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

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ABSTRACT

Sole ulcers are one of the most severe pathologies causing lameness in dairy cows and are associated with abnormal behavior and impaired production performance. However, little is known about how or whether lameness caused by sole ulcers affects the cow systemically. This study compared hematology profile, leukocyte gene expression, and physiological responses [metabolite, cortisol, the endogenous steroid hormone dehydroepiandrosterone (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 24 h of sole ulcer diagnosis. Leukocyte counts were obtained using an automated cell counter, cortisol and DHEA concentration by ELISA, and plasma haptoglobin, urea, total protein, creatine kinase, and glucose were analyzed on an Olympus analyzer. Expression of 16 genes associated with lameness or stress were estimated using reverse transcription-PCR. Data were analyzed using the MIXED procedure in SAS software (version 9.3; SAS Institute Inc., Cary, NC). Lame cows had a higher neutrophil percentage, a numerically lower lymphocyte percentage, and tended to have a higher neutrophil:lymphocyte ratio than sound cows. 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-1 α* , *IL-1 β* , *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 *MMP-13* mRNA has been found in cows with impaired locomotion and thus could be implicated in development of claw horn disorders.

Key words: lameness, sole ulcer, immune, gene expression, stress

INTRODUCTION

Lameness is the most serious welfare problem facing 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 pathologies causing lameness, are noninfectious in nature and occur when claw horn formation is disrupted. The underlying tissue then becomes inflamed and sometimes exposed (Murray et al., 1996). Cows are particularly vulnerable around the time of parturition; changes in hormone levels cause increased vascular permeability, increasing the risk of edema and ischemia in the hoof (Nocek, 1997; Tarlton et al., 2002; 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 (Thoenes et al., 2005).

Lameness is associated with behavioral 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.

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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 hypothesized 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, several other physiological responses 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 dehydroepiandrosterone (**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 (Galvayan 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 (Weissman, 1990).

Neutrophils are phagocytic granulocytes of the innate immune system that respond to glucocorticoids (Weber et al., 2006). Because lame cows have higher cortisol levels than sound cows, 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 characterize the leukocyte profile, leukocyte gene expression, cortisol and DHEA 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 hypothesized 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 license (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 parlor 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 parlor. 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/adduction, speed, and head bob) using the system adopted by O'Driscoll et al. (2010). Each of the 5 aspects were scored from 1 (perfect) to 5 (extremely impaired). These 5 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, DIM, liveweight, and BCS was created. 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 venipuncture 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 hematological 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 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, urea, total protein, creatine kinase, and glucose were analyzed on an Olympus analyzer (Olympus AU 400, Tokyo, Japan) using reagents supplied by Olympus.

Leukocyte Isolation from Whole Blood

Thirty-six milliliters of blood collected into acid-citrate-dextrose (ACD) tubes (Vacutette, Cruinn Diagnostics, Dublin, Ireland) 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, resuspended 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 homogeniza-

Table 1. Details of cows that were clinically lame and had an ulcer (lame) and their partner cow that was healthy (sound)

Cow pair	Lactation no.		DIM		Liveweight (kg)		BCS ¹	
	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 ²	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

¹BCS [1 (too thin) to 5 (too fat)].

²NA = not available.

tion of the pellet in TRI Reagent and the subsequent addition of chloroform followed by precipitation using isopropanol and ethanol. The RNA was quantified using a Nanodrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE). The RNA quality was assessed on an Agilent 2100 Bioanalyser (Agilent Technologies Ireland Ltd., Dublin, Ireland), and only RNA samples with a RNA integrity number of greater than 9.0 were used. Samples were DNase treated and purified using an RNeasy mini kit (Qiagen Ltd., Crawley, UK). One microgram of total RNA per animal was reverse transcribed into cDNA using random hexamers and the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Dublin, Ireland) in a 20- μ L reaction and stored at -20°C .

Real-Time Reverse-Transcription PCR

Primers for candidate genes (Table 2) were designed based on bovine sequences obtained from the National Center for Biotechnology Information database using Primer3 software and were commercially synthesized (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 $E = -1 + 10(-1/\text{slope})$, where E is efficiency, was used where slope refers to the slope of the linear curve of cycle threshold (**C_q**) 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: β -actin, succinate dehydrogenase complex subunit A, and *GAPDH*. These were found to have an average stability value of $M = 0.51$. A normalization factor, calculated based on the geometric mean of the 3 reference genes, was used to normalize the expression of each gene of interest.

Reverse-transcription PCR (RT-qPCR) was performed using the Applied Biosystems 7500 FAST RT-PCR equipment v2.0.1 (Applied Biosystems). One microliter of cDNA was added to a 19 μ L of master mix which included 10 μ L of Fast SYBR Green I master mix (Applied Biosystems), 8 μ L of nuclease-free water, and 0.5 μ L each of forward and reverse primers at concentrations individually optimized for each primer set. The following RT-qPCR 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 **C_q** 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 **C_q** values were adjusted for calculated efficiencies before averaging of replicates. These values were then normalized to the reference genes followed by calculation of relative quantities to the highest **C_q** 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 analyzed, least squares means 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), DIM, and BCS 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 toward significance ($0.05 < P \leq 0.10$) are also presented. Data are presented as least squares means $\pm$ standard error.

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 Hematological 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 percentage than sound and had a numerically lower lymphocyte percentage. Lame cows also tended to have a higher neutrophil:lymphocyte (**N:L**) ratio than sound cows. No other differences were observed in leukocyte differential. No difference was observed between lame and sound cows with regard to platelet number, red

Table 2. Gene, sequence, amplicon size, and National Center for Biotechnology Information (NCBI) accession number of primers used to analyze gene expression by quantitative PCR¹

Gene ²	Forward (F) or reverse (R)	Sequence 5' → 3'	Amplicon size (bp)	Primer efficiency (%)	NCBI accession no.
Cytokine gene expression					
<i>IL-1α</i>	F	TACCCATGACAGCAGTTGGA	132	92	NM_174092
	R	CAGCAGCAGCAAACCTGAGAC			
<i>IL-1β</i>	F	CAGTGCCTACGCACATGTCT	167	90	NM_174093
	R	CCAGGGATTTTTTGTCTCTG			
<i>IL-2</i>	F	AGAACCTCAAGCTCTCCAGGATGC	150	121	NM_180997.2
	R	TGGGGTTTCAGGTTTTTGTCTGGAGC			
<i>IL-4</i>	F	AGCAGACGTCTTTGCTGCCCC	163	98	NM_173921.2
	R	CACAGAACAGGTCTTGCTTGCCA			
<i>CXCL8</i>	F	TGGGCCACACTGTGAAAATTC	92	102	O'Loughlin et al., 2011
	R	CCTTCTGCACCCACTTTTCC			
<i>IL-10</i>	F	ACAGGCTGAGAACCACGGGC	175	105	NM_174088.1
	R	GACACCCCTCTCTTGAGCTCACT			
<i>CSF-2</i>	F	CTCCGCACCTACTCGCCAC	164	109	NM_174027.2
	R	GGCACGTTGGTTCCTGGGAGTC			
<i>IFN-λ</i>	F	TTCAGAGCCAAATTGTCTCC	205	108	O'Loughlin et al., 2011
	R	AGTTCATTATGGCTTTGCGC			
<i>TNF-α</i>	F	TGGAGGGAGAAGGGATTCTT	140	105	AF011926
	R	CCAGGAACTCGCTGAAACTC			
General gene expression					
<i>MMP-9</i>	F	CCCGACCCGAGTCGATGCAA	213	109	NM_174744.2
	R	GCGGCCACAAGGAACAGGCT			
<i>MMP-13</i>	F	CCTCTGGTCTGTTGGCTCACGC	172	101	NM_174389.2
	R	GCTCCTGGGTCTCTGGAGTGGT			
<i>CCR-5</i>	F	CCCTACGAGAAATCTCTCGAGTC	150	93	NM_001011672.2
	R	CCAGCGAGTAGAGTGGGGCA			
<i>GR-α</i>	F	CCATTCTGTTCACGGTGTG	132	105	AY238475
	R	CTGAACCGACAGGAATTGGT			
<i>Fas</i>	F	AGTTGGGGAGATGAATGCTG	171	104	NM_174662
	R	CCTGTGGATAGGCATGTGTG			
Haptoglobin	F	TGGTCTCCCAGCATAACCTC	185	92	BC109668
	R	AGGGTGGAGAACCACCTTCT			
<i>CD62L</i>	F	CCGATTGCTGGACTTACCAT	194	93	NM_174182
	R	CCAAGTCCACACCCCTTCTA			
Reference gene					
<i>ACTB</i>	F	ACTTGCGCAGAAAACGAGAT	123	103	BT030480
	R	CACCTTCACCGTTCCAGTTT			
<i>SDHA</i>	F	AACTGCGACTCAACATGCAG	132	94	NM_001034034
	R	TGTCGAACGTCTTCAGATGC			
<i>GAPDH</i>	F	GGGTCATCATCTCTGCACCT	176	102	DQ402990
	R	GGTCATAAGTCCCTCCACGA			

¹The primers for reverse-transcription PCR candidate genes were obtained using Primer3 software and based on bovine sequences obtained from the NCBI database.

²*CXCL8* = IL-8; *CSF-2* = colony stimulating factor-2; *TNF-α* = tumor necrosis factor α; *MMP-9* = matrix metalloproteinase-9; *MMP-13* = matrix metalloproteinase-13; *CCR-5* = C-C chemokine receptor-5; *GR-α* = glucocorticoid receptor α; *CD62L* = L-selectin; *ACTB* = beta-actin; *SDHA* = succinate dehydrogenase complex subunit A.

blood cell count, hemoglobin concentration, hematocrit percentage, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration.

in sound (3.68 ± 1.56) cows, but no significant difference was found.

Cortisol and DHEA

Serum cortisol concentration was higher in lame (9.84 ± 0.95 ng/mL) than in sound cows (6.80 ± 1.17 ng/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

Haptoglobin, Glucose, Protein, Urea, and Creatine Kinase

Lame cows tended to have higher haptoglobin levels than sound cows (2.3 ± 0.2 vs. 1.8 ± 0.2 mg/mL; $P \leq 0.1$) as well as tending to have higher glucose levels (3.13 ± 0.09 vs. 2.89 ± 0.12 mmol/L; $P \leq 0.1$). Protein levels were also higher in lame than sound cows (89.6 ± 1.2 vs. 84.8 ± 1.2 g/L; $P \leq 0.01$). Contrarily, lame cows

Table 3. Comparison of total leukocyte, neutrophil and lymphocyte number, neutrophil:lymphocyte (N:L) ratio, eosinophil and monocyte number in cows diagnosed with sole ulcers and no other pathology, and sound cows

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

had lower urea levels than sound (4.35 ± 0.27 vs. 5.17 ± 0.26 μ mol/L; $P \leq 0.05$), and we observed no difference in creatine kinase levels between lame and sound cows (875.91 ± 161.07 vs. 850.03 ± 159.31 μ mol/L).

Real-Time RT-qPCR Cytokine Gene Expression

No difference was observed 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 cows (1.52 ± 0.25 vs. 0.97 ± 0.25 ; $P \leq 0.1$). In all other cases where differences were detected (*IL-1 α* : $P = 0.05$; *IL-1 β* : $P = 0.1$; *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 cows (Figure 2).

General Immunological Gene Expression

No difference was observed in expression of matrix metalloproteinase (*MMP*)-9 or chemokine receptor 5

(*CCR5*). Relative expression of *MMP-13* ($P \leq 0.01$), *GR- α* ($P \leq 0.1$), *Fas* ($P \leq 0.05$), haptoglobin ($P \leq 0.1$), and *CD62L* ($P \leq 0.01$) 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 hypothesized that the pain and stress associated with lameness (Whay et al., 2003) have 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 noninfectious 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 hypothesized 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 N:L 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

Figure 1. Serum cortisol and dehydroepiandrosterone (DHEA) concentrations in cows that either had an ulcer (lame) or were otherwise healthy (sound). * indicates means differ by $P \leq 0.05$; ** indicates means differ by $P \leq 0.05$.

Figure 2. Differences in cytokine relative gene expression in leukocytes of cows that either had an ulcer (lame) or were otherwise healthy (sound). * indicates means differ by $P \leq 0.1$, ** by $P \leq 0.05$. Values were normalized to reference genes followed by calculation of relative quantities to the highest cycle threshold value.

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;

Figure 3. Differences in gene expression in leukocytes of cows that either had an ulcer (lame) or were otherwise healthy (sound). * indicates means differ by $P \leq 0.1$, ** by $P \leq 0.05$, *** by $P \leq 0.01$. Values were normalized to reference genes followed by calculation of relative quantities to the highest cycle threshold value.

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 cows, 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 to be 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, subacute, 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.

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 cows 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 total serum protein in lame animals to understand further the pathophysiology of the disorder.

Although little information is available 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 (Weissman, 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 pres-

ence 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 (Lischer et al., 2002; Bicalho and Oikonomou, 2013). 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. 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 reference 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 to 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 3 reference genes improves the reliability of the normalization of the data (Derveaux et al., 2010).

Cytokines are small proteins made and released by cells of the immune system that affect the behavior 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 generalized immune and stress response. 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-1 α , and tended to have greater expression of IL-1 β and *CXCL8* mRNA, as well as greater mRNA expression of the anti-inflammatory cytokine IL-10, and tended to have less expression of the pro-inflammatory cytokine CSF-2. This indicates probable regulation of an innate immune response to a sole ulcer (Opal and DePalo, 2000).

Both IL-1 α and IL-1 β , 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-1 β can increase expression of *CXCL8* mRNA in leukocytes (Fujishima et al., 1993), and both IL-1 β 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 upregulated in bovine leukocytes, and is hypothesized to contribute to neutrophilia (Buckham Sporer et al., 2008; O'Loughlin et al., 2011). 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 upregulated in the leukocytes of lame cows compared with sound cows, 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 keratinization 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 upregulated 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) also found that

even in cows that were not clinically lame, but with impaired locomotion, leukocyte *MMP-13* mRNA was upregulated. Matrix metalloproteinases 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 fibers 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). The *MMP-13* also has a role in the activation of other MMP 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-1 α and IL-1 β 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 sole ulceration, but rather a result of the inflammatory process.

The *GR α* mRNA is abundantly expressed in neutrophils, yet downregulated 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 *GR α* mRNA. In fact, Buckham-Sporer et al. (2007) and O'Loughlin et al. (2011) found increased expression of *GR α* 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 is involved in migration of neutrophils to sites of inflammation (Kansas, 1996). However, *CD62L* mRNA expression was downregulated in neutrophils when cattle were exposed to a glucocorticoid challenge test, and this downregulation correlates with peak neutrophilia (Weber et al., 2001). The neutrophilia was hypothesized 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 decrease in neutrophil *CD62L* expression after transportation was followed by a surge in expression after 9 h. These authors hypothesized that after the initial drop in expression, a rebound increase occurs as a result of young neutrophils being released from bone marrow. Chao et al. (1997) also identified a triphasic 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.

The *Fas* gene encodes a transmembrane receptor protein, which is an apoptosis promoter (Nagata and Golstein, 1995). The finding that *Fas* mRNA expression levels were higher in lame cows than sound was surprising, because 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., 2011). 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 N:L ratio.

CONCLUSIONS

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 generalized 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.

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