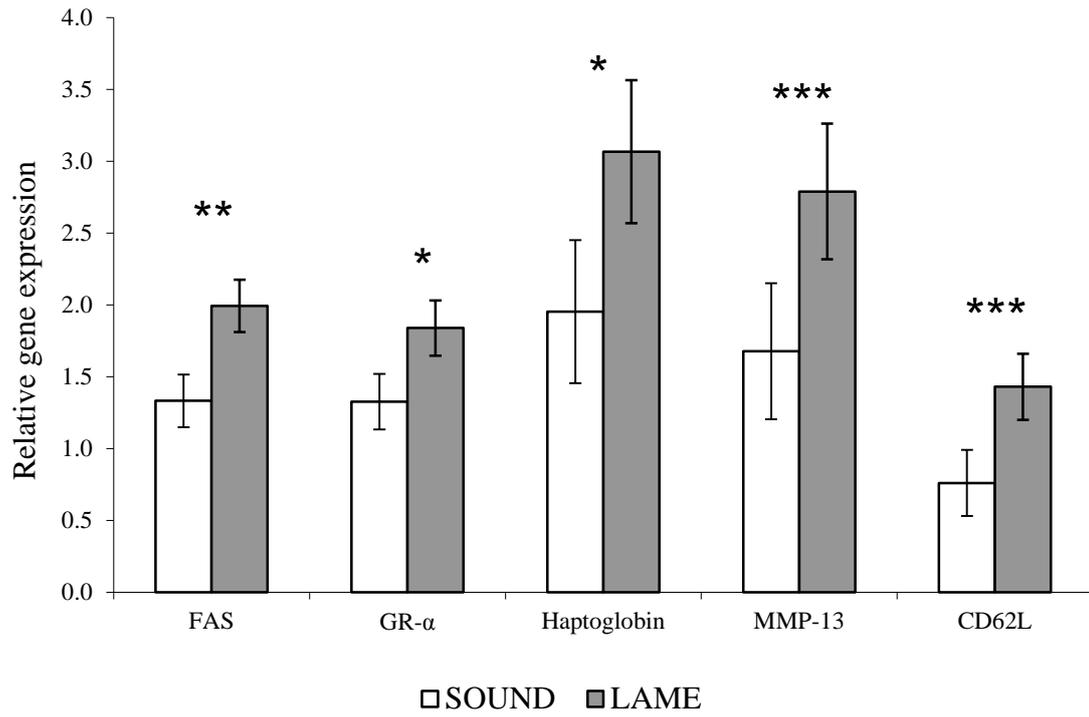23   by calculation of relative quantities to the highest Cq value.

34

24
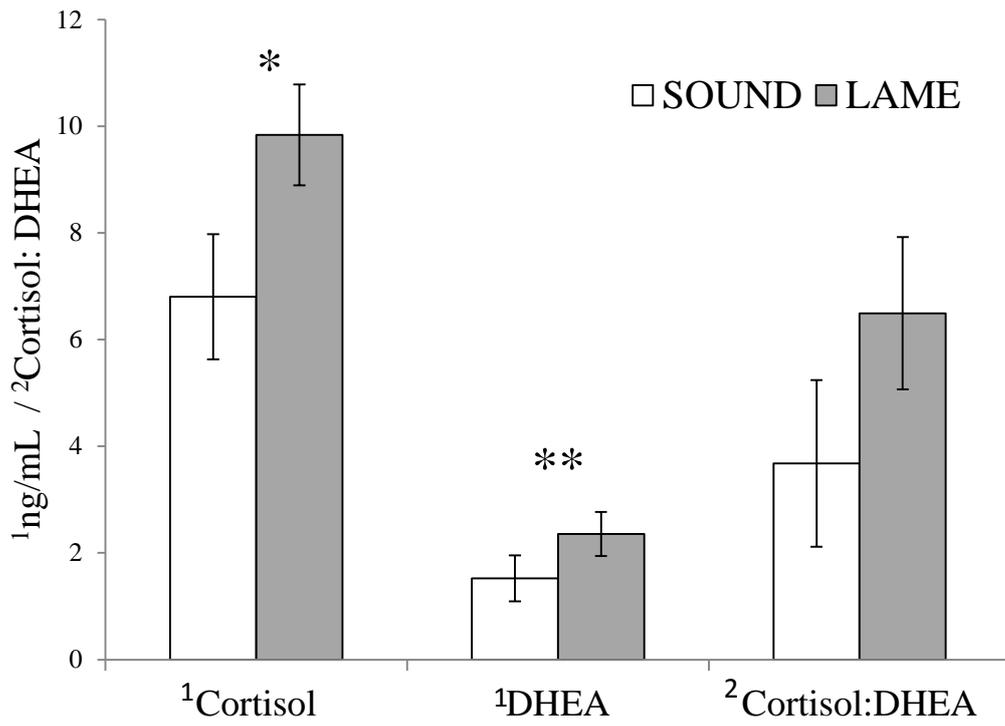
Figure 1. O'Driscoll et al.

26

35

27
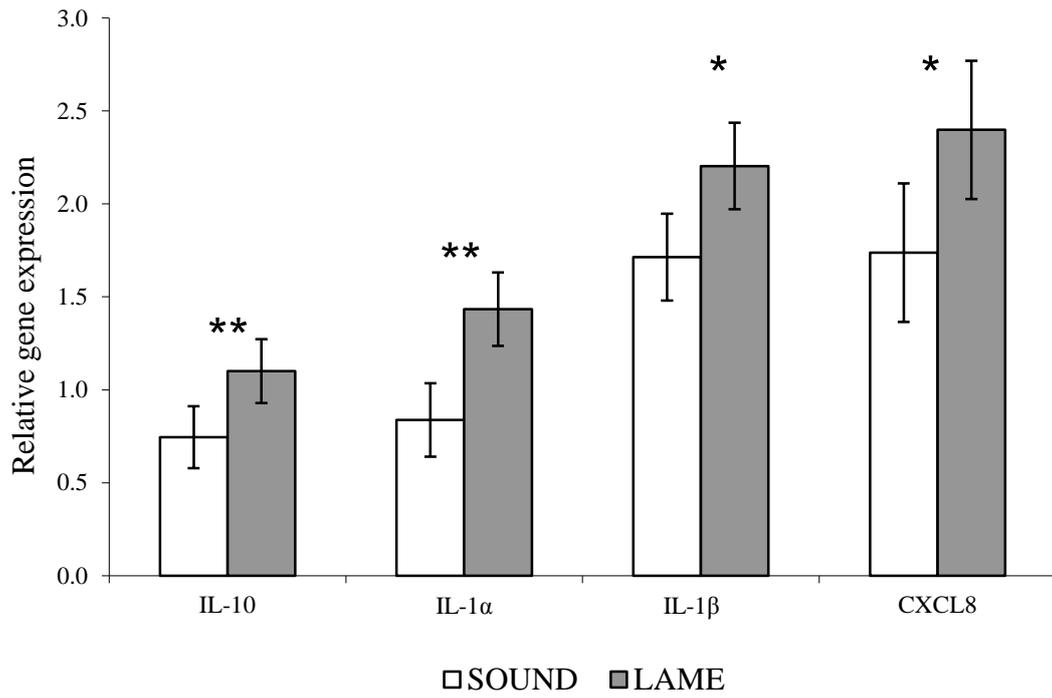
Figure 2. O'Driscoll et al.

36

29

Figure 3. O'Driscoll et al.