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PATRICIA ELIZA DE ALMEIDA

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DISCOVERING BIOMARKERS OF PAINFUL INFLAMMATORY FOOT LESIONS IN LAME DAIRY CATTLE

By

Patricia Eliza de Almeida

A DISSERTATION

Submitted to
Michigan State University
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ABSTRACT

DISCOVERING BIOMARKERS OF PAINFUL INFLAMMATORY FOOT LESIONS IN LAME DAIRY CATTLE

By

Patricia Eliza de Almeida

Lameness is a principal health issue and probably the single most common cause of distress in dairy cattle. Current methods used for screening lameness rely on qualitative scoring systems that are time-consuming, unreliable, non-sensitive, and require great skill on the part of the herdsman. This dissertation encompasses a series of studies aimed to discover biomarkers to assist with diagnosis of painful inflammatory foot lesions in lame dairy cows.

The first study investigated the use of a pressure plate for early detection of lameness. Peak vertical force (PVF) was measured using a pressure plate on 7 Holstein heifers that were deemed sound, on the basis of a subjective lameness scoring system. Feet were then inspected during trimming and revealed no lesions in 3 heifers and hairy heel wart (HHW) lesions in the other 4 heifers. Sound heifers demonstrated significantly higher PVF ($5.32 \pm 0.62 N.kg^{-1}$; $P = 0.01$) and better right-left hindlimb PVF symmetry (1.04 ± 0.02; $P = 0.03$) than those with HHW (3.64 ± 0.82 $N.kg^{-1}$ and 0.77 ± 0.16). Thus, a pressure plate was sensitive to detect gait abnormalities in heifers with HHW lesions that were undiagnosed by subjective means.

In the second study, blood serum and peripheral blood mononuclear cells (PBMC) were collected from 8 lame (i.e., inflammatory foot lesions and sickness behaviors) and 8

sound Holstein cows. The abundance of IL-1β, MMP-9, L-selectin, GRα and POMC was investigated in PBMCs via absolute quantitative real-time RT-PCR. Serum cortisol and dehydroepiandrosterone (DHEA) concentrations were also measured. Lame cows demonstrated a 23% decrease in serum DHEA ($P = 0.01$), a 79% increase in cortisol ($P = 0.10$), and a 65% higher cortisol:DHEA ratio ($P = 0.06$) compared to sound cows. No differences in PBMC expression of the candidate genes were found.

In the third study, BOTL5 microarrays were interrogated with the PBMCs cDNA samples from previous study in an 8-array balanced design. Samples from one lame and one sound cow were directly compared with each other on individual arrays. Statistical analysis of the resulting fluorescence intensity data revealed 31 putatively differentially expressed genes in lame versus sound cows ($P < 0.05$). Validation of 15 of these genes, whose protein products are known to be important in immune response, inflammation, and pain, was carried out using relative quantitative real-time RT-PCR. Up regulation ($P \leq 0.05$), expressed as fold increase (± SEM) in lame relative to sound cows, was confirmed for IL-2 (12.68 ± 1.47), IL-10 (2.39 ± 0.55), MMP-13 (10.44 ± 1.14), and CCR5 (5.26 ± 1.05) gene expression. Similarly, GM-CSF-R-alpha (2.30 ± 0.63) and IL-4 (2.06 ± 0.59) gene expression showed a tendency ($P = 0.10$) for up regulation in lame compared to sound cows.

In addition to vertical ground reaction force measured by pressure plate and circulating levels of DHEA and cortisol:DHEA ratio, PBMC co-expression of the genes mentioned above appear to be promising potential biomarkers for diagnosis of painful inflammatory foot lesions in lame dairy cattle. Further studies should verify the universality and clinical utility of these results.

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To my parents Maria Eliza and Fernando and my brother Daniel

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"Out beyond the ideas of right and wrong, there is a field. I'll meet you there." –

Mowlana Jalaluddin Rumi.

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LIST OF ABBREVIATIONS

°C	Degrees Celsius
μg	Microgram
ACD	Acid Citrate Dextrose
ACTH	Adrenocorticotrophic Hormone
AVP	Arginine Vasopressin
BLASTn	Basic Local Alignment Search Tool – nucleotide
BM	Body Mass
BOTL	Bovine Total Leukocyte
bp	Base Pairs
CCR5	Chemokine Receptor-5
CD	Cluster of Differentiation (e.g., CD4, CD62L)
CD62L	L-Selectin
cDNA	Complementary DNA
CINC-2	Cytokine-induced Neutrophil Chemoattractant-2
cm	Centimeter
CNS	Central Nervous System
CRF	Corticotrophin Releasing Factor
DGR	Dorsal Root Ganglia
DHEA	Dehydroepiandrosterone
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside Triphosphate

DTT	Dithiothreitol
E. coli	*Escherichia coli*
ECM	Extracellular Matrix
EIA	Enzyme Immuno Assay
EST	Expressed Sequence Tag
FDR	False Discovery Rate
fg	Fentogram
FL	Left Forelimb
FR	Right Forelimb
g	Gram
GATA-3	GATA binding protein 3
Gi/o	Inhibitory G-protein
GM-CSF-R-α	Granulocyte Macrophage-Colony Stimulator Factor-Receptor-alpha
GR	Glucocorticod Receptor
GRF	Ground Reaction Force
GRα	Glucocorticoid Receptor-alpha
HHW	Hairy Heel Wart
HPA	Hypothalamic-Pituitary-Adrenocortical
Hz	Hertz
ICAM-1	Intracellular Adhesion Molecule-1
IFN-γ	Interferon-gamma
IgG	Immunoglobulin G
IL	Interleukin (e.g., IL-1β, IL-6)

KC Keratinocyte-derived Chemokine

KC Keratinocyte-derived Chemokine

kDa KiloDaltons

kg Kilogram

LH Left Hindlimb

LOESS Local-Weighted Regression and Smoothing Scatterplots

log Logarithmic

LPS Lipopolysaccharide

M Molarity

m Meter

$MgCl_2$ Magnesium Chloride

min Minutes

MIP-2 Macrophage Inflammatory Protein-2

ml Mililiter

MMP-13 Matrix Metalloproteinase-13

MMP-9 Matrix Metalloproteinase-9

mRNA Messenger Ribonucleic Acid

N Newton

N/kg Newton per kilogram

NCBI National Center for Biotechnology Information

NGF Nerve Growth Factor

NSAIDs Non-Steroidal Anti-Inflammatory Drugs

OA Osteoarthritis

PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Reaction
PCR	Polymerase Chain Reaction
pg	Picogram
POMC	Pro-opiomelanocortin
PVF	Peak Vertical Force
Q-RT-PCR	Quantitative real-time Reverse Transcription Polymerase Chain Reaction
RA	Rheumatoid Arthritis
RH	Right Hindlimb
RL	Hind Hindlimb
RNA	Ribonucleic Acid
RR	Right Hindlimb
SEM	Mean Standard Error
SLE	Systemic Lupus Erythematosus
STAT4	Signal Transducer and Activator of Transcription 4
STAT6	Signal Transducer and Activator of Transcription 6
TGF-β	Transforming Growth Factor Beta
T_H0	Naïve T helper
T_H1	T helper type 1
T_H2	T helper type 2
TIGR	The Institute for Genomic Research
TIMP	Tissue Inhibitor of Metalloproteinase
TNF-α	Tumor Necrosis Factor-Alpha

Treg	Regulatory T cell
Tris-HCL	Tris-Hydrochloric Acid
US	United States
USDA	United States Department of Agriculture

INTRODUCTION

Lameness in dairy cattle refers to the abnormal gait used by animals as they attempt to alleviate or avoid pain from lesions and inflammation in the affected limb or back area (Scott, 1989; Hardie, 2000). Lameness not only severely compromises the welfare of affected animals due to pain and discomfort (Webster, 1986), but also has a real negative economic impact on the dairy industry. Estimated annual lameness incidence in adult dairy cows based upon studies conducted in the United States and Europe ranges from 4 to 56% (Leech et al., 1960; Prentice and Neal, 1972; Eddy and Scott, 1980; McLennan, 1988; Clarkson et al., 1996; Whitaker et al., 2000; Wells et al., 1993). This widely differing estimate is suggested to be due to issues with sensitivity of lameness detection within individual herds: how lameness is defined and by who (Whay et al., 1997). Lameness is suggested to cost approximately US$350 per clinical episode (Greenough and Weaver, 1997). This remarkable economic impact is due to direct losses with treatment and control (Moore et al., 2001), and indirect losses due to impaired reproductive performance (Sprecher et al., 1997), decreased milk yield (Warnick et al., 2001), increased involuntary culling rate (Rajala-Shultz and Grohn 1999), and decreased carcass value of culled cows (Arendonk et al., 1984; Booth et al., 2004). Realizing the economic importance of lameness, its high incidence, and severe implications to the welfare of cows, strategies to objectively diagnose, treat, and improve lameness outcomes are of paramount importance.

Qualitative biomechanical applications in cattle, such as scoring systems, have been adopted by the dairy industry for lameness screening, whereas the more quantitative

techniques have rarely been used (e.g. Herlin and Drevemo, 1997; Rajkondawar et al., 2002b; Tasch and Rajkondawar, 2004). Lameness scorings are based on qualitative assessments of kinematic measures (descriptors of motion) during standing and walking. This strategy is time-consuming, unreliable, non-sensitive, and requires great skill on the part of the herdsman (Whay et al., 1997; O'Callaghan et al., 2003; Wells et al., 1993; Kopcha et al., 2003). Furthermore, deviations in locomotion, the focus of scoring systems, might be associated with factors other than pain, including damage in locomotor system (muscles, ligaments, nerves) (Greenough et al., 1981), natural variation among cows, and physical constraints such as a distended udder (Flower et al., 2006). As a consequence of inappropriate lameness screening, 30 to 50% of cows are suggested to remain undiagnosed (O'Callaghan et al., 2003; Kopcha et al., 2003), treatment is often delayed or neglected, and the pathologies that underlie lameness (e.g., sole ulcer, white line disease, interdigital dermatitis) are likely to become chronic with unfavorable prognoses (Logue et al. 1998). To overcome some of the limitations imposed by the subjectivity of current lameness screening methods, alternative strategies able to objectify lameness diagnosis and enhance its accuracy and sensitivity are needed.

Lameness encompasses locomotor changes due to alterations in the limb loading profile, and in the force distribution within and between the limbs (Goodship et al., 1983; Morris and Seeherman, 1987; Merkens et al., 1988). These changes are manifested by asymmetries in temporal and spatial kinematics (geometry of motion), as well as in kinetics (force distribution variables between the limbs). As a result of technological innovations in biomechanical research, tools that enable objective measurement of kinematics and kinetic variables (e.g. joint angle, ground reaction force) are available for

field studies. For example, the vertical component of ground reaction forces (GRF), which is often used to characterize altered locomotion in lame horses (Clayton et al., 2000) and cows (Scott 1989; Rajkondawar et al., 2002b), can, now, be measured using pressure plates. This device measures pressure under foot using optoelectrical techniques such as light reflection or pressure sensitive electrodes. Compared to force plates, which measures the direction and magnitude of the ground reaction forces beneath the foot, pressure plates offer better portability and lower cost. However, the use of pressure plates to detect lameness in dairy cattle has never been documented.

In addition to changes in locomotion, lame cows manifest sickness response type behaviors. These behavioral changes are: increased lying time, altered social behavior, decreased food-intake (Singh et al., 1993; 1994; Sauter-Louis et al., 2004), and hyperalgesia (increased sensitivity to noxious stimuli) (Whay et al., 1998). Sickness response is a phenomenon triggered by the recognition of anything foreign to the host (Hart, 1988). It is initiated by the immune system but orchestrated and partially created by the brain (Maier and Watkins, 1998; Hart, 1988; Kent et al., 1992). Sickness response includes physiological, behavioral, and hormonal responses designed to enhance the chances of host survival (**Table 1**).

Table 1 Changes in various systems as part of the sickness response.

System	Changes
Behavioral	Decreased social interaction and exploration Decreased sexual activity Decreased food and water intake
Physiological	Fever Alterations in plasma ions to suppress minerals required by bacteria and viruses to replicate Increase in white blood cell replication Increased sleep
Hormonal	Increased release of classic hypothalamo-pituitary-adrenal [HPA] Increase release of sympathetic hormones

Although the mechanism underlying sickness behaviors in lame cows is not characterized, it may be triggered by injury and inflammation often present at the claw(s). Lesions in the claw horn constitute 90% of all lameness cases (Murray et al., 1996) and involve exposure to microorganisms (e.g., spirochetes and *Fusobacterium necrophorum*), loss of horny sole and uncovering of the corium, laminar inflammation, and laminar tissue necrosis. These lesions most likely trigger innate immunity and the release of proinflammatory cytokines by peripheral immune cells (e.g., macrophages). These cytokines are crucial for the development of sickness response and allow communication between peripheral tissues and the central nervous system (CNS) (Watkins and Maier,

2005). Consequently, a pronounced increase in the activity of the HPA axis is expected to occur (Buckingham, 1996; Turnbull and Rivier, 1999) and culminates in the release of steroid hormones such as cortisol and dehydroepiandrosterone (DHEA) from the adrenal cortex (Besedovsky et al., 1986; Jessop, 1999). Despite the occurrence of sickness behaviors in lame cows, physiological changes consistent with sickness response, such as altered steroid hormones, have not been documented. Therefore, the potential of using circulating concentrations of HPA axis related hormones as candidates to differentiate lame from sound cows is worthy of investigation.

In addition to changes in hormones that reflect HPA axis activation, immune cells also are important sources of information about organismal health (Dunnes, 2005). For example, peripheral blood mononuclear cells (PBMCs) are responsible for a comprehensive surveillance of the body for signs of infection and disease. PBMCs have been used as sources of biomarkers to identify ongoing autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, type I diabetes, and multiple sclerosis (Maas et al., 2002), and non-autoimmune diseases such as renal cell carcinoma in humans (Burczynski et al., 2005), Johne's disease (Coussens et al., 2004a; 2004b), trypanosomiasis (Hill et al., 2005), and tuberculosis (Meade et al., 2006) in livestock. Considering the previous use of PBMCs for the diagnosis of a variety of diseases and the cells' vital role as triggers of sickness response, these ciculating mononuclear leukocytes might carry transcriptome-level information regarding the presence of inflammatory events at the cow's claw and thus aid lameness screening in dairy herds.

Because of limitations imposed by the subjectivity of current methods used for lameness screening in dairy cows and the consequent negative impact to cattle welfare

and the dairy industry, the main objective of this Ph.D. dissertation research was to discover candidate biomarkers with potential to assist with objective diagnosis of lame cows suffering from inflammatory and painful foot lesions. This was accomplished by completing three related studies, which were entitled: i) Early detection of lameness in heifers with hairy heel warts using a pressure plate; ii) Depressed DHEA and increased sickness response behaviors in lame dairy cows with inflammatory foot lesions; and iii) Signature gene expression of peripheral mononuclear cells in lame dairy cows with inflammatory foot lesions. These studies are described in Chapters Two, Three and Four of this dissertation.

The overall hypothesis explored in this dissertation was that lame cows with painful inflammatory foot lesions have behavioral, biomechanical and physiological changes that are amenable to objective measurement. This hypothesis was tested under the following sub-hypotheses:

i) a pressure plate is a sensitive tool for early detection of lameness in heifers suffering from *papillomatous digital dermatitis* compared to Sprecher's (1997) lameness scoring system;

ii) circulating concentrations of key inflammation regulating steroids (cortisol and DHEA) and indicators of PBMC activation (gene expression for IL-1β, CD62L, MMP-9, POMC and GRα) are altered in lame cows showing sickness behavior and foot lesions compared to healthy sound cows;

iii) cows suffering from inflammatory and painful type of lameness derived from foot lesions have a signature gene expression profile of PBMC that is distinguishable from that of healthy sound cows.

CHAPTER ONE

A REVIEW OF THE LITERATURE

I. LAMENESS IN DAIRY CATTLE

Today's dairy cows face different environmental and management challenges relative to the cows of the past. High energy rations, confinement on concrete, constant exposure to corrosive conditions, conformation defects, and perhaps even increased body size are some risk factors that increase the probability of "production diseases". Production diseases are traditionally induced by management mistakes made by the modern dairy industry to maximize profit (Markusfeld, 2003). For example, inappropriate management of clinical digital diseases in cows increases the risk of metabolic disorders (Enting et al., 1997); providing cows with inappropriate space for resting and and improper hygiene (Sogstad et al., 2006) increase the incidence of lesions at the tarsus and teat injuries which predispose to lameness and subclinical or clinical mastitis (Østerås and Lund, 1988; Rajala-Schultz and Gröhn, 1999). Despite an increased awareness of such factors, high levels of production diseases in dairy herds have persisted into the 21st century.

Lameness in cattle is a debilitating condition, which is often associated with tissue damage, pain, and discomfort, and manifests as an inability to walk normally (O'Callaghan, 2002). Worldwide, 60% of a herd may become lame at least once a year (Vermunt, 2005). Consequently, economic losses and cow welfare issues are alarming concerns for the dairy industry. Problems such as inaccurate lameness diagnosis, the paucity of licensed veterinary products available for lameness treatment, and producer

desensitization to lameness occurrence in their herd may exacerbate the problem (Wells et al., 1993; Mill and Ward, 1994; Whay et al., 2003, O'Callaghan, 2002). Hence, research targeting prevention, diagnosis, and treatment of lameness are timely and paramount.

A. Pain and welfare

Lame cows do suffer from behavioral-modifying pain and prolonged discomfort, in spite of the widespread belief amongst the general public that cattle are relatively insensitive to pain (O'Callaghan, 2002). This belief stems from cattle's instinctively stoical nature, a survival strategy used by prey species in the wild to divert the attention of a predator away from a sick or injured animal. This makes the identification of injury or disease in cattle difficult until a condition is at an advanced stage (O'Callaghan, 2002). Furthermore, the inattention to cattle's pain might be due to a general lack of understanding regarding ethical questions. Ethical questions posed by society and philosophers are often considered as merely emotional outbursts by the livestock industry, a means of threatening agricultural jobs, current livestock practices, and thus the sustainability of animal agriculture. As a consequence, concerned citizens on both sides of the debate tend to be irrational about animal welfare issues. This is confounded by the fact that animals are not part of the vocal constituency and are thus unable to press for a reasonable resolution to the problems surrounding their welfare (Rollin, 1989).

The physiological mechanisms associated with pain perception are complex and remain poorly understood (Hutchinson et al., 2004). Several biological factors contribute to a substantial inter-individual variability in nociception (Hardy et al., 1967). However,

despite the difficulties in determining if an animal is in pain, lame cows do have an altered (exacerbated) pain indicative of primary hyperalgesia (increased sensitivity to noxious stimuli in the vicinity of an injury) for a period up to 28 days following apparent lameness resolution compared to sound cows (Whay et al., 1998). The hyperalgesia phenomenon occurs due to a cascade of sensitizing events that follow tissue injury. For example, damaged cells and primary afferent fibers release a number of chemical mediators, including substance P, neurokinin A, and calcitonin gene-related peptide which have direct effects on the excitability of sensory and sympathetic fibers (Siddall and Cousins, 1997; Woolf, 1989). Moreover, immune cells that migrate to the injury site have a role in modulating pain (Watkins and Maier, 2000) because they contribute to sensitization of peripheral nerve system by releasing inflammatory mediators such as proinflammatory cytokines, reactive oxygen and nitrogen species (i.e., free radicals), norepinephrin, bradykinin, histamine, potassium ions, serotonin, and products from the cyclo-oxygenase and lipoxygenase pathways of arachidonic acid metabolism (Dray, 1995; Levine et al., 1993; Siddall and Cousins, 1995; 1997). These molecules act synergistically rather than individually, generating what is often referred to as a 'sensitizing soup' that effectively lowers the response threshold for nociceptive fibers (Aδ and C) activation which results in hyperalgesia (Treede et al., 1992).

Considering that lame cows do experience pain, welfare issues in dairy systems must be carefully considered. Lame cows display behavioral changes such as increased lying time, decreased food-intake, hyperalgesia, and altered social behaviors (Singh et al., 1993; 1994; Galindo and Broom, 2002; Sauter-Louis et al., 2004; Whay et al., 1998). These modifications might prevent lame animals from meeting their range of 'needs' that

bring equilibrium to functional systems. A 'need' is defined as a deficiency in an animal that can be remedied by obtaining a particular resource or responding to a particular environmental or bodily stimulus (Fraser and Broom, 1990). If an animal is in need, its motivational state is affected so that behavioral and physiological coping responses that remedy the need can be made. Coping responses allow the animal to control and maintain mental and physical stability. It includes normal regulation of body state and emergency responses, such as high adrenal activity, heart rate, or flight activity, which requires more energy expenditure and hence is used only when the animal predicts that normal regulatory actions will be inadequate (Broom, 1991). Owing to the lame animal's impaired state and inability to properly cope with its environment, lameness represents a serious animal welfare concern (Broom, 1986) that must be addressed.

B. Economic impacts

Lameness continues to be one of the largest financial drains on the dairy industry. Lameness in dairy cows is second only to mastitis in terms of its detrimental effect on herd productivity (Esslemont and Kossaibati, 1996). In the United States, lameness costs on average between US$300 to $400 per incident (Greenough and Weaver, 1997). The United States Department of Agriculture (USDA) reports a national incidence rate of 12% (Morrow, 2002), which is equivalent to 9 million dairy cows (Amstutz, 1985; Greenough and Weaver, 1997), and amounts to an annual financial loss of approximately US$300 million. As a result of its high incidence, prominent economic losses, and the pain and discomfort that lame animals experience, lameness has been identified as a

principal health issue by the Animal Welfare Information Center of the USDA (Morrow, 2002).

The ultimate cost of a case of lameness is attributable in part to the costs of treatment and control methods (Britt et al., 1996; Shearer and Hernandez, 2000; Hernandez et al., 1999). However, numerous indirect costs that substantially increase losses from lameness also must be considered. For example, lameness causes profound decreases in milk yield (Rajala-Schultz et al., 1999; Warnick et al., 2001; Hernandez et al., 2005). In an examination of the impact of clinical lameness (including sole ulcers, white line disease, interdigital dermatitis and papillomatous digital dermatitis) on the milk yield of dairy cows, Green et al. (2002) found that milk loss per affected cow averaged 360 kg with a 95% confidence interval that ranged from 160 to 550 kg. Similarly, Hernandez et al. (2002) reported a 10% decrease in milk production in lame cows with interdigital phlegmon compared to sound cows and also demonstrated a linear relationship between increasing degree of lameness and decreasing milk yield among cows in their second or later lactations (Hernandez et al., 2005). Furthermore, lameness impairs reproductive performance (Lucey et al., 1986; Collick et al., 1989; Lee et al., 1989; Hernandez et al., 2001) with lame cows having longer calving-to-conception intervals than sound cows (Hernandez et al., 2005). Lame cows also have an increased susceptibility to other diseases such as mastitis (Peeler et al., 1994), and increased risk for involuntary culling (Collick et al., 1989; Esslemont and Kossaibati, 1997). A recent study by Hadley et al. (2006) demonstrated that the most common reason for culling across 10 states of the U.S.A. were injury related conditions (26.9%), reproductive problems (18.9%), low production (12.8%), mastitis (12.1%) and mortality (10.6%). In the

National Animal Health Monitoring System Dairy 1996 Study, lameness alone was reported as the reason for culling 15% of dairy cows sent to slaughter.

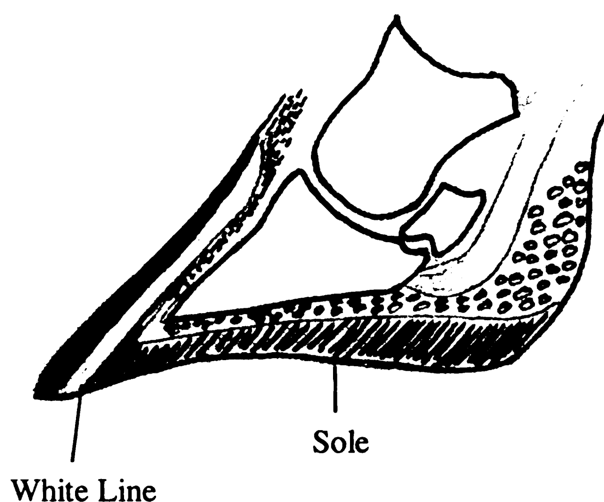
C. Epidemiological considerations

There is high variability in reported incidence of bovine lameness and few studies have been performed on its prevalence in the dairy cow population, especially in high-producing Holstein cows (Espejo et al., 2006). Lameness incidence estimates of between 12% (Morrow, 2002) to 50% (Kopcha et al., 2003; Espejo et al., 2006) have been made in the United States. This variability in lameness incidence is reflected in worldwide estimates, such as the 0% to 50% incidence reported by Harris et al. (1988) in Australia, 9% to 50% in the Netherlands reported by Barkema et al. (1994), and 5% to 70% reported in the United Kingdom by Eddy and Scott (1980) and Hedges et al. (2001). Difficulty in defining clinical lameness in dairy cows may in part explain this high variability in reported incidence. Parlor workers, farm managers, veterinarians, and research workers have been used to identify lame cows both within (Barkema et al., 1994; Clarkson et al., 1996) and between (Lucey et al., 1986; Hedges et al., 2001) farms. Part of the variation also may be attributed to the different skills of personnel responsible for identifying lame cows. Large variability also occurs in the incidence and types of lameness across farms (Barkema et al., 1994; Hedges et al., 2001). The imprecise definition of lameness (Martin et al., 1987) and the use of subjective methods for lameness screening (Thomsen and Baadsgaard, 2006) cause misclassification: lame cows defined as non-lame and vice versa. These factors also may cause downward bias in identifying whether or when a cow becomes lame (Martin et al., 1987). If true the

impacts of lameness on cow welfare, production, and economic loss are likely to be significantly underestimated.

Almost 90% of lameness incidents arise from abnormalities of the claw (Weaver, 2000) with an occurrence of 92% of lesions in the hind feet and 65% in the lateral claw (Murray et al., 1996). The most common lesions and abnormalities are white line disease, sole ulcer, toe ulcer, heel erosion, double sole, and sole hemorrhage (Russell et al., 1982; Murray et al., 1996; Kopcha et al., 2003). Sole ulcers (40%) and white line lesions (29%) are suggested to be the predominant diseases of horn, and digital dermatitis (40%) is the most common skin disease (Murray et al., 1996). Lesions of the claw horn (**Figure 1.1**) are suggested to be the most intractable cause of lameness, which develop initially as hemorrhages of the sole and white line and can progress to solar ulceration and white line separation (Murray et al., 1996; Vermunt and Greenough, 1996). Abnormalities on a claw can be inter-correlated (Manske et al., 2002). For example, animals with heel horn lesions are likely to show erosive dermatitis while animals with sole hemorrhages are also likely to have white-line disease on the same claws (Manske et al., 2002). Furthermore, ulceration of the sole and toe, white line disease, and heel erosion are usually associated with a general condition termed laminitis (Greenough et al., 1981), which results from insult to the vascular tissue of the foot. The correlation between lesions suggests that factors impairing the structural integrity of the claw are associated with multiple pathologies.

Figure 1.1 Most common sites of lameness lesions within the claw: white line and sole. Drawing by P.E. Almeida.



Detailed studies of cohorts of animals (Leonard et al., 1996, Leach et al., 1997) and epidemiological investigations (Clarkson et al., 1996) have identified several potential risk factors contributing to lameness. Lameness is likely affected by a complex array of factors (Vermunt and Greenough, 1994; Manske et al., 2002; Cook, 2003), which include animal behavior and social interaction among animals (Leonard et al., 1996), body conformation, contagious agents, diet, genetics, housing systems, hygiene, and management (Bergsten, 2001). For example, foot and leg disorders that result in lameness tend to increase with more confined management systems and increased production (Bergsten, 2001). A number of these factors, either alone or in conjunction with one another, influence the severity and the prevalence of lameness (Hirst et al., 2002). However, the relationships between these factors have not been well studied with regard to their impact on lameness, and thus the etiology of lameness is poorly understood.

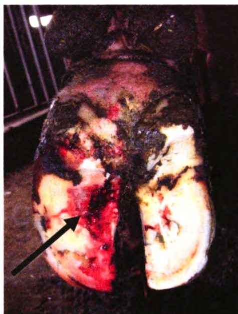
Among all housing systems, solid concrete floors are associated with an increased prevalence of hoof lesions when compared with straw yards around first calving (Webster, 2002), straw yards for milking cows (Somers et al., 2003), slatted-concrete

floors (Frankena et al., 1992), and rubber slats (Hultgren and Bergsten, 2001). Increased hoof lesions are associated with reduced lying times (Leonard et al., 1996), discomfort lying, and the presence of high steps and slopes in housing (Philipot et al., 1994). Moreover, freestall barns appears to be more detrimental to hoof health compared with tie-stall barns or straw yards (Cook, 2003; Sogstad et al., 2005), and there is convincing evidence that the incidence of claw horn lesions might be greater for cows housed in cubicles than in straw yards (Livesey et al., 1998; Webster, 2001). Studies in Wisconsin have shown that cows in sand-based stalls have a lower prevalence of lameness than cows in mattress-based stalls (Cook, 2003; Cook et al., 2004). This difference may be attributed in part to the physical surface of the lying and walking areas (Bergsten and Frank, 1996; Faull et al., 1996) and in part to the fact that cows in straw yards spend more time lying down (Singh et al., 1993).

Hemorrhagic lesions of the sole (**Figure 1.2**) and white line typically start to develop around the time of parturition and tend to be most severe in the early weeks of lactation (Bergsten and Frank, 1996; Leach et al., 1998; Livesey et al., 1998; Offer et al., 2000). A typical dairy cow or heifer that develops claw horn lesions during the first weeks of lactation will have experienced marked changes in both housing and feeding. For example, cows move from pasture or dry-cow accommodations in a straw yard into cubicle house, and eat more starch and protein through concentrate ration feeding. These peripartum animals also will have experienced major hormonal changes associated with physiological processes of parturition and the onset of lactation. Therefore, any resolution of the problem of claw horn lesions in early lactation requires an analysis of the relative

importance of these three risk factors: housing, nutrition, and parturition itself (Webster, 2002).

Figure 1.2 Hemorrhagic sole ulcer lesion on the lateral claw of a cow's left hindlimb (Picture by P.E. Almeida at the Kellogg Biological Station Dairy Center – Michigan State University).



The profound physiological changes that occur around calving and lactogenesis, likely, influence connective tissue metabolism. In particular, blood concentrations of the hormones estrogen and relaxin are increased during calving, are these regulate matrix remodeling (Winn et al., 1994). As the name suggests, relaxin is associated with increased laxity of connective tissues, particularly those of the birth canal. However, its influence is systemic and is known to slacken other structures, such as cruciate ligaments in humans (Blecher, 1998). Estrogen is considered to have an anabolic influence on connective tissue metabolism, via induction of TGF-β (Ashcroft et al., 1997). It has pro-fibrotic influence in hormone-replacement therapy, and may increase the risk of fibrotic diseases such as scleroderma (Beebe et al., 1997). Contrarily, estrogen is also reported to

reduce fibroblast proliferation and collagen synthesis in cruciate ligaments (Liu et al., 1997). Therefore, either or both of these hormones are suggested to contribute to the pathogenesis of claw horn lesions and that the events associated with calving and the onset of lactation are primary factors in the etiology of lameness in dairy cattle (Tarlton et al., 2002).

Also, increased matrix metalloproteinase (MMP) activity in the circulation (Weber et al., 2006b) and in circulating leukocytes (Burton et al., 2005) occurs with the onset of labor in association with the need for reproductive tract and pelvic ligament softening, and may also contribute to lameness susceptibility early postpartum. Indeed, biochemical, biomechanical, and histological changes in feet of primiparous cows support this hypothesis (Tarlton et al., 2002). In that study, histological changes were observed as early as two weeks prepartum (**Figure 1.3**) and were accompanied by an increase in laxity of the connective tissue supporting the third phalanx within the hoof (**Figure 1.4**). Regional variations in connective tissue strength of the hoof are associated with metabolic and compositional differences in total protein, collagen, proteoglycan, fat, and water content, as well as the key regulatory molecules that mediate changes in them leading to altered structure and composition of the connective tissue, the MMPs and their natural tissue inhibitors the TIMPs. In turn, reduced suspensory support for the third phalanx is expected to lead to greater loading of the sole, and may cause bruising and pain. However, when external forces on the sole are greatest, such as when heavy (e.g., pregnant) animals stand for long periods on concrete, these will be most severe, and may progress to a sole ulcer. The swelling and distortion of the laminae and corium are likely

to increase lateral pressure on the hoof wall, and therefore predispose to white line separation (Tarlton et al., 2002).

Figure 1.3 Histological appearance of the laminated region of the anterior hoof wall of hind lateral claw. Corium (Co) stained red, and horn (Ho) stained yellow, (a) maiden heifer, (b) two weeks prepartum, (c) four weeks postpartum, (d) twelve weeks postpartum. Maiden heifer laminae were straight with narrow corium. A widening and distortion of the interdigitating laminae at four and twelve weeks can be seen. An apparent reduction in overall collagenous matrix of the corium with increased matrix thickening is shown (arrows). From Tarlton et al. (2002)

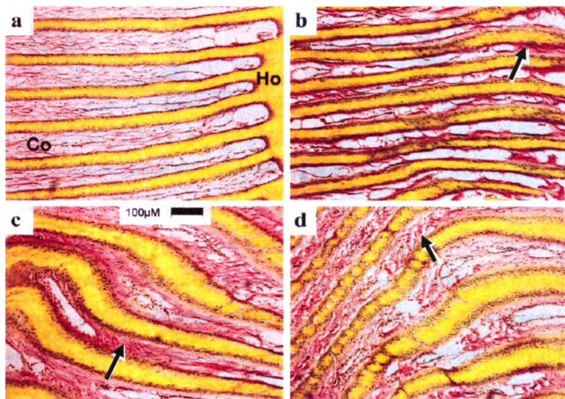
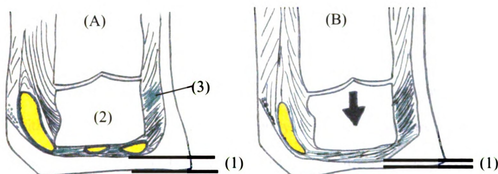


Figure 1.4 Transverse section of a cow's claw (A) without and (B) with distal displacement of the pedal bone. (1) Vertical displacement of the corium on the inner surface of the horn capsule. The dark arrow on (B) indicates the force acting on the pedal bone (2) that causes its sinking. The suspensory apparatus (3) is the main structure responsible for suspending the pedal bone. Modified by P.E. Almeida from Lischer et al. (2002).



In addition, supplementation with minerals and vitamins seem to affect the occurrence of lameness. For instance, supplementation with biotin is associated with a reduction in lameness, especially white line disease (Hedges et al., 2001; Amory et al., 2006). In addition to nutrition (e.g., increase period of feeding corn silage) (Faye and Lescourret, 1989), farms that have foot baths and hoof-trimming apparatus are reported to have less lameness than farms that do not have these features available (Amory et al., 2006).

Finally, although much of the feet and leg health is associated with environmental effects (e.g., management and housing), studies have revealed genetic sources of variation in diseases of the foot and leg (Distl et al., 1990; Huang and Shanks, 1995). For examples, traits such as foot angle, hoof diagonal, and rear leg set have shown genetic associations with certain foot diseases (Distl et al., 1990). Therefore, genetic selection

may represent an important strategy to decrease the incidence of lameness in dairy cows (Boettcher et al., 1998).

D. Treatment

People who have been trained to trim cows' feet and treat lame cattle (i.e. foot trimmers and veterinary surgeons) play an important role in lameness management on many farms (O'Callaghan et al., 2004). Their perceptions and working practices when dealing with lame cattle are likely to be inherently related to the welfare of the lame cow (O'Callaghan, 2002). Treatment of claw horn lesions usually involves physical restraint of the affected limb while excessive or defective horn is removed. In this scenario, the sensitive corium is often exposed (Blowey, 1993) so pain is an important consideration. As foot trimmers do not have the training or authority to administer anesthetics during treatment of foot lesions they cannot alleviate the acute pain associated with this procedure. The options available to the foot trimmer when presented with diseased feet are to advise veterinary attention or to proceed despite legal restrictions. Those who choose to proceed rely on good trimming technique and unloading of weight bearing by diseased claws to limit pain (O'Callaghan et al., 2004).

Once lameness occurs, limited treatments are available for dairy cows. Unlike foot trimmers, veterinary surgeons can prescribe and administer analgesic, anesthetic, or sedative drugs to alleviate pain or stress. Pharmacological interventions that produce pain relief mainly by restoring nociceptive sensitivity to its resting state (Dayson and Marks, 2003; Huxley and Whay, 2004; Skarda, 1996) are difficult to use in the dairy industry because of government food and drug regulations. Only one local anesthetic drug,

procaine hydrochloride (Willcain; Arnolds Veterinary Products Ltd) and two non-steroidal anti-inflammatory drugs (NSAID), ketoprofen (Ketofen® 10%; Merial Animal Health Ltd, Essex, UK) and flunixin meglumine (Meflosyl 5%; Fort Dodge Animal Health, or Banamine; Schering-Plough Animal Health, Middlesex, UK) are currently licensed for use in lactating cattle. However, despite legal requirements to prevent unnecessary pain, and evidence that administration of NSAID in addition to normal treatments for lameness in cattle results in significant reduction in the level of hyperalgesia after treatment (Whay et al., 1998), the pain control potentially afforded by these drugs is not fully utilized by veterinary surgeons when treating lame cattle (O'Callaghan et al., 2004). Limited use of analgesics in cattle may be due to low client expectation regarding pain control and reluctance to withhold milk (which is unnecessary for Ketoprofen). As such, the principal approach to treating lameness is by claw trimming (**Table 1.1**).

Table 1.1 Treatment practices for common lameness pathologies (Greenough, 1987).

Pathology	**Etiology**	**Treatment**
Interdigital phlegmon (foot rot)	Claw injury and *Fusobacterium necrophorum*	Systemic antibiotics
Interdigital dermititis	*Dichelobacter nodosum*	Cleansing and topical antibiotics
Digital dermititis (hairy heel wart)	Unknown	Cleansing and topical oxytetracycline
Pododermititis circumscripta (sole ulcer)	Claw injury, laxity of suspensory apparatus due to physiological changes around parturition	Therapeutic trimming, elevation of the sound claw
White line disease	Mechanical injury, foreign body, maybe secondary to sole ulcer, high energy feed	Cleansing and drainage, therapeutic trimming

Trimming for treatment is different than for prevention of lameness. Where preventative trimming aims to optimize weight distribution within the hoof minimizing strain injuries (Toussaint-Raven et al., 1985), therapeutic trimming is carried out to clean the injured area from necrotic tissue and expose the lesion to facilitate healing and treatment delivery. The importance of trimming as a preventive method for claw horn lesions was demonstrated by Manske et al. (2002) where abnormal claw shape was strongly associated with sole ulcer (r^2= 0.41 at cow level)-suggesting the importance of maintaining a correct claw shape for the prevention of hoof-horn lesions.

Once the lesion has been treated, claw blocks, rubber coatings and bandages have all been used to aid recovery. For instance, corrective trimming for ulcers involves creating a steep slope of the horn around the ulcer, taking care not to damage the corium. This procedure is performed to avoid entrapment of dirt in the ulcer area. Moreover, the elevation of the sound claw, by application of a claw block to relieve all weight bearing

from the affected area and facilitate healing (Greenough, 1987), is also an important treatment practice.

II. LAMENESS ASSESSMENT IN DAIRY CATTLE

Lameness alters locomotion such that the supporting or swinging limb periods, or both, are shortened, so that the stride length is reduced. Reduction in the supporting limbs period is most common with acutely painful lesions such as sole abscess (Phillips, 1998). When the swinging limb period is shortened increased abduction or adduction often occurs as the animals attempt to decrease load bearing by a particular part of the hoof. Some unevenness of gait is evident before and after each lameness incident, and in total the animal's gait may be affected for three months, with clinical lameness being apparent for an average of eight weeks (Phillips, 2002).

There are several methods available for lameness assessment, ranging from the more qualitative (e.g., lameness scoring scales) to the more quantitative biomechanical approaches (e.g., automated motion analysis systems), each which detects abnormalities of locomotion. Qualitative biomechanical applications in cattle have been adopted by the dairy industry for use in the field, whereas the more quantitative techniques have rarely been used.

The deviations in gait observed in lame cattle are to avoid pain associated with injuries on the feet and legs (Whay et al., 1998). However, much of the variation reported for both objective and subjective measures of gait also could be due to factors other than pain, including damage in locomotor system (muscles, ligaments, nerves) (Greenough et al., 1981), natural variation in gait among cows, and physical constraints to normal gait

such as a distended udder (Flower et al., 2006). The obvious method for determining which gait attributes are specifically associated with pain would be to observe the effect of an analgesic on behavior of an animal with impaired gait (Rutherford, 2002). Unfortunately, little work on this issue has been published for cattle. Therefore validation of gait measures that correlate with pain as objective measurement techniques to diagnosis of lameness associated with tissue injury and pain, are urgently needed. Similarly, available information derived from studies performed in horses and broilers employed subjective and objective diagnostic approaches to evaluate the potency of analgesics (Owens et al., 1995; McGeown et al., 1999) but failed to focus specifically on the gait characteristics associated with pain. Objective measures of gait changes with significant correlation to pain could be used for both treatment development and monitoring. In addition these measures could be able to assist with screening of cows suffering from lameness type pain and welfare issues.

A. Qualitative lameness assessment

There are several qualitative methods for assessing lameness using locomotion scoring scales. These scales are based on qualitative assessments of kinematic measures, which are descriptions of motion during standing and walking that range from a few measures of back posture and stride length (Sprecher et al., 1997; see **Figure 1.5**) to a combination of back posture, leg abduction, limb weight bearing, joint flexion, relative front to hind foot placement, head carriage and 'smoothness' of gait (Welsh et al., 1993). Other scales use a combination of these measures (e.g. Morrow, 1966a; b; Manson and Leaver, 1988a; b; Whay et al., 1997; Kestin et al., 1992; Wells et al., 1993; May and

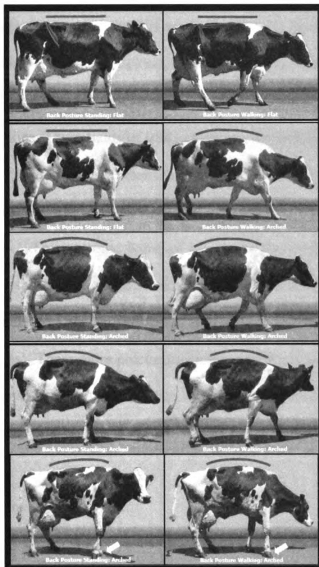
Wyn-Jones, 1987). The behavioral measures used by the various scoring scales to establish the severity of lameness may also indicate the painfulness of the condition, such as a humped back, unwillingness to move, and changed stride length of the lame limb. Scoring scales often show good correlation to other measures such as activity level (O'Callaghan et al., 2003). The relationship between lameness scoring and fertility (Sprecher et al., 1997) suggests that such systems may be useful predictors of the systemic differences between severities of lameness. However, although lameness scoring scales benefit from simplicity and ease of use in the field, these scales are subjective in nature and hinder lameness diagnostic accuracy.

Overall, lameness scorings have varied between (inter)- and within (intra)-observer reproducibility of diagnosis depending on the type of lameness lesion involved and the lameness score system in question. As with any subjective technique, scoring systems can vary in reliability. For example, numerical rating scales has been shown to correctly discriminate healthy cows from those with sole ulcers ($R^2 = 0.73$) in 92% of cases with reasonable intra- and inter-observer reliabilities ($R^2 \geq 0.64$) (Flower and Weary, 2006). However, other studies report correct diagnosis of only 22% (van Eerdenburg et al., 2003) and 48% (Whay et al., 1997) of lameness cases. As an aggravating factor, even the same observer may not score the gait of a cow the same on 2 occasions. For example, O'Callaghan et al. (2003) reported that a trained observer rescoring the gait of 129 cows was consistent for only 56% of observations. In addition, a lack of agreement between observers also has been reported; O'Callaghan et al. (2003) found only 37% agreement in the scores of 2 observers, and Winckler and Willen (2001) found 68% agreement among scores of 3 observers. Moreover, subjective lameness

scoring systems were demonstrated to lack sensitivity to detect subtle changes in posture and weight bearing (Flecknell and Molony, 1997; Whay et al., 1997; O'Callaghan et al., 2003).

By using lameness scoring scales, producers misdiagnose lameness in 50 to 55% of the cases (Wells et al., 1993). Others have found that the probability of observer and expert agreement on exact estimates of 9 classes of gait scores ranged between 25 and 47% and rose to 80% if one class difference was permitted between observer and expert (Engel et al., 2003). Observer training inconsistently improved the agreement between observer and expert estimates, but the probability of agreement on exact score estimates remained low at 47% (Engel et al., 2003). With these pitfalls in mind, methods of objective, automated scoring may enable earlier, more accurate detection of lameness which could lead to more efficacious practices that reduce animal welfare issues in commercial dairy units.

Figure 1.5 Example of a locomotion scoring scale (Sprecher et al., 1997). During standing and walking, based on back posture, and stride length where appropriate, cows can be graded from sound (score 1) to severely lame (score 5). Courtesy of Zinpro® Corporation, Eden Prairie, MN and used with permission from the company.



1 – Standing and walking flat

2 – Standing flat and walking arched

3 – Standing and walking arched. Gait is short strided

4 – Standing and walking arched. Cow favors one or more legs/feet

5 – Standing and walking arched. Cow demonstrates an inability, or extreme reluctance to bear weight on one or more limbs/feet

B. Quantitative lameness assessment

Quantitative assessment of locomotion is based on the use of biomechanical techniques. Biomechanics is the study of the causes and description of motion. This is referred to as gait analysis when the mode of locomotion, principally walking, is used. Taking a broader definition, biomechanics can encompass all motions, including eating, bullying, lying, standing, walking, trotting, cantering, galloping and transitions between motions. Three fields of study comprise biomechanics: kinetics is the study of the forces that cause motion; kinematics is the description of motion caused by forces, and neuromuscular analysis is the study of the functional anatomy of the animal.

To overcome some of the limitations and subjectiveness of lameness scoring scales, objective and technologically advanced methods may be used to improve accuracy of measures for lameness detection (e.g., stride length, stance time, and weight-bearing forces). These more precise measures, which will be expanded upon below, may provide means for effective detection of lameness, treatment monitoring, and development of lameness therapies.

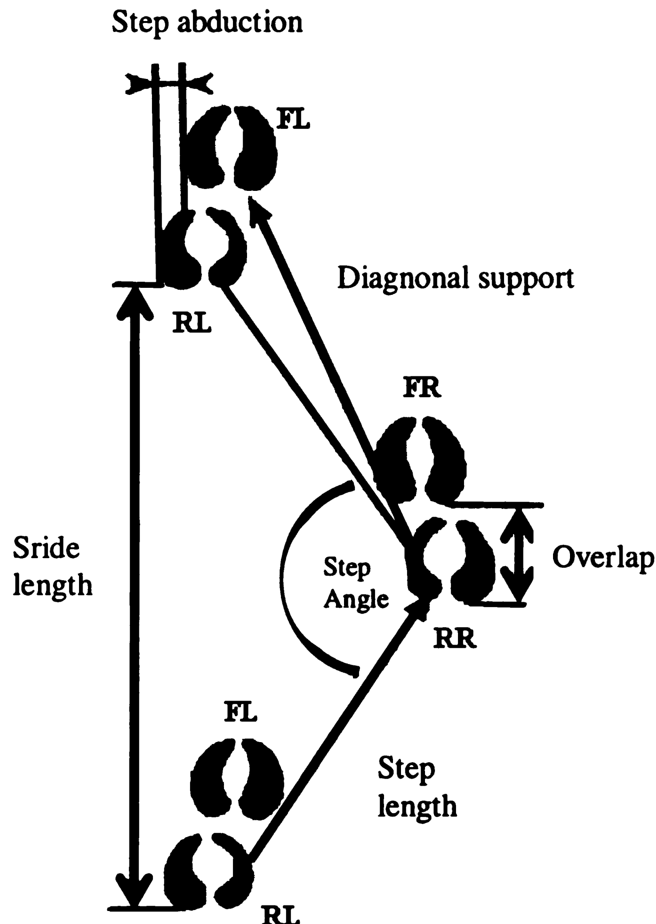
i. Kinematics

Kinematic variables that describe motion principally include linear and angular measurements of position (e.g. distance from an origin, or joint angle), displacement (change in position), velocity (rate of change of displacement) and accelerations (rate of change of velocity). At walk, for example, the position of a cow's feet (illustrated in **Figure 1.6**) enables the measurement of a number of other stride parameters (e.g. step abduction, overlap, stride length, step length) (Telezhenko et al., 2002). These parameters

can be used to objectively measure locomotor changes that occur with lameness and therefore assist with lameness diagnosis (Herlin an Drevemo, 1997; Flower et al., 2005). Kinematics also can be useful to objectively evaluate the effect of different types of flooring on cow's locomotion. For example, Telezhenko et al. (2002) identified kinematic differences between slatted and solid floors, where slatted floors were suggested to impair locomotion and require more energy.

Additional kinematic variables that can be recorded in such analyses include timing of events, trajectories of positions, jerk (rate of change of acceleration) and snap (rate of change of jerk). In fact, an infinite number of potential kinematic variables are possible, depending on which position is selected on the animal. Typically, positions are constrained to segment end points, bony landmarks, and centers of rotation of joints, which together still provide a vast number of potential variables. These variables can be measured using basic equipment (e.g. ruler and stopwatches to measure distances and speeds; goniometers to measure joint angles), consumer equipment (e.g. digital cameras in combination with picture software) or specialized equipment (e.g. automated motion analysis systems, accelerometers, force platforms).

Figure 1.6 Stride parameter measurements (modified by P.E. Almeida from Telezhenko et al., 2002). In addition to the six variables indicated, step asymmetry was measured as the difference between two consecutive steps. FL = left forelimb; RL = right hindlimb; FR = right forelimb; RR = right hindlimb.



ii. Kinetics

Several methods of can measure forces both directly and indirectly. Direct measurements can be made using force platforms, force transducers, pressure plates and strain gauges. Indirect measurements of forces can be obtained mathematically from theoretical muscle-force models, or through inverse dynamics of accelerometer or other kinematic data. The ability to measure forces is important as the stance phase of the limb has been considered the most susceptible time to injury occurrence rather than during the

swing phase of the limb (Peloso, 1994) and also an important indicator of locomotor abnormalities associated with lameness and pain (Rushen et al., 2006), as cows tend to remove the weight from the injured leg while walking and standing (Scott, 1989; Neveux et al., 2006).

The most common device for measuring forces, particularly those exerted between the hoof and ground, is a force platform. This instrument is embedded flush in the ground and measures forces in all three orthogonal directions (i.e. vertical, anterior-posterior, medio-lateral). A force platform collect data on loading forces, including ground reaction forces, loading rates, location of force (in combination with knowledge of foot position), and stance times. The peak vertical force is used most frequently. For adult cows, the ratio of fore- to hindlimb peak vertical forces is similar between studies, ranging from 4.95 to 5.61 body-mass (BM) in the forelimb and 3.64 to 4.96 BM in the hindlimb (**Table 1.2**).

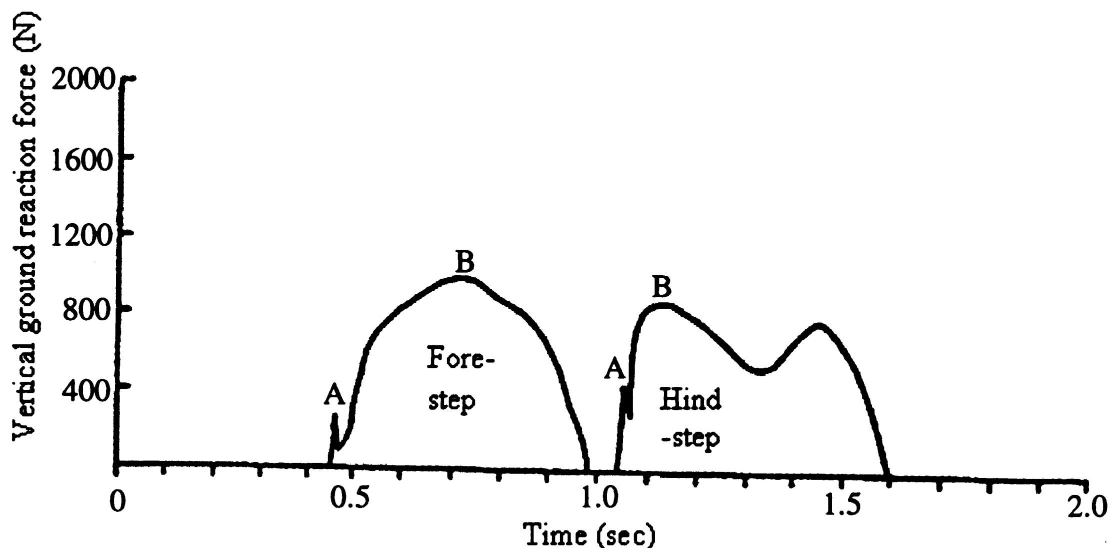
Table 1.2 Comparison of studies measuring peak vertical forces in sound cows. N = Newton; n = number of animals included in the study; BM = body mass.

Peak vertical force				Body mass	n	Breed	Reference
Fore (BM)	Hind (BM)	Fore (N)	Hind (N)	(kg)			
5.61	4.96	3537	3132	631	9	Holstein-Friesian	van der Tol et al. (2005)
5.48	4.57	3301	2753	602	8	Friesian	Scott (1989)
4.95	3.64	3324	2444	671	9	Holstein-Friesian	van der Tol et al. (2003)
5.42	4.25	N/A	N/A	N/A	3	Holstein	Rajkondawar et al. (2002)

In six sound maiden heifers, force platform readings were recorded monthly for 6 months (Scott, 1988). The vertical force in the front limbs produced a modal pattern and experienced a peak value of approximately 62% of body weight. In the hindlimbs the pattern was bimodal and the peak values were approximately 50% of body weight. These

fore- and hind-limb patterns are illustrated in **Figure 1.7** where, in addition to small impact spikes, a short time difference between the limbs typical of sound animals can be seen. In addition, Scott (1989) demonstrated that peak vertical force is linearly increased with body mass both intra- and inter-cow, which supports the recommendation to account for different animal masses by normalizing peak vertical forces by dividing by body mass (Clayton and Schamhardt, 2001).

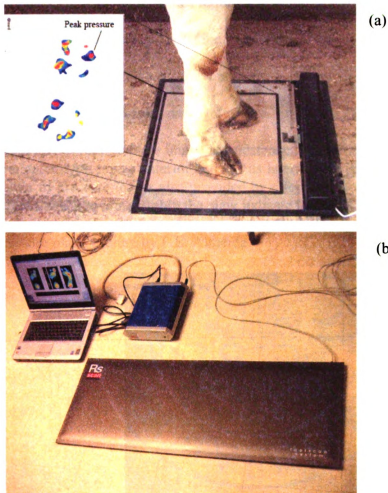
Figure 1.7 Vertical force for fore- and hind-limbs for a sound animal (from Scott, 1988). Note the modal forelimb, short time difference between limb contacts, bimodal hindlimb, lower hindlimb values and impact spikes for each limb, all of which might be typical of sound animals. A = impact spike; B = peak vertical ground reaction force.



In another study, ground reaction forces in 4 lame and 8 sound Friesian cattle were measured (Scott, 1989). Peak vertical force was considered to be a sensitive measure for detecting lameness, which was often reduced in the lame limb. The potential of force plate technology to detect lameness has resulted in the commercialization of a lameness detection system for the dairy industry. This device is called the SoftSeparator (Tasch and Rajkondawar, 2004). It focuses on one variable (i.e. vertical force) and uses an algorithm to separate the data for several animals moving over the device at one time.

An alternative to a force plate is a pressure plate, which can also be used to measure vertical force. Typical systems possess 1 or 4 sensors per cm^2, cover 20 x 20 to 100 x 200 cm areas and collect data from each sensor at between 40 and 500 observations per second. A photograph of two systems and the pressure distribution of the four front claws of a standing cow are illustrated in **Figure 1.8**. Pressure plates measure the pressure exerted between the ground and areas under each claw for sound cattle standing, walking and pre-post trimming (van der Tol et al., 2002; 2003; 2004, respectively – used in combination with a force platform underneath to provide calibration). Analysis of each claw could involve weight distribution, peak pressure, and location of peak pressure. Using the six regions of the claw (Smilie et al., 1999), the pressure within each region could also be determined. Although high pressure concentrations have been proposed to cause ulcers (Toussaint-Raven et al., 1985; Greenough and Weaver, 1997), no empirical studies have been performed that correlate pressure measurements to lesions.

Figure 1.8 Photograph of (a) an animal standing on a pressure plate system (Tekscan, Boston, MA; 1.44 sensors per cm^2, 34 x 47cm area and 40 Hz sampling rate). Insert shows the pressure plate profile with colors ranging from low (blue) to high (red) pressures, and the point of peak pressure is indicated; (b) a RSscan pressure plate (Footscan Scientific version, RSscan International, Olen, Belgium; 0.39 sensors per cm^2; 100 x 40 cm, 250 Hz). Pictures by P.E. Almeida.



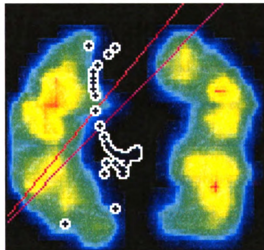
(a)

(b)

In comparison to force plates, pressure plates are less expensive and more portable. The pressure plate provides a map of vertical pressure whereas a force plate provides the net force, which is equivalent to the center of pressure profile (**Figure 1.9**). A force plate also provides horizontal forces, which in combination with vertical forces provides an even more sensitive measure to detect lameness (Scott, 1989). A pressure plate has proven to provide a quick, accurate and sensitive means to identify trimming

effects in horses (van Heel et al., 2004), to develop normative data for time-related changes in humans (de Cock et al., 2002) and to evaluate locomotion in cattle (van der Tol et al., 2002; 2003; 2004). Further work evaluating its effectiveness in detecting lameness early in cattle is still required. Placing it across the width of a single-file alley may provide valuable information. The device would need to be covered with a thin rubber mat for disguise, to prevent it from becoming slippery when wet, and to offer additional protection from damage.

Figure 1.9 Pressure plate image at midstance for a cow's left forelimb at walk. Image is orientated with top as anterior and left as lateral. The path of the net force, known as the center of pressure that is also provided by a force platform, is included (dotted black and white line). Red areas within the claw indicate the areas receiving more pressure. Red and pink lines represent force vectors from other feet in simultaneous contact with the pressure plate. Picture by P.E. Almeida.



The principles of kinetic measurements have been covered above. Variations on the applications include the use of force shoes (Rollot et al., 2004) and pressure insoles (Judy et al., 2001). In addition, strain gauges exist that usually consist of a material whose electrical resistance changes in response to a deformation (Verteramo and Seedhom, 2004). The feasibility of these techniques on live cattle is limited in

comparison to the other kinetic measures, partially owing to the need to attach the devices to the animals.

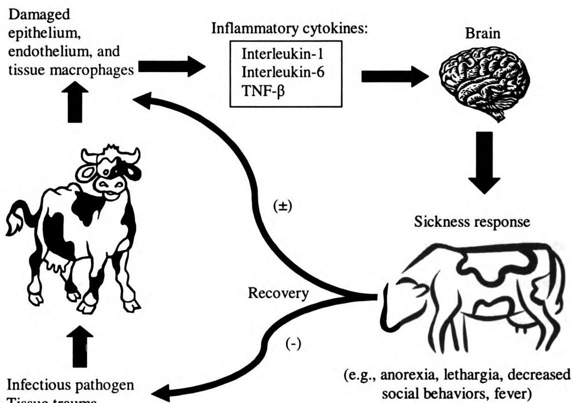
II. SICKNESS RESPONSE

In addition to gait changes, lameness can induce a classical sickness response in cattle that is related to neuroendocrine and immune reactions to infection and inflammation (e.g., fever and profound physiological and behavioral changes). These changes are adaptive, evolutionarily old, and occur across a wide range of species as an attempt to decrease body activity and so decrease energy expenditure to save energy for use in the production of fever which often facilitates recovery (Kent et al., 1992).

Sickness response classically includes body temperature, endocrine (activation of the hypothalamic-pituitary-adrenal axis and sympathetic nervous system) and behavioral changes (e.g., decreased social interaction and decreased food and water intake). Although changes in pain responsiveness have not been considered in the classic view of sickness, hyperalgesia (increased sensitivity to noxious stimuli) is now considered a natural part of the sickness response because recuperative behaviors supportive of healing would be produced by enhanced pain (Watkins et al., 1995a; 1995b). As well as behavioral changes (e.g., increased lying, decreased eating, and decreased social behaviors), cows with lameness present hyperalgesia that lasts up to 28 days after treatment (Whay et al., 1997; 1998). This hyperalgesic state supports the premise that the changes in gait, characteristic of lameness, are likely due to enhanced pain sensitivity at the injury site (i.e., foot).

The explanation for the behavioral changes observed as part of sickness response can be explained simplistically by assuming that infectious pathogens make their way to the brain where they are detected by neurons. However, many pathogens that elicit sickness behavior upon infection do not target the brain. Evidence now indicates that infectious pathogens induce sickness response by stimulating mononuclear phagocytic cells at the injury site to produce soluble proteins called pro-inflammatory cytokines (Kent et al., 1992). The pro-inflammatory cytokines interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) are synthesized in both brain and periphery. Systemic or central injection or recombinant pro-inflammatory cytokines induce a full-blown sickness behavior syndrome similar to that observed in infected animals. In essence, the immune system uses cytokines to convey information to the brain and other physiological systems about tissue injury and infection (Hart, 1988) (**Figure 1.10**).

Figure 1.10 Phagocytic cells produce pro-inflammatory cytokines when activated by pathogens. The cytokine molecules act in the brain to re-organize the animal's behavioral priorities. The resulting sickness behavior syndrome is an adaptive response that enhances the animal's immunological defenses (±) and inhibits (-) proliferation of the pathogen. Thus, sickness behavior enhances disease resistance and promotes recovery. Adapted by P.E. Almeida from Johnson (2002) and Hart (1988).



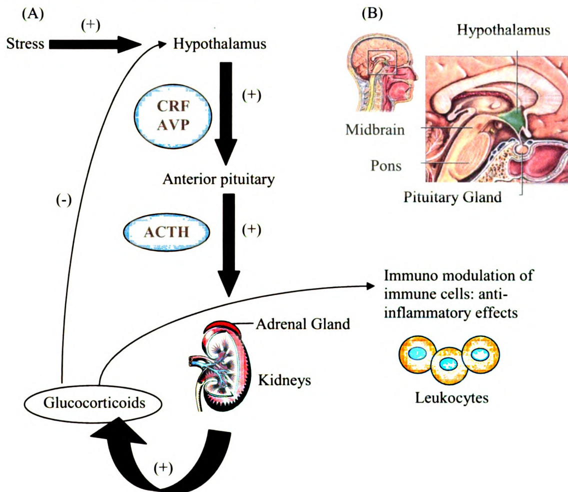
A. Mediators of the HPA axis responses to acute immune insults

As mentioned above, the proinflammatory cytokines released during immune activation relay information about the events in the periphery to the brain (hypothalamus) (Tracey, 2002), which then takes measures to re-establish "homeostasis" as quickly as possible by "adaptive responses". The central nervous system effector of this response is the "stress system" with its main components, the corticotrophin-releasing factor

(CRF)/arginine-vasopressin (AVP) and locus ceruleus-noradrenaline (LC-NA)/autonomic (sympathetic) neurons of the hypothalamus and brain stem (Chrousos, 2000). Activation of the HPA axis and the LC-NA/autonomic system result in systemic elevations of glucocorticoids and catecholamines (CAs), respectively, which act in concert to maintain homeostasis. Stress influences the immune response primarily through the HPA axis system. Activation of the HPA axis culminates in secretion of glucocorticoids (**Figure 1.11**), that are recognized by glucocorticoids receptor molecules in cells of numerous organs, including immune cells. For example, because cytokines can activate hypothalamic-pituitary release of glucocorticoids, in turn, glucocorticoids suppress further cytokine synthesis and thus inhibiting inflammation (Besedovsky et al., 1986).

The HPA response to acute immune insults is driven primarily by the mediators released from activated immune/inflammatory cells (Buckingham et al., 1996; Turnbull and Rivier, 1999). The cytokines IL-1β, IL-6 and TNF-α, which are released sequentially from activated macrophages in response to, for example, endotoxin, are known to be particularly important in this regard. They amplify the inflammatory response locally, influence the adaptive immune response, and serve as signals of an inflammatory response to the CNS. The role of T-cell-derived cytokines in activating the HPA axis has received less attention, although IL-2 triggers ACTH release from the pituitary gland in vivo (Turnbull and Rivier, 1999).

Figure 1.11 Overview of the HPA axis system. (A) Stress stimulates (+) the hypothamalus to CRF and AVP that induce (+) the release ACTH by the anterior pituitary. This culminates with the release of steroid hormones (e.g. glucocorticoids) by the adrenal gland cortex and a negative feedback (-) to hypothalamic activation. Furthermore, glucocorticoids also modulate inflammatory responses by acting on immune cells via glucocorticoid receptors. (B) Anatomical location of the hypothalamus and pituitary gland. Brain picture is from the American Accreditation HealthCare Commission (A.D.A.M., Inc) website (www.urac.org - http://z.about.com/d/p/440/e/f/19239.jpg).



B. Mechanism of cytokine activation of the HPA axis

The mechanisms by which pro-inflammatory cytokines activate the HPA axis and increase glucocorticoid secretion are complex and varied, including both centrally mediated actions and actions at the levels of the pituitary gland and the adrenal cortex.

Several lines of evidence suggest that IL-1, IL-6 and TNF-α act synergistically in the neuroendocrine system, as they do in the immune system, and thereby drive the release of ACTH and glucocorticoids by potentiating each other's actions in a complex cascade of events (Buckingham et al., 1996; Turnbull and Rivier, 1999).Because there is no arterial blood supply to the anterior pituitary gland, cytokines released into the circulation only reach the hypophyseal portal capillaries in the median eminence (ME) of the tuber cinereum via the anterior hypophyseal arteries (Porter et al., 1983). Cytokines are large polypeptides (15 kDa) and, therefore, are unlikely to diffuse across the blood-brain barrier; however, they diffuse into the ME because there is little or no blood-brain barrier there. While the evidence that cytokines act at the hypothalamic level to increase the secretion of CRF and AVP is compelling, the ability of cytokines released from peripheral cells (e.g., macrophages) to gain access to targets in the CNS has been questioned. It has been argued instead that cytokines may enter the brain via: (a) fenestrated regions of the capillary endothelium; (b) areas in which the permeability is increased by e.g. local inflammation; or (c) an active transport system (Buckingham et al., 1996; Turnbull and Rivier, 1999). Another route of communication that must be mention is that proinflammatory cytokines also can be produced by glial cells under infection or inflammatory stimuli, and these brain cytokines can be critical in the mediation of sickness responses (Wieseler-Frank et al., 2005).

C. HPA mediated changes in the immune system

Stress has been defined as a state in which the organism confronts a novel, threatening, or challenging situation, or when the metabolic or physical status is

compromised (Zinder and Dar, 1999). The activation of a global stress response during tissue injury and infection (e.g., lameness) can profoundly influence the function of the immune system indirectly, through activation of the HPA axis, and directly through local modulatory cytokine actions on inflammatory responses (Chrousos, 2000). The general immunosuppressive and anti-inflammatory effects of stress are well known (Elenkov and Chrousos, 1999). However, stress does not simply suppress all aspects of immunity, but rather results in wide changes in acute and chronic immunocompetence and even exaggerated responsiveness of certain components of the innate immune/inflammatory reaction (Yeager et al., 2004).

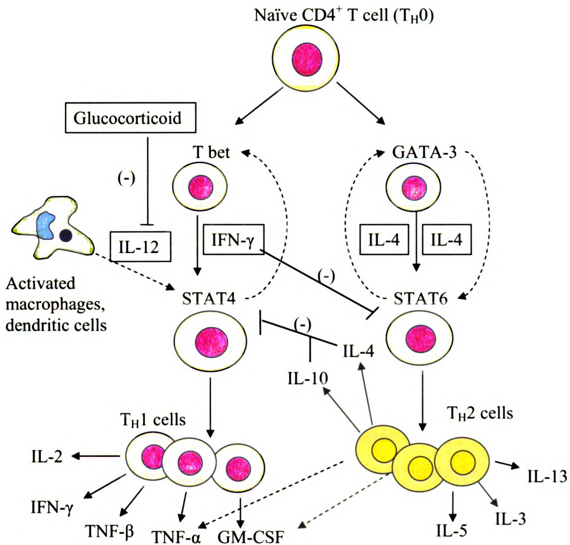
The stress induced release of hypothalamic CRF leads ultimately to adrenal secretion of glucocorticoids, epinephrine and norepinephrine into the peripheral circulation, which in turn influence local and systemic immune/inflammatory reactions in major ways. Glucocorticoids modulate immune/inflammatory reactions through their cognate receptor (GR) present in the cytoplasm of target immune cells (Weber et al., 2006a). For example, glucocorticoids prevent the transendothelial migration of leukocytes from the circulation into extravascular fluid space, reduce the accumulation of monocytes and granulocytes at inflammatory sites, and suppress the transcription and action of many cytokines and inflammatory mediators in immune and immune accessory cells (as reviewed in Chrousos, 2000). In addition, glucocorticoids influence the development, course, and pathology of certain allergic, autoimmune/inflammatory infections, and neoplastic diseases, predominately by stimulating T_H2 and limiting T_H1 based immune responses, which will be expanded below.

Naive T_H0 cells can differentiate to at least two functional classes of cells during an immune response (Mosmann et al., 1986) — T_H1 cells, which are classified as such, based upon their secretion of interferon-gamma (IFN-γ) and T_H2 cells, which instead secrete interleukin-4 (IL-4) (Abbas et al., 1996; Ho and Glimcher, 2002). Hereafter, we refer to IFN-γ and IL-4 as effector cytokines. T_H1 cells are responsible for inducing cytotoxic T cells for cell-mediated immunity, whereas T_H2 cells are responsible for inducing B cells for humoral (antibody-based) immunity. As well as their protective roles in host defense, both subsets of T_H cell have been implicated in pathological responses. For example, T_H1 cells can mediate organ-specific autoimmunity (Murphy and Reiner, 2002) and T_H2 cells have been implicated in the pathogenesis of asthma and allergy (Murphy and Reiner, 2002). The final composition of the T_H-cell response to antigen can, therefore, determine whether the outcome of infectious, inflammatory and autoimmune responses is favorable or unfavorable.

The process of differentiation from uncommitted T_H0 cell to a mature T_H1 or T_H2 cell is highly plastic. Many factors influence the decision to become a T_H1 or T_H2 cell. The cytokines IL-12 and IL-4, acting through signal transducer and activator of transcription 4 (STAT4) and STAT6, respectively, are key determinants of this outcome (Abbas et al., 1996; Ho and Glimcher, 2002). Antigen dose, co-stimulator factors such as adhesion molecules (e.g., ICAM-1), and other non-cytokine factors also have crucial roles in determining the dominance of a particular T_H cell type response (Kohlmeier et al., 2006). How each signal influences the differentiation process is an area of active investigation and, often, lively controversy.

In situations of stress, glucocorticoid acting through GR in antigen-presenting cells (APCs) directly suppresses transcription of the main inducer of T_H1 responses, IL-12 (Elenkov et al., 1996; Blotta et al., 1997) (**Figure 1.12**). Since IL-12 is extremely potent in enhancing IFN-γ and inhibiting IL-4 synthesis by T cells, the inhibition of IL-12 production may represent a major mechanism by which glucocorticoids affect the T_H1/T_H2 balance, that is, by enhancing humoral (T_H2) and suppressing cell mediated (T_H1) immunity (Wilder, 1995; Elenkov et al., 1996; 1998; Ramirez et al., 1996). The cytokines produced by differentiated effector T cells function to activate macrophages and B lymphocytes in the effector phases (eliminate antigen and activate other immune cells) of cell-mediated and humoral immunity.

Figure 1.12 Development of T_H1 and T_H2 type cell subsets. Cytokines produced in the innate immune response to microbes or early in adaptive immune responses influence the differentiation of naïve $CD4^+$ T cells into T_H1 or T_H2 cells. IL-12, made by activated macrophages and dendritic cells, induces T_H1 cell development through a STAT4-dependent pathway. IL-4, which may be produced mainly by T cells themselves, favors induction of T_H2 cells through a STAT 6 dependent pathway. The transcription factor Tbet, produced in response to IFN-γ, amplifies T_H1 responses, and GATA-3 is critical for T_H2 differentiation. Other cytokines that may influence helper T cell differentiation are not shown. However, IFN-γ and IL-4 are the main differentiating cytokines for T_H1 and T_H2, respectively. Glucocorticoid suppresses IL-12 and consequently, T_H2 type immune response is favored. Adapted by P.E. Almeida from Abbas and Litchman (2003) and Tizzard (2004).



Other adrenal steroids, such as dehydroepiandrosterone (DHEA) and its sulphate metabolite (DHEA-S), released in response to ACTH during the stress response, also have immunomodulatory properties. These include increased mitogen-stimulated IL-2 production by T cells (Daynes et al., 1990; Suzuki et al., 1991), diminished TNF-α and IL-6 production by macrophages (Di Santo et al., 1996; Straub et al., 1998), inhibition of natural killer cell differentiation (Risdon et al., 1991), and inhibition of mitogen-induced lymphocyte proliferation (Padgett and Loria, 1994). DHEA is also considered to possess anti-glucocorticoid activity (Kalimi et al., 1994) though its antagonistic mechanism of action is unclear (Dillon, 2005) that include production of T_H1 cytokines (Suzuki et al., 1991; Daynes et al., 1995) and down regulation of T_H2 (Daynes et al., 1995; Tabata et al., 1997). There also is evidence that DHEA(S), or closely related steroids, alters the T_H1/T_H2 cytokine balance (Tabata et al., 1997). The specific alterations in the balance of T_H1 and T_H2 cytokines seem to be model-specific. Rook et al. (1997) have shown that DHEA or androstenediol improve survival in a mouse model of *Mycobacterium tuberculosis* infection. Delayed hypersensitivity response was improved and IL-2 was increased, while IL-4 was decreased in the DHEA and androstenediol treated animals (Rook et al. 1997). These authors propose that DHEA and androstenediol stimulate T_H1 cytokine release, which may be of importance in successful response to disease.

Plasma concentrations of DHEA(S) are lower in chronic inflammatory conditions such as systemic lupus erythematosus (SLE), inflammatory bowel disease, rheumatoid arthritis, and polymyalgia rheumatica (Straub et al., 1998; 2002) than in health humans. Moreover, circulating concentrations of DHEA(S) correlate inversely with those of cortisol in the pathological conditions mentioned above (Straub et al., 1998; 2002).

Increased circulating levels of cortisol have been demonstrated in sheep (Ley et al., 1994) and horses with severe lameness (Galey et al., 1991; Clarke et al., 1982), but it is still uncertain whether these alterations in cortisol concentration are only a result of the severity of the disease or also plays a role in its etiology. These results have not been replicated in cows with lameness (Ley et al., 1996). However, in other situations involving pain and stress, including branding, castration, and dehorning (Lay et al., 1992; Robertson et al., 1994; Stafford et al., 2002; McMeekan et al., 1998), cows demonstrate elevated circulating cortisol concentrations. In addition, circulating DHEA concentrations are altered (decreased) during castration stress in pigs (Carroll et al., 2006). Similar to cortisol, information regarding serum DHEA concentrations in lame dairy cows is lacking.

D. Pro-algesic versus analgesic actions of immune cells

Immediately following tissue injury, inflammatory and pain mediators are delivered by the circulation (e.g., bradykinin), and by local tissue macrophages and dendritic cells that are activated. The inflammatory response is then amplified by migration of leukocytes into the inflamed tissue, by production of inflammatory mediators, including cytokines (TNF-α, IL-1 and IL-6), chemokines (e.g., keratinocyte-derived chemokine [KC], macrophage inflammatory protein-2 [MIP-2] and cytokine-induced neutrophil chemoattractant-2 [CINC-2]), and nerve growth factor (NGF), as well as by tissue acidification (Rittner et al., 2005). Leukocytes are the source not only of hyperalgesic agents but also of analgesic mediators. Among the best-characterized and clinically relevant of these are the endogenous opioid peptides and their receptors. Other

analgesic mediators include anti-inflammatory cytokines (IL-4, IL-10 and IL-13) as well as somatostatin and the endocannabinoids (Rittner et al., 2005).

In later stages of inflammation, cytokines (e.g., IL-4, IL-10 and IL-13) are produced by infiltrating leukocytes that limit inflammation and counteract hyperalgesia (Cunha and Ferreira, 2003). Cellular sources of IL-4, IL-10 and IL-13 for analgesia include mast cells and T_H lymphocytes. The analgesic actions of IL-4, IL-10, and IL-13 are apparent in models of rat paw inflammation as well as in a model of peritonitis and knee-joint incapacitation induced by zymosan (Cunha and Ferreira, 2003). Thus, during inflammation, analgesic cytokines counteract the effects of the proinflammatory hyperalgesic cytokines generated in the early stages of the inflammatory response.

Moreover, peripheral opioid receptors synthesized in dorsal root ganglia (DRG) at the spinal cord are intra-axonally transported to the peripheral nerve endings (Mousa et al., 2001). Upon activation by opioid ligands, opioid receptors couple to inhibitory G-proteins (Gi/o), which leads to inhibition of calcium and (or) sodium channels, and to a decreased level of neuronal cyclic adenosine monophosphate. Consistent with these effects, opioids attenuate the excitability of nociceptors, the propagation of action potentials, and the release of excitatory proinflammatory neuropeptides (substance P, calcitonin gene-related peptide) from central and peripheral nociceptor endings (Stein et al., 2003). All of these mechanisms result in analgesia.

Three families of opioid peptides are well characterized: endorphins, enkephalins, and dynorphins. Each binds to all three opioid receptors (*mu, kappa, delta*) but with varying affinities. The opioid family derives from a single gene that is transcribed, translated, and then processed into three peptides, pro-opiomelanocortin (POMC), pro-

enkephalin, and pro-dynorphin. All opioid peptides are expressed by leukocytes (Rittner et al., 2005; Mousa et al., 2004), but endorphins deriving from processed POMC protein have been studied most extensively.

The opioid-containing leukocytes are primarily T- and B-lymphocytes, granulocytes, and monocytes/macrophages (Mousa et al., 2001; Rittner et al., 2001; Cabot et al., 1997). These opioid-bearing cells migrate and accumulate in inflamed tissue (Machelska et al., 1998, 2002, 2004; Brack et al., 2004; Brack and Stein, 2004), via activation of adhesion molecules (e.g., CD62L) and chemokines (e.g., CXCR2 ligands KC, MIP-2 and CINC-2). During stress stimulation (e.g., swim stress, surgery) or local injection of corticotropin-releasing factor (CRF), leukocytes secrete opioids, which bind to their receptors on peripheral sensory nerve endings (Stein et al., 1990; Stein, 1993; Stein, 1996; Schäfer et al., 1996). The magnitude of endogenous analgesia is reported to be proportional to the number of opioid-containing polymorphonuclear cells (during early inflammation) and mononuclear cells (during late inflammation) at the injury site (Brack et al., 2004; Machelska et al., 2003). Moreover, the percent increase in proliferation of human peripheral blood immune cells incubated with morphine ex vivo is highly correlated with the subjects' tolerance to noxious cold stimuli (Hutchinson et al., 2004). These data present the immune system as a potential novel source of cells in which to investigate novel biological markers of pain tolerance. Similarly, the β-endorphin concentration in peripheral blood mononuclear cells has been suggested as a diagnostic tool for painful conditions such as fibromyalgia (Panerai et al., 2002) and acute myocardial infarction (Buratti et al., 1998), and certainly has the potential to be used as a

diagnostic tool for important painful conditions occurring in livestock, such as lameness in dairy cows.

IV. DIAGNOSTIC BIOMARKERS OF DISEASE

A biomarker is a measurable indicator of a specific biological state, particularly one relevant to the risk of contraction of disease or the presence and stage of disease (Rifai et al., 2006). Biomarkers can be used clinically to screen for, diagnose or monitor the activity of disease, and to guide molecularly targeted therapy or assess therapeutic responses to drug treatment (Etzioni et al., 2003). The utility and importance of biomarkers has been recognized by substantial funding from public and private organizations, and efforts for biomarkers discovery are now commonplace in both academic and industrial settings.

Recent developments in areas of research such as gene expression microarrays, proteomics, and immunology offer new approaches to disease screening (Henson et al., 1999). Consequently, biomarkers have become exceedingly refined. For example, genomics researchers have moved beyond one-gene-at-a-time analyses, and are now profiling thousands of gene transcripts to generate entire patterns of information that can be used for a range of clinical purposes, including disease classification or diagnosis, prognosis, and treatment monitoring and surveillance. The quest for a single biomarker for a particular disease has the illusion of analytical simplicity, but makes little sense from a biological perspective. It is not surprising that, to date, we have failed to find an accurate single marker for foot and leg diseases associated with lameness in livestock, which comes in hundreds of types and stages and is a product of several factors from

environmental to genetics, to nutrition and so on. Instead, why not take advantage of the very complexity of the disease and search for several indicators that, if measured simultaneously, may constitute a biomarker?

A. Biomarker discovery

Biomarkers can take any form, and as a consequence a variety of strategies have been adopted for their discovery (**Table 1.3**). The potential for genomic biomarkers to improve diagnosis, prognosis, treatment decisions and long-term outcomes can be substantial, and provide an insight in disease etiology that is unprecedented. Dissection of global changes in gene expression during pre-disease states, disease progression, and following clinical treatment can provide great insight into disease mechanism and treatment management (Chabas et al., 2001; Lock et al., 2002).

Global changes in gene expression during pre-disease states, disease progression, and following clinical treatment can provide great insight into disease mechanism and treatment management. For example, early investigations distinguished acute myeloid and acute lymphoblastic leukemia cells using gene expression profiling (Golub et al., 1999). Subsequent studies have used microarray technology to predict outcomes in breast and ovarian cancers (Berchuck et al., 2005; Huang et al., 2003). Additionally, classification of diffuse large B-cell lymphomas on the basis of gene expression profiles can identify clinically significant subtypes of cancer and the new classification has significant prognostic implications (Alizadeh et al., 2000). Examination of systemic lupus erythematosus (SLE) using microarray technology identified a subgroup of patients who may benefit from new therapeutic options (Rus et al., 2002; Baechler et al., 2003).

Table 1.3 Different approaches for biomarker discovery. Adapted from Yun-Fu et al. (2005) and Crebs (2006).

Role	**Key activity**	**Sources of discovery**	**Validation endpoints**
Diagnostic	Differentiates healthy from a specific disease state	Expression analysis, epidemiology studies, mechanism of disease studies	Correlation with a specific disease state
Prognostic	Predicts the likely course of disease	Expression analysis, epidemiology studies, mechanism of disease studies	Correlation with a clinical outcome
Stratification	Prior to administration of a drug compound, predicts which patients will respond or suffer from adverse effects	Pre-clinical studies, clinical trials	Correlation with a clinical response to a specific drug in controlled clinical trials
Pharmacodynamic and pharmacokinetic	Tracks a drug's *in vivo* activity at different concentrations	Metabolite analysis, animal models	Correlation with the concentration or activity of a drug in animal and human studies
Efficacy and outcome	Monitors the beneficial effects of a specific drug on an intended target or condition	Molecular targets, clinical trials	Correlation with the activity of a drug in clinical trials with placebo controls
Toxicity	Indicates potentially harmful effects of a drug on any unintended cellular processes, cells, tissue, or organs	Toxicology studies, immunohistochemistry, clinical trials	Correlation with the concentration or activity of a specific drug in clinical trials

B. Biomarker validation

Validation of the potential biomarkers can be achieved using an independent set of samples (Koike et al., 2005). Furthermore, a second methodology platform can be used to confirm initial findings. For example, if mass spectrometry is used for biomarker discovery, a different, more experienced platform can be used to validate and develop a diagnostic assay (Constans, 2005). Similarly, gene expression profiles can be validated using a new patient cohort and also performing real-time reverse transcriptase PCR to support the initial findings of a microarray-based gene expression profile (Gordon et al., 2005).

C. Issues with biomarker research

As microarrays were one of the first technologies used for large-scale discovery of genomic biomarkers, the field has made strides in developing standards for both research and clinical applications. The promising results of microarray technology in areas including cancer, cardiovascular, and inflammatory diseases raise the issue of whether and how this tool could be used in other areas (i.e., lameness). However, several issues remain to be addressed to improve confidence in new biomarker discoveries and enable comparability of datasets, including, sample quality, inter-platform differences, user variability (e.g., hybridization techniques), and data analysis.

An obvious use of microarrays is to discover, in a "holistic" manner, genes that are either up- or down regulated under various scenarios of interest. The high-dimensionality of microarray data with examination of thousands of transcripts in a relatively small number of samples raises the potential concern of creating false-positive

results. In addition to simple fold-changes, which fail to account for variability of expression between groups, more advanced statistical tests based on multiple testing were established, including the concept of the false discovery rate (FDR) which offers a sensible balance between the number of true and false-positives and basically yields probability values with higher stringency (Storey and Tibshirani, 2003). Given concerns over microarray methodology, there is a demand that results of key changes in transcript levels be confirmed by independent methods. When the goal of microarray analysis is gene discovery, usually independent methods such as quantitative real-time RT-PCR or Western blots (for protein analysis) have been used to reproduce results of up- or down regulated genes.

As many of these technologies produce large outputs, another major challenge is data analyses and bioinformatics. Separating true signals associated with a specific phenotype from background may be difficult for a variety of reasons, including the heterogeneity of the sample tissue, number of genes and clinical measurements, strength of the signal, confounding factors, and genetic heterogeneity of the disease. While classical biostatistical methods continue to be used, they often can not achieve the desired power due to the large number of variables collected from relatively small sample sizes (Allison et al., 2006), especially when studying complex diseases (e.g., lameness) on a heterogeneous population (e.g., dairy cattle) using sub-optimal experimental designs suited to the species being studied (e.g., farm level).

V. DISSERTATION'S HYPOTHESES

H_1: A pressure plate is a sensitive tool for early detection of lameness in heifers suffering from *papillomatous digital dermatitis* compared to Sprecher's (1997) lameness scoring system.

H_2: Circulating concentrations of key inflammation regulating steroids (cortisol and DHEA) and indicators of PBMC activation (gene expression for IL-1β, CD62L, MMP-9, POMC and GRα) are altered in lame cows showing sickness behavior and foot lesions compared to healthy sound cows.

H_3: Cows suffering from inflammatory and painful type of lameness derived from foot lesions have a signature gene expression profile of PBMC that is distinguishable from that of healthy sound cows.

CHAPTER TWO

Early detection of lameness in heifers with hairy heel warts using a pressure plate

I. ABSTRACT

Lameness is an indicator of pain and suffering, is a welfare concern and has economic impacts on the dairy industry. Subjective locomotion scoring is unreliable for detecting mild cases of lameness in dairy herds. Undetected lameness can progress to a more serious and painful state with unfavourable prognosis. The aim of this study was to conduct an investigation on the use of a pressure plate for early detection of lameness in dairy heifers compared to a subjective visual scoring system. Seven heifers deemed sound on the basis of a visual scoring system were walked through a chute where an RSscan pressure plate was disguised on the floor. Claws were inspected during trimming and revealed either no lesions (n = 3) or hairy heel warts on at least one hind claw (n = 4). Peak vertical force (PVF) and right-left hindlimb PVF symmetry were calculated. Sound heifers demonstrated significantly higher PVF (5.32 ± 0.62N.kg^{-1}; P = 0.01) and better right-left hindlimb symmetry (1.04 ± 0.02; P = 0.03) than those with hairy heel warts (3.64 ± 0.82 N.kg^{-1}; 0.77 ± 0.16). Using a pressure plate, gait abnormalities from foot lesions, that were undiagnosed using a subjective lameness scoring system were detected. Early detection of lameness is vital to reduce dairy industry losses and to improve animal welfare.

II. INTRODUCTION

Lameness refers to an abnormal gait caused by painful lesions of the limbs or back as the cow attempts to alleviate or avoid pain in the affected area (Scott, 1989) by

reducing propulsion, reducing her speed of walking, arching her back and lowering her head. The annual lameness incidence rate in adult dairy animals, depending on farm, location and year of study, ranges from 4 to 56% (Booth et al., 2004), and costs average US$350 per incident (Greenough and Weaver, 1997). The most common lesions associated with lameness are white line disease, sole and toe ulcers, heel erosion, double sole, sole hemorrhage and papillomatous digital dermatitis (hairy heel warts) (Murray et al., 1996).

The main strategy used for lameness detection in dairy cattle consists of subjective visual scoring that is based on qualitative assessments of kinematic measures. Kinematic measures are descriptions of motion, during standing and walking, ranging from a few measures of back posture and stride length (Sprecher et al., 1997) to a combination of back posture, leg abduction, limb weight bearing, joint flexion, relative front to hind foot placement, head carriage and 'smoothness' of gait (Welsh et al., 1993). Unfortunately, the subjective nature of visual scoring often results in high inter-observer variability (Engel et al., 2003), especially in detecting subtle changes in posture and weight bearing characteristic of mild cases of lameness (O'Callaghan et al., 2003). This issue results in a large number of lame cows going undiagnosed and therefore treatment is neglected, particularly in early stages of the condition when prognosis is favorable (Logue et al., 1998). Therefore, methods of locomotion analysis that offer greater accuracy without the biases that are inherent in a subjective analysis require investigation as a means to improve lameness assessments in dairy cattle.

Changes in the vertical component of ground reaction forces have been successfully used as indicators of lameness in horses (Clayton et al., 2000) and cattle

(Scott, 1989). Vertical ground reaction force can be measured using a pressure plate, which offers better portability and lower cost compared to force plates. However, to date, limited attempts have been made to objectively assess lameness in dairy cattle (e.g. Herlin and Drevemo, 1997; Rajkondawar et al., 2002a; Tasch and Rajkondawar, 2004) and information regarding the potential use of pressure plates for diagnosis is lacking.

The objective of this study was to perform a preliminary investigation on the potential of a pressure plate as a tool for early detection of lameness in heifers suffering from hairy heel warts and to determine the sensitivity of this device compared to a subjective lameness scoring system (Sprecher et al., 1997).

II. MATERIALS AND METHODS

All procedures of the experiment were approved by the All-University Committee on Animal Use and Care at Michigan State University. Seven Holstein heifers (age: 1.8 ± 0.6 years, weight: 645 ± 60 kg), deemed sound by an experienced veterinarian on the basis of a visual lameness scoring system (Sprecher et al., 1997) were used in this study, which was performed in early spring at a commercial dairy farm in Michigan, USA. A pressure plate (Footscan Scientific version, RS*scan* International, Olen, Belgium) able to measure vertical ground reaction force at a sample frequency of 250 Hz was used for data collection. The pressure plate measured 100 cm x 40 cm with 8192 conductive pressure-sensitive polymer sensors each of 0.39 cm^2. The plate, and a surrounding board of the same thickness measuring approximately 250 x 100 cm were placed level in a concrete pathway. This pathway was located in a barn adjacent to the heifers' home pen, which was used to guide heifers to a trimming chute. The pressure measuring device was

covered and disguised with a non-slip carpet. Heifers were walked down the pathway where measurements started at initial contact of the fore foot touching the plate. Measurements from both fore and hindlimbs of one side of an animal were collected in a single trial. Trials had duration of approximately 5 seconds. Data collection continued until three good trials from each hindlimb were obtained for each heifer. A trial was considered good when the hindlimb being analyzed was fully on the pressure plate throughout its stance phase and the animal was walking at a constant speed (i.e. no obvious visual signs of slowing down or speeding up) with no unusual body movements (e.g. no stumbling or abnormal head placement).

On the following day, trimming was performed according to the farm's standard operating procedure and the claws were inspected. Lesion type and severity were recorded. Heifers were then divided into a group with no foot lesions (n = 3) and a second group with hairy heel wart lesions on at least one hind foot (n = 4). Trials from heifers from these two groups were then subjected to analysis. Only one trial per hindlimb from each heifer had acceptable quality to be included in the analysis. A trial was considered acceptable when vertical ground reaction force exhibited a bimodal pattern on a force-time graphical display (e.g. Scott, 1988; Rajkondawar et al., 2002a; 2002b). Insufficient trials were recorded from fore limbs from each heifer to analyze the data; hence the fore limbs were omitted from the analysis.

Peak vertical force (PVF) was normalized by body mass (kg) and presented for each hindlimb and as an average of both hindlimbs ($N.kg^{-1}$) and right-left hindlimb PVF symmetry were calculated. Differences in these variables between groups were analyzed using SPSS statistical software (SPSS Inc., Chicago, Illinois, USA) at an alpha level of

0.05. As data were normally distributed (Kolmogorov-Smirnov $P > 0.05$), independent Student t-tests for unequal (Levene's $P < 0.05$) and equal (Levene's $P > 0.05$) variance were used as necessary.

IV. RESULTS

Heifers with no foot lesions demonstrated significantly better right-left hindlimb peak vertical force symmetry than heifers with foot lesions (1.04 ± 0.02 vs 0.77 ± 0.16; $P = 0.04$). In addition, the presence of hairy heel wart lesions significantly reduced the PVF (averaged over right and left) compared to no lesions (3.64 ± 0.82 N.kg^{-1} vs 5.32 ± 0.62N.kg^{-1}; $P = 0.01$) (**Table 2.1**). The subjective visual scoring system used in this study was unable to detect lameness in heifers with hairy heel wart lesions.

V. DISCUSSION

A search for tools that can provide accuracy and account for biases inherent of subjective analysis is timely. In this study, gait abnormalities associated with hairy heel wart lesions undetected by Sprecher's et al. (1997) lameness scoring system were successfully revealed using a pressure plate. A lame animal reduces the load in the affected limb as means of reducing pain. This study shows that the animal's efforts at load-reducing, which are often subjectively and unreliably assessed using visual scoring systems, can be assessed objectively by measuring the peak vertical force using a pressure plate. The demonstration of reduction in PVF in heifers with hairy heel wart lesions supports the assertion that quantitatively measurable changes occur with lameness, as previously demonstrated in cows (Rajkondawar et al., 2002a; 2002b; Scott,

1989) and horses (e.g. Merkens and Schamhardt, 1988) at the walk. Similarly, right-left limb PVF symmetry changes were revealed to be a promising measure to detect lameness associated with hairy heel wart lesions in heifers, which agrees with findings in horses with experimentally induced lameness (Merkens et al., 1988).

To be considered an auxiliary diagnostic tool, data collection must be practical, reliable, suit operating procedures in dairy farm settings and provide measures that can be easily understood by people who are not necessarily knowledgeable in biomechanics. Although the changes in the peak vertical force observed in this study characterized the presence of hairy heel warts, further studies should be carried out to assess the sensitivity of PVF to detect other important lameness conditions such as white line disease and sole ulcers. Furthermore, the analysis of a single trial per limb might not be a sensitive enough data capture method to detect lameness due to the impacts of high intra and inter-animal variability. The variation observed between the three trials per limb within an animal suggests that the magnitude of locomotion variation in heifers might be high and methods to minimize it should be implemented. Overall, locomotion variability can be minimized by using an average of multiple trials as a representative of the gait pattern, reducing inter-trial walking velocity variation, and/or collecting data from both right and left limbs simultaneously with a larger pressure plate. In horses, force variables are considered to be quite stable, and analysis of three to five trials is accepted to provide a representative gait pattern (Schamhardt, 1996). However in cattle such information is unknown. If several trials are deemed necessary to provide representative gait pattern, the practicality of using pressure plates for lameness assessment in dairy settings must be re-assessed and technology would have to evolve to improve reliability of pressure plate measures.

Owing to the importance of maintaining a constant walking velocity to achieve reliable pressure plate analysis (Khumsap et al., 2002), especially when data from right and left limbs are collected in different trials, alternative ways to control cattle walking velocity should be developed to improve detection of lameness in dairy systems.

VII. CONCLUSION AND ANIMAL WELFARE IMPLICATIONS

This study has shown that a pressure plate offers opportunities to objectively detect lameness related gait abnormalities in heifers suffering from hairy heel warts. Further validation of the vertical ground reaction forces and an approach for pressure plate data collection is an important next step to develop this technique further as an objective tool for the early detection of lameness in dairy cattle. Moreover, technology will evolve to enable sources of variability during pressure plate data capture to be minimized, for instance, the use of larger plates that would allow data collection from right and left body sides simultaneously. Quantitative analysis of gait offers a means for early and reliable detection of lameness that contributes to improvements in lameness prognosis, reduction in dairy industry losses, and ultimately, to enhanced animal welfare.

Table 2.1 Mean peak vertical force (PVF) and right-left hindlimb PVF symmetry of heifers (645 ± 60 kg) with (n = 4) and without (n = 3) hairy heel wart lesions. Values marked with the same symbol were significantly different. * (P = 0.04) indicates PVF differences between RH and LH of heifers with lesions; ** (P = 0.01) indicates PVF mean differences between hindlimbs heifers with and without lesions; *** (P = 0.04) indicates PVF symmetry mean differences between hindlimbs of heifers with and without lesions. RH = right hindlimb; LH = left hindlimb.

Group	PVF RH ($N.kg^{-1}$)	PVF LH ($N.kg^{-1}$)	PVF symmetry
Lesions	3.08 (0.29) *	4.19 (0.82) *	
Mean PVF	3.64 (0.82) **		0.77 (0.16) ***
No lesions	5.36 (0.60)	5.28 (0.76)	
Mean PVF	5.32 (0.62) **		1.04 (0.02) ***

CHAPTER THREE

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CHAPTER THREE

Depressed DHEA and increased sickness response behaviors in lame dairy cows with inflammatory foot lesions.

I. ABSTRACT

Lameness is a multifactorial condition influenced by the environment, genetics, management and nutrition. Detection of lameness is subjective and currently limited to visual locomotion observations which lack reliability and sensitivity. The objective of this study was to search for potential biomarkers of inflammatory foot lesions that underlie most cases of lameness in dairy cows, with a focus on the sickness response and relevant endocrine, immune and behavioral changes. Serum and peripheral blood mononuclear cells (PBMC) were collected from eight sound and eight lame high-producing Holstein cows. Immune cell activation was investigated in PBMCs using a candidate gene approach in which the expression of pro-opiomelanocortin, interleukin-1beta, L-selectin, matrix metalloproteinase-9 and glucocorticoid receptor-alpha was measured via quantitative real time-RT-PCR. Endocrine changes were investigated by monitoring serum concentrations of cortisol and dehydroepiandrosterone (DHEA). Additionally, systematic behavioral observations were carried out to characterize a behavioral profile associated with a sickness response typical of this condition. Lame cows showed significantly lower eating ($P = 0.004$) and ruminating ($P = 0.05$) behaviors and higher incidence of self-grooming ($P = 0.04$) compared to sound cows. Lame cows also showed a 23% decrease in serum DHEA ($P = 0.01$) and 65% higher cortisol:DHEA ratio ($P = 0.06$) compared to sound cows. However, no significant differences were found in candidate gene expression between lame and sound cows. Serum DHEA concentration

and cortisol:DHEA ratio are promising objective indicators of inflammatory foot lesions in dairy cattle and may be useful as diagnostic targets for animals in need of treatment.

II. INTRODUCTION

Lameness in dairy cows refers to the abnormal gait used by the animals as an attempt to alleviate or avoid pain from lesions and inflammation in the affected limb or back area (Scott, 1989; Hardie, 2000). Lameness is not only perceived to affect the welfare of the animals, but also has a high economic impact on the dairy industry with costs averaging US$350 per clinical episode (Greenough and Weaver, 1997). These costs include its treatment and control (Moore et al., 2001), impaired reproductive performance (Sprecher et al., 1997), decreased milk yield (Warnick et al., 2001) increased culling rate, and decreased carcass value of culled cows (Arendonk et al., 1984). Estimated annual lameness incidence in adult dairy cows ranges from 4 to 55% depending on farm, location, and year of study (Booth et al., 2004). This widely differing estimate is suggested to be due to the sensitivity of lameness detection within individual herds, how it is defined and by whom (Whay et al., 1997). The highest incidence of lameness is found to occur soon after calving (Offer et al., 2000) and claw horn lesions in the hindlimbs represent about 90% of all lameness. Sole ulcers (40%) and white line lesions (29%) are suggested to be the predominant diseases of horn, and digital dermatitis (40%) is the most common skin disease (Murray et al., 1996). Realizing the economic importance of lameness, its high incidence and implications to animal welfare due to pain and discomfort, strategies to objectively diagnose, treat, and improve lameness outcomes are urgently needed.

Lameness screening accuracy has been hindered by subjectivity, by low between- and within-observer reproducibility of diagnosis (Wells et al., 1993; Engel et al., 2003), and variable sensitivity and response of individual animals to pain (Hardy et al., 1967). Currently, there are no reliable measures available to objectively screen cows for the presence of lameness. Lameness diagnosis has been performed using subjective visual scoring systems that focus on changes in locomotion. In addition to their unreliability, these systems are not sensitive to detect mild cases of lameness (O'Callaghan et al., 2003) because lesion severity is the predominant factor that leads to deterioration of locomotion (Whay et al., 1997). As a consequence of detection unreliability, 30 to 50% of cows are suggested to remain undiagnosed (Kopcha et al., 2003; Clarkson et al., 1996) such that their lameness tends to progress to a more serious and/or chronic condition that often has a compromised prognosis (Logue et al., 1998). Furthermore, the multifactorial pathogenesis of lameness exacerbates the need for biomarkers that are not only able to assist with diagnosis but also able to help better understand lameness etiologies.

In addition to changes in locomotion, lame cows display increased lying time, decreased food-intake and altered social behaviors (Singh et al., 1993; 1994; Galindo et al., 2002; Sauter-Louis et al., 2004). These behavioral modifications are intrinsic of a psychoneuroimmunological event known as 'sickness response'. This event requires a bi-directional communication between the immune system and the brain. During the sickness response several changes in behavior and physiology occur to facilitate host survival during infection and tissue injury (Hart, 1988). The behavioral changes noted in lame cows support the notion that the immune system is involved in the pathogenesis of this condition. In fact, tissue injury and infection, which are common elements of

lameness in dairy cows, are known to unleash the signals necessary for immune activation. Inflammatory mediators released during immune activation (e.g., pro-inflammatory cytokines) relay information to the brain (hypothalamus) about the events in the periphery (Tracey, 2002). As a consequence, the brain initiates anti-inflammatory mechanisms that culminate in the release of cortisol and dehydroepiandrosterone [DHEA] by the adrenal cortex (Besedovsky et al., 1986). These anti-inflammatory steroids down regulate the expression of inflammatory genes (e.g. proinflammatory cytokines) in circulating leukocytes via steroid hormone receptors, especially glucocorticoid receptors (GRα) (Jessop, 1999).

In response to tissue injury and infection, ruptured cells (e.g., mast cells, neutrophils) release intracellular proteins that trigger cytokine production (e.g., proinflammatory cytokines - IL-1β, TNF-α, IL-6) In addition, vascular mast cells and macrophages release products (e.g., histamines, eucosanoids, tryptases, pre-formed TNF, cytokines, chemokines) that result in vasodilation and fluid extravasation (swelling) (Tracey, 2002). Leukocytes migrate from the vasculature to the injury site via adhesion molecules, an important one being L-selectin (CD62L), and enhance inflammation (Venturi et al., 2003). Neutrophils recruited to the injury site undergo degranulation, phagocytosis, and respiratory burst (Nathan, 2002) that result in release of oxidants and metalloproteinases (e.g., matrix metalloproteinase-9 [MMP-9]), both of which can damage otherwise healthy tissue and up-regulate the inflammatory response (Di Girolamo et al., 2006). Lymphocytes and monocytes recruited to the injury site can help abate pain by synthesizing and delivering neuropeptides (e.g., pro-opiomelanocortin [POMC]-derived β-endorphin) (Mousa et al., 2004). POMC is up-regulated in these

peripheral blood mononuclear cells (PBMC), the predominant endorphin-containing cell types at later stages (> 96h) of acute inflammation (Rittner et al., 2001).

Limited and unsuccessful attempts have been made to detect physiological changes (e.g., cortisol as in [Ley et al., 1996]) in cows suffering from lameness. Leukocytes are known to carry gene expression signatures characteristic of other physiological (Burton et al., 2005; Madsen et al., 2004; Weber et al., 2006a) and disease states (Coussens et al., 2004a; 2004b; Hill et al., 2005; Meade et al., 2006). Therefore, gene expression of these cells, along with the endocrine changes in response to brain activation, may provide biomarkers for diagnosis of common inflammatory foot lesions that result in dairy cattle lameness. We hypothesized in the current study that circulating concentrations of key inflammation regulating steroids (cortisol and DHEA) and indicators of PBMC activation (gene expression for IL-1β, CD62L, MMP-9, POMC and GRα) would be altered in lame cows showing sickness behavior and foot lesions compared to sound cows with no sickness behavior or foot lesions. Our objective was to explore changes in key sickness behaviors and neuroendocrine-immune physiology as potential biomarkers of lameness in dairy cows.

III. MATERIALS AND METHODS

A. Subjects

The study subjects were 16 Holstein cows with mean weight, age and days in lactation of 1467 ± 114 kg, 4.5 ± 0.25 years, and 183 ± 65 days, respectively. Use of animals for this study was approved by the Michigan State University (MSU)'s All University Committee on Animal Use and Care (AUF# 05/04-079-00). All cows were

owned by MSU, housed in a freestall housing unit built with a 10 degree slope, and fed and cared for according to the standard operating procedures of MSU's Kellogg Biological Station Dairy Center research facility. Lame cows (n = 8) selected for study met two criteria suggesting that the animals were in pain: (i) abnormal gait and back posture while walking and standing (**Figure 3.1a**), and (ii) presence of visible lesions on at least one hindlimb at feet inspection (**Figure 3.1b**). The causes (i.e., sole ulcers, footrot, sole bruising and interdigital dermatitis) and duration of lameness were not determined and likely varied from cow to cow. Instead, lame cow selection was intended to comprise a snap shot of clinical lameness in the herd at time of sampling, which is representative of what may occur in health monitoring programs on commercial farms. Sound cows (n = 8) had normal (straight) back posture (**Figure 3.1c**) and no visible foot lesions (**Figure 3.1d**). None of the cows on the study presented with pathological conditions other than lameness and none of the lame cows were treated prior to blood samples collection.

B. Blood collections and isolation of serum and PBMCs

Blood samples were obtained from animals during mid morning via jugular venipuncture, using 2.5-cm 14-gauge needles (Fisher Scientific, Pittsburgh, PA). This study was performed during the summer. Blood (~130 ml per cow) intended for PBMC isolations was collected into 50 ml centrifuge tubes (BD Biosciences, San Jose, CA) that contained 4 ml of acid-citrate dextrose (ACD) (Weber et al., 2004) as an anticoagulant, and immediately placed on ice. An additional 8 ml of blood per cow was collected into vacutainer tubes (10 ml tubes; BD Biosciences, San Jose, CA) containing no

anticoagulant. Serum was harvested (1000 x g at 4°C for 20 min) from these tubes within an hour of blood collection and stored at -80°C until use for steroid analyses.

PBMCs were prepared from the ACD anticoagulated blood as previously described (Coussens et al., 2002). Briefly, blood tubes were centrifuged at 4°C for 30 min at 1150 x g and resulting buffy coats containing the PBMCs were transferred into PBS layers (32 ml) that were overlaid on 10 ml cushions of Percoll (1.084 g/ml; Sigma Chemical Co. St. Louis, Mo) in new 50 ml tubes. Buffy coats from 3 blood collection tubes per cow were added to each Percoll gradient. The Percoll tubes were centrifuged at 500 x g for 40 min at room temperature to pellet erythrocytes and contaminating granulocytes away from the PBMCs (lymphocytes and monocytes), which remained floating on the top of the Percoll gradient. The PBMCs were then retrieved and transferred to a series of new 50-ml conical tubes and washed once with 20 ml of cold PBS. Final cell pellets were collected by centrifugation for 10 min at 800 x g at 4°C. The cell pellets were suspended in 4 ml of TRIzol reagent (Invitrogen, Carlsbad, CA) and incubated for 10 minutes at room temperature to ensure lysis of PBMCs and preservation of their released RNA. This mixture was then frozen at -80°C until use for RNA isolation (see below). Time from blood sampling until PBMC lysis in TRIzol averaged 3 h. All procedures for PBMC preparations were done using sterile reagents and supplies.

C. RNA isolation

Total RNA was isolated from PBMCs according to the TRIzol manufacturer's instructions (Invitrogen) and treated with RQ1 RNase-Free DNase (Promega, Madison, WI) as described previously (Madsen et al., 2004). RNA concentration and purity were

determined with a ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) and Agilent 2100 Bioanalyzer (Quantum Analytics Inc., Foster City, CA), respectively. cDNA was synthesized from 2 µg of total RNA per sample using Superscript II RNaseH- Reverse Transcriptase (Invitrogen).

D. Real time RT-PCR standard curve preparation

Quantification of mRNA can be either relative or absolute. Absolute quantification of transcript abundance allows the precise determination of copy number per cell number or unit mass of tissue. However, it requires the construction of standard curves from various known dilutions of PCR-derived amplicons for each candidate gene under study (Bustin, 2000). Standard curves for use in the quantitative real time RT-PCR assays of the current study were created as recently described by Madsen-Bouterse et al. (2006). Briefly, PCR amplicons for IL-1β, GRα, POMC, MMP-9 and CD62L were developed from bovine-specific PCR primers (**Table 3.1**) that were designed using OLIGO Primer Analysis Software (Molecular Biology Insights, Inc. Cascade, CO). A standard curve for a control gene (β-actin) was also developed to check for sample loading consistency across lameness groups and wells of the real time PCR plates. The PCR reaction mixtures contained 1X PCR buffer, 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 M forward primer, 0.2 M reverse primer, 35 ng cDNA template (from bovine PBMCs), and 1.0 U per reaction Taq DNA polymerase (Invitrogen), brought to a final volume of 25 ml with sterile Milli-Q water. Reaction conditions used to generate the PCR amplicons were as follows: denature at 95°C for 5 min followed by 35 cycles of 95°C for 30 seconds (s) (denature), 52°C for 30 s (anneal), and 72°C for 30 s (extend), and a final extension at

72°C for 10 min. The resulting single band amplicons for each candidate gene were gel purified, ligated into the pGEM-T Easy vector (Promega), and the recombinant plasmids were transformed into JM109 competent *E. coli* cells (Promega). Positive clones containing the IL-1β, GRα, MMP-9, POMC, CD62L and β-actin cDNA inserts were selected by blue/white colony screening and confirmed by DNA sequence analysis (MSU Research Technology Support Facility). Plasmids from white colonies were isolated using the Mini-prep Plasmid DNA Isolation kit (Promega). Additional amplicons for IL-1β, GRα, POMC, MMP-9, CD62L and β-actin were then generated by PCR using the respective plasmids as template and primers shown in **Table 3.1**, and gel purified prior to dilution for their use as template to develop the quantitative real-time RT-PCR standard curves.

E. Quantitative real-time RT-PCR analysis of PBMC gene expression

As described in Madsen-Bouterse (2006), five to six concentrations of each amplicon (1×10^{-18} to 1×10^{-13} g), or 20 ng of test PBMC cDNA were added as templates for the quantitative real-time RT-PCR analyses, which utilized the SYBR Green PCR Master Mix system for real time fluorescence detection in a PE7700 thermal cycler (Perkin Elmer Applied Bioscience; Foster City, CA). The real time RT-PCR primers for IL-1β, GRα, POMC, MMP-9, CD62L and the β-actin control gene are shown in **Table 3.2** and were created using the Primer Express™ software (version 2.0, Applied Biosystems). Amplification efficiency, observed as parallel slopes of the amplification curves, was similar between the standard curve amplicon and test PBMC cDNA within gene, and the standard curves were always linear ($R^2 > 0.9$; **Figure 3.2**). IL-1β, GRα,

POMC, CD62L and β-actin mRNA abundance in samples from lame and sound cows was determined using the equation for each standard curve. All reactions, including a template negative control (no cDNA template) were run in triplicate.

F. Cortisol and DHEA assays

Serum concentrations of anti-inflammatory steroids were determined using two commercial kits: (i) the Cortisol Correlate-EIA (Assay Designs, Ann Arbor, MI), and (ii) the DHEA Correlate-EIA kits (Assay Designs) following the manufacture's instructions. A serum dilution factor of 1:16 was used for the cortisol assay and of 1:50 for the DHEA assay. All samples per assay were processed on single plates. Assay sensitivity for cortisol was 56.7 pg/ml and for DHEA was 2.9 pg/ml.

G. MMP-9 assay

MMP-9 total activity in serum of lame and sound cows was measured using a gelatin zymography assay as in (Weber et al., 2006a). Briefly, serum volumes equivalent to 50 μg of total protein per sample were loaded into each lane of one-dimensional SDS-polyacrylamine gels impregnated with 0.25% (w/v) gelatin (Precast 10% Zymogram Ready Gel, Biorad, Hercules, CA). Gel electrophoresis was conducted at 65V for 30 min, then 120 V for an additional 60 min. Subsequently, gels were washed twice for 30 minutes in 2.5% (v/v) Triton X-100 to remove SDS, and then incubated in 50mM Tris (pH 7.5), 5mM $CaCl_2$ and 0.02% Na N_3 at 37°C for 18 h to enable MMP-9 activity (i.e., degradation of the gelatin). Gels were stained with Coomassie brilliant blue stain in 25% methanol, 10% glacial acetic acid for 60 min to visualize clear bands of digested gelatin.

Band images were collected on the Fluor-S MultiImager (BioRad) and the density of clearings at the 92 KDa marker (indicating MMP-9) analyzed by scanning densitometry (GS-710 Calibrated Imaging Densitometer and Multi-Analyst Software; Biorad).

H. Observation of sickness behaviors

Focal sampling and continuous recording were used to collect maintenance, feeding, social and grooming behavior performance (**Table 3.3**). Briefly, focal sampling means observing one individual animal for a specified amount of time and continuous recording means observing all instances of its behavior at that time given frame – usually for several different categories of behavior (Martin and Bateson, 1993). During a period of 3 consecutive days and starting on the day of blood collection, 2-minute observations were made in 15-minute intervals for a total of 4 hours per day (96 minutes of total observation time).

I. Statistical analysis

Statistical analyses of steroid and mRNA abundance and behavioral data sets were performed using the MIXED procedure of SAS (SAS Institute, Cary, NC), with a model that included lameness group as the fixed effect and cattle age (in days), weight (in kg), day in lactation (in days), and pregnancy status (yes, no) as covariates. Lesion types were not accounted for in the statistical model because these were not diagnosed. Data sets were checked for parametric statistical assumptions (e.g. normality and equal variances) before statistical analysis using the One-Sample Kolmogorov-Smirnov procedure and Levene's test (Ott and Longnecker, 2002). Logarithmic transformation

was performed on variables not meeting the assumptions for parametric statistical analysis (i.e., gene expression data sets) prior to statistical analysis. Statistical significance of the lameness group effect was considered when $P \leq 0.05$, and a significant tendency was considered when $0.05 < P \leq 0.10$.

IV. RESULTS

A. Behavioral observations in lame versus sound cows

As expected, lame cows showed classical sickness responses in that they had significantly decreased eating ($P = 0.01$; 8.85 ± 2.22 min), ruminating ($P = 0.01$; 18.41 ± 3.34 min), and increased self-grooming ($P = 0.05$; 3.91 ± 0.71 min) behaviors compared to sound cows (17.36 ± 2.03 min; 28.57 ± 3.06 min; 1.13 ± 0.65 min, respectively) (**Table 3.4**).

B. Serum steroids in lame versus sound cows

Lame and sound cows demonstrated significantly different ($P \leq 0.05$) concentrations of the anti-inflammatory steroid DHEA. DHEA was approximately 23 % lower ($P = 0.01$) in lame (543.2 ± 34.2 g/ml) compared to sound (702.5 ± 37.1 pg/ml) cows (**Figure 3.3a**). Although serum cortisol of lame cows (2690.7 ± 579.2 pg/ml) was approximately 49% higher than that of sound cows (1391.8 ± 583.7 pg/ml), this difference was not statistically significant ($P = 0.16$) (**Figure 3.3b**). The ratio of cortisol:DHEA has been suggested as being more informative than isolated cortisol values (Butcher et al., 2005), largely due to the antagonist actions of DHEA on cortisol actions (Kalimi et al., 1994). In the current study, the serum cortisol:DHEA ratio was

65% higher (P = 0.06) in the lame (5.44 ± 1.12) than the sound (1.88 ± 1.13) cows (**Figure 3.3c**), suggesting that endocrine regulation of inflammation was somewhat out of balance in the lame animals.

C. PBMC gene expression in lame versus sound cows

The cDNA for 4 of the candidate genes studied (POMC, IL-1β, CD62L, and GRα) was readily amplified in real time RT-PCR reactions and resulting mRNA abundances were thus quantified for statistical analysis using the corresponding linear standard curves. Results showed that in this group of animals, no differences were detectable in mRNA abundances for any of these 4 genes between lame and sound cows (**Table 3.5**). Expression of the control gene, β-actin, also was not different from sample to sample (or well to well), as expected. Somewhat surprisingly, the expression of MMP-9 was so low in the PBMCs as to preclude its detection, even in PBMCs from the lame cows, using this real time RT-PCR approach. Therefore this data set was omitted from statistical analysis and we concluded PBMC MMP-9 gene expression did not differ significantly between lame and sound cows.

D. MMP-9 activity in serum of lame and sound cows

Despite lack of detectable gene expression for MMP-9, this enzyme is normally secreted during inflammation by activated neutrophils and PBMCs and can thus be detectable in serum using gelatin zymography. However, similar to MMP-9 mRNA abundance in the PBMCs, the activity of this proteolytic enzyme in serum was too low in most serum samples to be measured accurately by scanning densitometry and this data set

was omitted from statistical analysis. Because detection of a related, constitutively expressed matrix metalloproteinase (MMP-2) showed equal band intensities across all samples (representative zymography gel shown in **Figure 3.4**), neither sample loading error nor assay conditions appeared to be the cause of the variably low MMP-9 activity in the sera. Thus, like PBMC MMP-9 gene expression, we concluded that serum MMP-9 activity was not different between lame and sound cows.

V. DISCUSSION

The current study showed that lame cows exhibited behavioral changes consistent with the sickness response and which occurred in conjunction with clear changes in the neuroendocrine system but, surprisingly, with little impact on expression of classical inflammatory and pain relieving genes in PBMCs. However, the low number of biological replicates, multiple etiologies, and unknown chronicities of the lameness included in this study likely hindered statistical power and need to be carefully considered in future studies. Moreover, despite the obvious sickness response in severely lame cows, the localized nature of the inflammatory process that underlies such lameness cases might represent a limitation to finding changes in circulating PBMCs. Nonetheless, the systemic endocrine changes observed here are novel and should be expanded upon and explored further as potential candidate markers of lameness due to inflammatory foot lesions.

The sickness response consists of a myriad of immune (e.g., fever and hyperalgesia), behavioral (e.g., increased sleep and decreased locomotion, sexual behavior, exploration, and food and water intake), and endocrine (e.g., release of classic

stress hormones from the sympathetic nervous system and hypothalamic-pituitary-adrenal axis [HPA]) changes that are mainly driven by proinflammatory cytokines released from damaged tissue and activated immune cells (Buckingham, 1996). Activation of the HPA axis increases adrenocorticotrophic hormone (ACTH) synthesis in the pituitary gland that stimulates the cortex of the adrenal glands to release stress steroids, especially glucocorticoid (cortisol) and androgen (DHEA) (Bauer, 2005; Butcher et al., 2005). Both of these steroids are anti-inflammatory, but cortisol is highly immunosuppressive (Cupps and Fauci, 1982) while DHEA has clear immune protecting activities (Sacco et al., 2002). In fact, there is evidence that DHEA has an 'anti-glucocorticoid' effect in animals and humans (Svec and Porter, 1998). DHEA has also been described to increase resistance to viral and bacterial infections (Loria et al., 1996; Zhang et al., 1999), inhibit the production of IL-6 thereby reversing immunologic defects during aging (Du et al., 2001; Daynes et al., 1993), restore immune function after thermal and trauma-hemorrhage injury, and reduce mortality rates from septic challenge (Ben-Nathan et al., 1999; Marx et al., 2003). DHEA concentration is reported to fall during severe infection (Rook et al., 1997) and chronic inflammatory diseases such as systemic lupus erythematosus, inflammatory bowel disease, rheumatoid arthritis and polymyalgia rheumatica (Masi et al., 1984; Deighton et al., 1992; Lahita et al., 1987; Straub et al., 1998; Dillon, 2005). DHEA also is dramatically reduced during ageing humans in conjunction with the onset of increased risk of immune dysfunction and inflammatory disease (Buckingham et al., 1996). It was thus not surprising that serum DHEA concentration was lower in the lame compared to the sound cows, reflecting the chronic inflammatory nature of the lameness cases included in this study. The reason for the DHEA depression during chronic

inflammation is unknown, however, it is assumed that this may lead to a heightened state of tissue damage because DHEA normally inhibits expression of potent proinflammatory cytokines such as TNF-α and IL-6 (Danenberg et al., 1992; Araghi-Niknam et al., 1997; Di Santo et al., 1996; Kimura et al., 1998). Furthermore, the unaltered cortisol concentration found in lame cows supports the premise that a reduced serum DHEA is often coupled with stable or increased serum glucocorticoid concentrations (Straub et al., 2002). The cortisol:DHEA imbalance observed in the lame cows could represent a cause or an effect of this painful inflammatory disorder. Whether cause or effect, the cortisol:DHEA ratio appears to be a candidate diagnostic tool for inflammatory foot lesions and associated lameness worthy of further testing.

The activation of the pituitary-dependent adrenal response, characterized by changes in DHEA and sickness behaviors, provides evidence for the fact that the stimuli from the inflammatory foot lesions in lame cows does indeed reach the central nervous system to stimulate anti-inflammatory and/or healing signals. Therefore, the lack of changes in the inflammatory gene expression observed in PBMCs was surprising and unexpected due to the importance of these genes as modulators of the immune-to-brain bi-directional communication and inflammatory network. However, unaltered expression of these particular genes might be explained by the lack of profound change in circulating cortisol in the lame versus sound cows. Elevated cortisol is one of the prerequisites for anti-inflammatory signaling in leukocytes that includes down-regulation of its own receptor (GRα) (Raubenheimer et al., 2006), and acute phase cytokines (e.g., IL-1 β) and neuropeptide (e.g., POMC derived β-endorphin) synthesis (Besedovsky et al., 1986). The unaltered levels of MMP-9 expression between lame and sound cows also was surprising

because MMP-9 is involved in tissue destruction during inflammation and it is known to mediate the extravasation of PBMCs to the injury sites (Constantinescu et al., 2001).

If indeed an inflammatory response is present in lame cows then circulating levels of other proinflammatory cytokines may be altered to enable immune-to-brain communication, which may have been represented here by the decrease in serum DHEA concentration and increase in sickness behaviors (i.e. reduced eating, altered locomotion and posture). Alternatively, the immune-to-brain communication could have been achieved via stimulation of the efferent vagus nerve (Maier et al., 1998) which, through the cholinergic anti-inflammatory pathway, controls inflammation in discrete and localized tissues where invasion and injury typically originate (Borovikova et al., 2000). Thus, circulating leukocytes might not be influenced by this route of immune-to-brain activity, possibly explaining the lack of differences in PBMC gene expression observed between lame and sound cows. On the other hand, it is also possible that PBMCs may not be good cellular indicators of well-localized inflammatory events, such as in lame cows with inflammatory foot lesions. Although information about the chronicity of foot lesions included in the study is unknown, the unaltered gene expression differences in lame versus sound cows might have been the result of the chronic nature of the foot lesions, which could have impaired or desensitized the immune system for pain control and healing induction. As such, a broader genome-wide search for gene expression biomarkers of the pathological conditions that underlie lameness likely will be needed in the future.

In conclusion, this study tested a variety of parameters as candidate biomarkers of inflammatory foot lesions in lame dairy cows exhibiting sickness behavior. However, the

sensitivity and specificity of the current results need to be tested in a larger sample of animals with varying etiologies and severities of foot lesions. Biomarker validation could also be expanded to testing PBMC and serum samples using more global screening platforms, such as those available for genomic and proteomic analyses. Nonetheless, the depressed serum DHEA concentrations and increased serum cortisol:DHEA ratios detected in this study appear promising for lameness detection and may indicate DHEA as a possible anti-inflammatory therapy for the treatment of clinical lameness. Results of this study thus suggest that, in combination with observations of locomotion and sickness behaviors, assessment of serum DHEA concentration (or the cortisol:DHEA ratio) might help target animals in need of pain relief and facilitate the monitoring of lameness therapies.

Table 3.1 Bovine gene-specific primers used to develop real time RT-PCR standard curves.

Primer	Length (bp)	Tm (°C)	Forward 5' to 3'	Reverse 5' to 3'
POMC	285	92	GGCCTGAGCTGGAGTATGG	CGGTAGGAGGGCAGAAGAGA
IL-1β	452	86	CTCTCCACCTCCTCTCACA	ACATTCTTCCCTTCCCTTCT
CD62L*	711	86	CCCAACAACAGGAAGAGTAAG	CCTATAGTTGCATATGTATCAAATTTTC
MMP-9	296	90	CAGACCTTTGAGGGCGAACT	TCGTCGAAGTGGGCATCTC
GRα	397	77	ATCACCAATCAGATACCAAAAT	CACACAGACTTTGGGCACTG
β-actin	781	91	AAGGCCAACCGTGAGAAGATG	TGCGGTGGACGATGGAG

* As in Weber et al., 2006a

Table 3.2 Bovine gene-specific primers used for quantitative real time RT-PCR.

Primer	Length (bp)	Tm (°C)	Forward 5' to 3'	Reverse 5' to 3'
POMC	73	81	TTGTCACGCTGTTCAAAAACG	CCCTCACTGGCCCTTCTTG
IL-1β	142	79	TAACTGGTACATCAGCACTTCTCAAA	GCTCGAAAATGTCCCAGGA
CD62L*	144	74	ACGGGAAAAAAGGATTACTATGGA	GCCTATAGTTGCATATGTATCAAATTTTC
MMP9*	73	81	TGTACGGCCCGAAGCT	CCATCGAAGGGATACCCATCT
GRα†	74	78	TGTGGTTTAAAGAGGGCCAAGA	TTCTACGTTCCCATCACTGAAAAG
β-actin*	74	82	GGCCGAGCGGAAATCG	GGCCATCTCCTGCTCGAA

* As in Weber et al., 2006a; † as in Burton et al., 2005.

Table 3.3 Behavior categories recorded and their definitions.

Behavior categories	Definition
Maintenance:	
Walking	Movement of legs with a change in space
Standing on 4 limbs	Supported by 4 feet
Standing on 3 limbs	Supported by 3 feet
Laid	Not supported by feet
Feeding:	
Eating	Head in head-gate leading to feed bunk
Drinking	Muzzle in water cup
Ruminating	Chewing
Social Grooming:	
Self-grooming	Licking or rubbing a part of its own body
Allo-grooming	Licking or rubbing another animal
Agonist interaction	Initiator of contactual behavior
Non-agonistic interaction	Receiver of contactual behavior

Table 3.4 Behavioral profile of lame (n = 8) and sound (n = 8) cows. LSMeans (± SEM) are represented as the duration (min) of each behavior over the time period studied (see Materials and Methods). * $P \leq 0.05$ for main effect of lameness group.

Behavior	Lame Time (min)	Sound Time (min)	*P*-value
Ruminating	18.41 ± 3.34 *	28.57 ± 3.06	0.05
Eating	9.09 ± 2.25 *	21.83 ± 2.45	0.004
Self-grooming	4.13 ± 0.73 *	1.48 ± 0.79	0.04
Laid	30.21 ± 6.05	37.43 ± 6.59	0.45
Standing on 3 limbs	6.56 ± 3.26	3.59 ± 3.55	0.70
Standing on 4 limbs	47.36 ± 8.03	37.17 ± 8.75	0.43
Walking	5.29 ± 1.36	2.24 ± 1.48	0.17
Drinking	1.65 ± 1.12	2.40 ± 1.22	0.67
Agonistic interaction	2.11 ± 1.41	1.61 ± 1.54	0.82
Non-agonistic interaction	0.67 ± 0.45	0.74 ± 0.49	0.92

Table 3.5 Quantitative real time RT-PCR analysis of PBMC mRNA abundance (in $\log_2$ fentograms, fg) of the inflammation regulating genes pro-opiomelanocortin (POMC), interleukin-1β (IL-1β), L-selectin (CD62L), and stress hormone glucocorticoid receptor alpha (GRα) gene. β-actin is shown as a control gene. Data represent LSMeans (± SEM) of mRNA abundance for lame (n = 8) versus sound (n = 8) cows.

Gene	Lame [mRNA] (Ln fg)	Sound [mRNA] (Ln fg)	*P*-value
POMC	2.80 ± 0.70	2.62 ± 0.78	0.95
IL-1β	0.68 ± 0.17	0.67 ± 0.24	0.26
CD62L	3.42 ± 1.00	3.40 ± 1.55	0.32
GRα	1.19 ± 0.30	1.03 ± 0.35	0.23
β-actin	10.26 ± 0.63	10.56 ± 0.55	0.72

Figure 3.1 General stance and foot lesion profiles of lame and sound cows. Lame cow: (a) arched back posture (courtesy of Zinpro®Corporation, Eden Prairie, MN) and (b) foot lesion. Sound cow: (c) straight back posture (courtesy of Zinpro®Corporation, Eden Prairie, MN) and (d) no visible foot lesions.

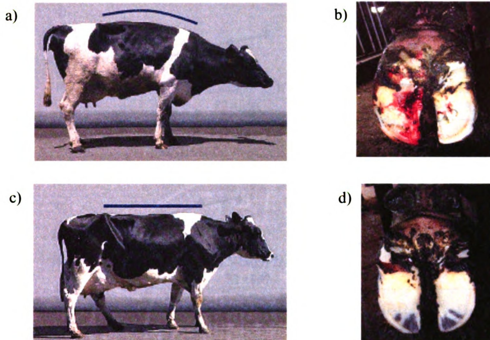


Figure 3.2 Standard curves used for absolute quantification of the expression of candidate genes.

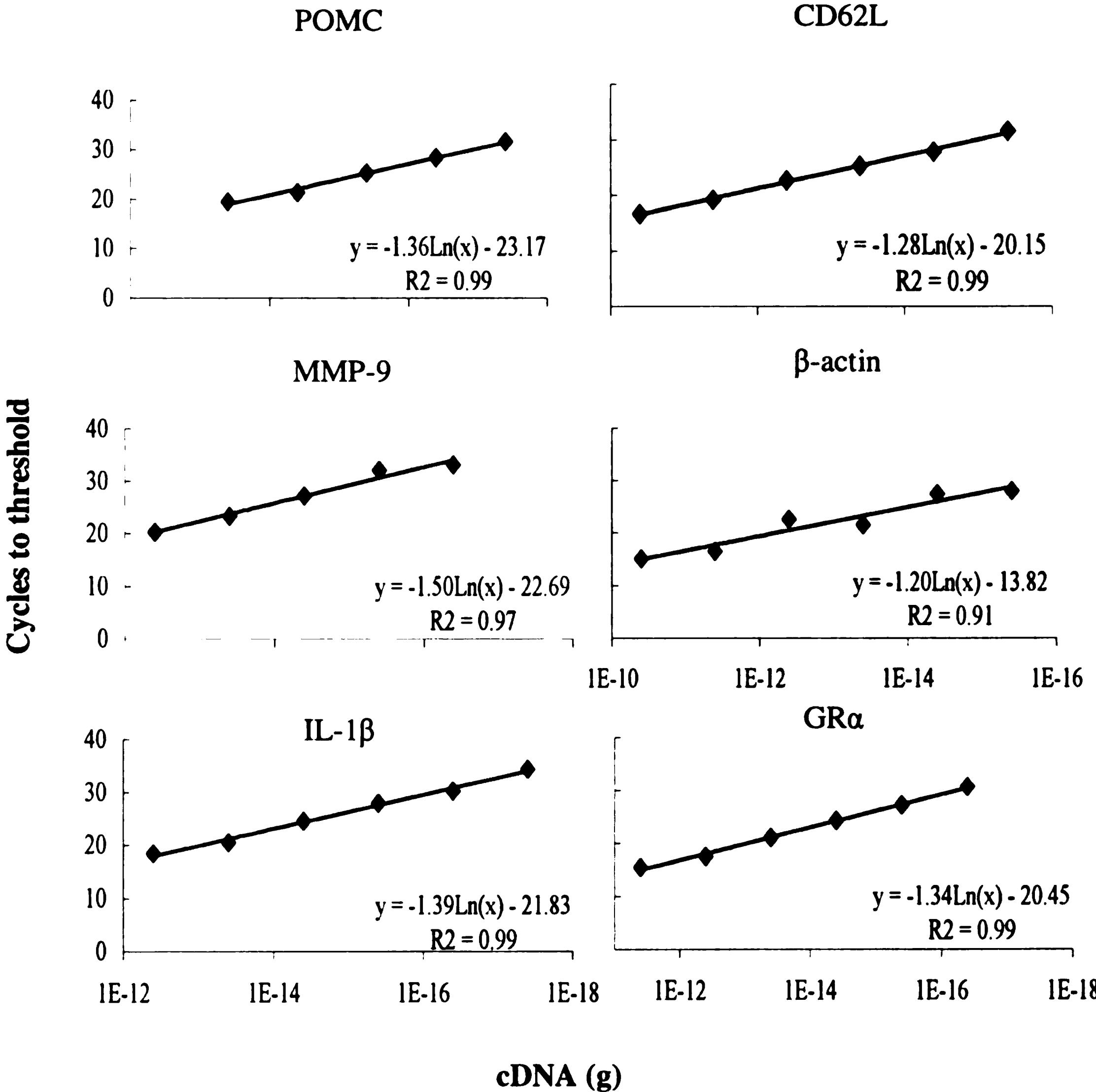


Figure 3.3 Cortisol (a), DHEA (b) concentrations, and cortisol:DHEA (c) ratio in sera of lame (n = 8) and sound (n = 8) cows. The bars represent LSMeans (± SEM) of lame (dark bar) and sound (white bar) cows. * $P = 0.01$ and † $P = 0.06$ for the main effect of lameness group.

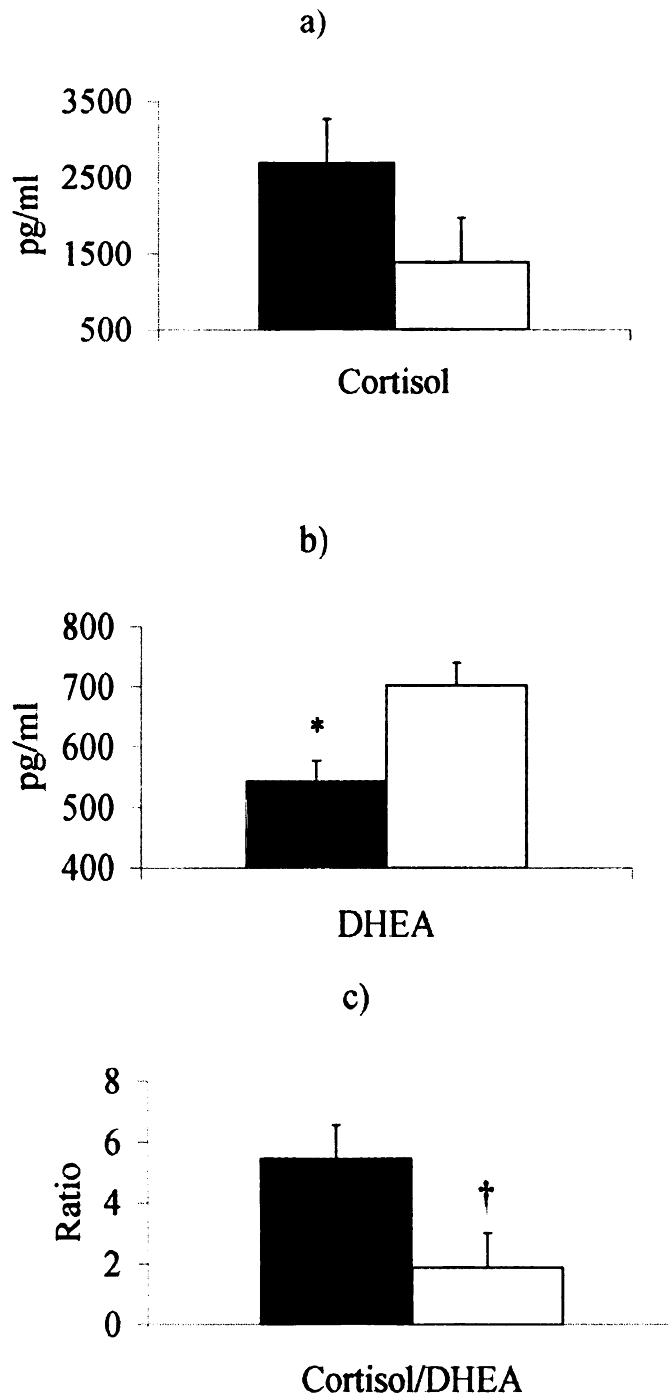
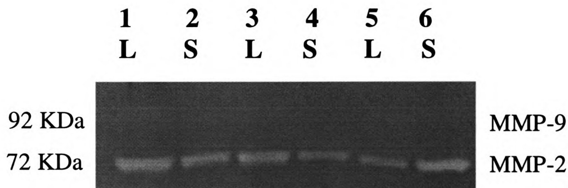


Figure 3.4 Representative gelatin zymogram of MMP-9 and MMP-2 activities in serum of lame and sound cows. Lanes 1, 3 and 5 are sera from lame (L) cows while lanes 2, 4 and 6 are sera for sound (S) cows. Little to no MMP-9 activity was detected in any sera despite clear and consistent MMP-2 activity in all sera.



CHAPTER FOUR

Signature gene expression of peripheral mononuclear cells in lame dairy cows with inflammatory foot lesions.

I. ABSTRACT

Lameness is a major health issue and likely the single most common cause of pain and discomfort in dairy cattle. Appropriate treatment is delayed or neglected due, in part, to lack of reliable diagnosis. Assessment of cows with lameness is currently limited to subjective visual scoring systems based on locomotion and posture abnormalities. These systems are unreliable to detect lameness, and therefore, a large number of cows remain lameness associated with painful inflammatory foot lesions in dairy cattle using microarray-based gene expression profiling of peripheral blood mononuclear cells (PBMC). BOTL5 microarrays spotted in duplicate with cDNA representing bovine immune response genes were interrogated with cDNA samples in an 8-array, balanced design. Samples from eight lame cows with foot lesions related-lameness and from eight sound cows were pair-matched by age, weight, days in lactation, and pregnancy status at time of PBMC collection and directly compared with each other on individual arrays. Statistical analysis of resulting fluorescence intensity data revealed 31 genes that were putatively differentially expressed in lame versus sound cows ($P < 0.05$). Of these, BLASTn analysis and gene ontology information showed that 28 genes had high similarity or homology to known human and/or rodent genes. Validation of 15 of these genes known to be important in inflammation and pain was carried out using relative quantitative real-time RT-PCR, which confirmed the up regulation of interleukin (IL)-2

(12.68 ± 1.47 fold increase) and IL-10 (2.39 ± 0.55 fold increase), matrix metalloproteinase-13 (MMP-13) (10.44 ± 1.14 fold increase), and chemokine C-C motif receptor-5 (CCR5) (5.26 ± 1.05 fold increase), in lame relative to sound cows ($P \leq 0.05$). Similarly, granulocyte-macrophage colony-stimulating factor receptor alpha chain precursor (GM-CSF-R-alpha) (2.30 ± 0.63 fold increase) and IL-4 (2.06 ± 0.59 fold increase) showed a tendency ($P = 0.10$) for up regulation in lame compared to sound cows. PBMC co-expression of IL-2, MMP-13, CCR5 and IL-10, and potentially IL-4 and GM-CSF-R-alpha appears to be a promising, objective signature of lameness-related inflammatory foot lesions in dairy cattle. In conclusion, this study revealed potential biomarkers of severe lameness in dairy cattle. In addition to boosting diagnostic reliability, the prospect of developing such biomarkers of lameness could help identify animals in need of pain relief and provide appropriate molecular targets for the development and monitoring of novel lameness therapies.

II. INTRODUCTION

Lameness in cattle is a debilitating condition which is often associated with tissue damage, pain, and discomfort and manifests as an inability to walk normally (O'Callaghan, 2002). This condition continues to be one of the largest financial drains on the North-American dairy industry, and one of the greatest concerns for animal welfare. Visual observation, the most commonly used method for lameness recognition, is time-consuming, unreliable, non-sensitive, and requires great skill on the part of the herdsman (Whay et al. 1997; O'Callaghan et al. 2003; Wells et al. 1993; Kopcha et al. 2003). Due to inappropriate diagnosis, treatment for lameness is often delayed or neglected and this

condition progresses to a more chronic or severe state that often has a compromised prognosis (Logue et al. 1998). Furthermore, common misconceptions regarding the ability of cattle to experience pain and the paucity of licensed veterinary products (O'Callaghan, 2002) might aggravate welfare problems. Attempts to develop objective methods for lameness diagnosis are limited.

The use of objective measures of locomotion alterations, such as through biomechanics, appears promising (e.g., van der Tol et al., 2004; Scott, 1989; Rajkondawar et al., 2002b). However, because of the multifactorial and etiopathogenesis complexity of lameness, a one-dimensional measure (e.g., gait change) may not provide enough accuracy and sensitivity to characterize all lameness types. Physiological measures to assist with lameness assessment are improving (e.g., cortisol:DHEA ratio as in Almeida et al., 2007 *in press*) and research aimed to expand the use of objective measures to enhance lameness detection accuracy, and ultimately, dairy cattle welfare, should be encouraged.

Although still in the early stages of research and development, genomic biomarker research can provide a comprehensive insight into pathophysiological processes as well as more precise predictors of outcome not previously attainable with traditional biomarkers (e.g., protein, biochemical) (Ginsburg and Haga, 2006). For instance, microarray-based gene expression profiling is a powerful approach for the identification of molecular biomarkers of disease, such as in human cancers (DePrimo et al., 2003) and inflammatory disease (Heller et al., 1997). Microarrays allow rapid measurement of the expression levels of thousands of transcripts in a single experiment and comparison of expression patterns across many samples (Lockhart et al., 1996). In

livestock, immune-based expression signatures for Johne's disease (Coussens et al., 2004a; 2004b), trypanosomiasis (Hill et al., 2005) and tuberculosis (Meade et al., 2006) have been discovered using microarray-based gene expression profiling.

Similar to the examples described above, a molecular diagnostic assay using a clinically accessible tissue, such as blood, could greatly assist with detection of painful inflammatory lameness in dairy cows. Gene expression profiling of peripheral blood mononuclear cells (PBMC) to assist with lameness diagnosis has never been documented. PBMCs are responsible for the comprehensive surveillance of the body for signs of infection and disease and therefore may be good sources of information about organismal status. For example, Vollmer-Conna et al. (2004) demonstrated a correlation between PBMCs derived proinflammatory cytokines and sickness behavior, while Maas et al., (2002) identified specific PBMC profiles in patients with autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, type I diabetes, and multiple sclerosis. Moreover, altered gene expression in PBMCs correlates with inflammatory bowel disease pathogenesis (Mannick et al., 2004).

In addition to altered locomotion, lame cows experience pain (Whay et al., 1997; Rushen et al., 2006) and show systemic signs consistent with sickness response including decreased food-intake (Almeida et al., 2007), increased lying time (Singh et al., 1993), altered social behaviors (Galindo and Broom, 2002), and hyperalgesia (Whay et al., 1997). The hypothesis of this study was that cows suffering from inflammatory and painful type of lameness derived from foot lesions have a signature gene expression profile of PBMC that is distinguishable from that of sound cows. The objective of this

study was to search for potential biomarkers of painful inflammatory foot lesions in lame dairy cattle using a microarray-based gene expression profiling approach.

III. MATERIALS AND METHODS

A. Subjects

The study subjects were 16 Holstein cows with mean weight, age and days in lactation of 1467 ± 114 kg, 4.5 ± 0.25 years and 183 ± 65 days, respectively. Use of animals for this study was approved by the Michigan State University (MSU)'s All University Committee on Animal Use and Care (AUF# 05/04-079-00). All cows were owned by MSU, housed in a freestall housing unit built with a 10 degree slope, and fed and cared for according to the standard operating procedures of MSU's Kellogg Biological Station Dairy Center research facility. Subject selection was performed as described in Almeida et al. (2007). Briefly, lame cows (n = 8) met the two selection criteria: (i) abnormal gait and back posture while walking and standing and (ii) presence of visible lesions on at least one hindlimb at feet inspection, suggesting that the animals were in pain. The causes (e.g., sole ulcers, footrot, sole bruising and interdigital dermatitis) and duration of lameness were not determined and likely varied from cow to cow. Instead, lame cow selection was intended to comprise a snap shot of clinical lameness in the herd at time of sampling, which is representative of what may occur in health monitoring programs on commercial farms. Sound cows (n = 8) had normal (straight) back posture and no visible foot lesions. Cows selected for this study did not show any pathological conditions other than lameness at time of sample collection. None of the lame cows were treated for lameness before blood sample collection.

B. Blood collections and isolation of PBMCs and RNA

Blood samples were obtained from one pair of random lame and sound cow per day for 8 days. All samples were collected at 9:00 am via jugular venipuncture, using 2.5-cm 14-gauge needles (Fisher Scientific, Pittsburgh, PA). Blood (130 ml per cow) intended for PBMC isolation was collected into 50 ml centrifuge tubes (BD Biosciences, San Jose, CA) that contained 4 ml of acid-citrate dextrose (ACD) as an anticoagulant (Weber et al., 2004) and immediately placed on ice. PBMCs were prepared from the ACD anticoagulated blood using a Percoll gradient as previously described (Almeida et al., 2007; Coussens et al., 2002). The cell pellets were suspended in 4 ml of TRIzol reagent (Invitrogen, Carlsbad, CA) and incubated for 10 min at room temperature to ensure lysis of PBMCs and preservation of their released RNA. This mixture was then frozen at -80°C until use for RNA isolation, which was performed according to the manufacture's instructions (Almeida et al., 2007).

C. Experimental design

The experiment included 8 cDNA microarray arrays (BOTL5) containing 3888 total spots with duplicate spots of 932 bovine EST clone inserts developed from a normalized bovine total leukocyte (BOTL) cDNA library and additional duplicate spots of 459 amplicons representing targeted cytokine, receptor, signal transduction molecule, transcription and growth factor, enzyme, cell cycle regulator and cellular component genes. A list of genes represented on the BOTL5 microarrays and their EST sequences can be found at http://www.cafg.msu.edu under the 'links' icon.

Arrays were interrogated with the labeled cDNA samples in a balanced design where each differently labeled sample was used equally often in the experimental layout as described in **Figure 4.1**. More specifically, four randomly selected lame cDNA samples were labeled with Cy3 and compared with cDNA samples from four sound cows (paired matched by age, weight, days in lactation and pregnancy status at time of PBMC collection) labeled with Cy5. The remaining 4 cDNA samples from lame cows were labeled with Cy5 and compared with cDNA samples from sound cows labeled with Cy3 using the same pair matching criteria.

D. Preparation of labeled cDNA and hybridization

All conditions for cDNA synthesis, dye labeling, array hybridizations and readings were as described previously (Coussens et al., 2002). Briefly, PBMC total RNA (12 μg) was converted to cDNA using the Atlas Powerscript labeling system (Biosciences Inc., Alameda, CA) according to the manufacturer's instruction, with minor modifications as described previously (Coussens et al., 2002). Reverse transcription was performed using Oligo $(dT)_{15-18}$ as primer. Following first-strand cDNA synthesis, cDNAs from each cow were split into two equal aliquots and these were differentially labeled using NHS-derivatized Cy3 or Cy5 dyes (Amersham Pharmacia Biotech; Piscataway, NJ). Labeled cDNAs were purified to remove unincorporated dyes, paired among cows according to the experimental design described in **Figure 4.1** to 10 μl using Microcon 30 spin concentrators using (Millipore Corp., Bedford, MA).

Microarray hybridizations were performed as described in Madsen et al. (2004). Briefly, concentrated Cy3 and Cy5 labeled probe cDNA was added to 110 μl of

hybridization buffer (SlideHyb-3, Ambion Inc., Alameda, CA) and incubated at 70°C for 5 min immediately before hybridization. Hybridizations were conducted for 18 h in a GeneTAC Hybridization Station (Genomics Solutions Inc., Ann Arbor, MI) using a step-down hybridization protocol (65°C for 3 h, 55°C for 3 h, 50°C for 12 h). Following hybridization, microarrays were washed within the hybridization station, followed by rinsing once in 0.3 M NaCl, 0.03 M sodium citrate and once in ddH_2O, and then immediately dried by centrifugation. Microarrays were scanned using a GeneTAC LS IV microarray scanner and GeneTAC LS software version 3.3.0 (Genomic Solutions, Inc., Ann Arbor, MI). Digital Genome Pro software version 2 (MolecularWare Inc., Cambridge, MA) was used to process microarray images, find spots, and finally to create reports of raw total spot intensities.

E. Microarray data analysis

Total fluorescence intensity values for Cy3 and Cy5 from each spot on the microarrays were imported into SAS (SAS/STAT software, SAS Institute, Cary, NC) and averaged across duplicate spots. Potential dye intensity biases in the microarray data sets were visualized using M (logCy3 - logCy5) vs. A ((logCy3 - logCy5)/2) plots as described in Madsen et al. (2004). Array-specific data normalization was then performed considering a locally-weighted regression and smoothing scatter plots (LOESS) procedure of SAS (SAS Institute, SAS/STAT Software version 9.1) to account for dye biases (Yang et al., 2002). The efficiency of LOESS normalization was assessed by monitoring M-A plots for data from each array before and after LOESS normalization. A two-step mixed model (Wolfinger et al., 2001) was used to analyze the LOESS-adjusted

log intensities. The first step involved array specific normalization and the second step involved gene-specific analyses to test for the effects of group (lame versus sound cows). The normalization model included the fixed effect of dye, as well as the random effects of array, patch x array, dye x array and dye x patch within array effects. The residuals from the first step were scale-normalized for differences in within array variability. The second step of the mixed model analysis consisted of gene-specific models for the aforementioned residuals. The gene-specific model included the fixed effects of dye and group as well as random effects of array, spot within array and cow within group. The analysis was performed with the MIXED procedure of SAS (Littell et al., 1996). In the gene-specific analysis, lameness effect was declared significant when $P \leq 0.05$ and tendency towards significance when $0.06 \leq P \leq 0.10$.

F. Validation of differential gene expression using quantitative real-time RT-PCR

The spotted cDNA sequences representing genes whose expression profiles were and tended to be significantly influenced by lameness in this analysis were matched to the nearest human and mouse homologs in TIGR by BLAST searching, facilitated by the Michigan State University Center for Animal Functional Genomics web site at http://www.cafg/msu.edu. Biological functions of these genes were determined through an extensive PubMed literature search (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). This information was used to determine which genes to select for validation of differential expression due to lameness. Laboratory-based validation of differential gene expression of 15 genes was performed on genes with documented importance to immune

response, inflammation and pain, and *P*-value of effect of lameness ≤ 0.05 in the microarray data analysis (**Table 4.1**).

Recent evidence indicates that quantitative information from arrays may be imprecise for transcripts showing small differences in expression, and therefore, overlook genes of significant interest (Chuaqui et al., 2002). Recent evidence suggests that anti-inflammatory cytokines (i.e., IL-10 and IL-4) modulate widespread pain in humans (Üçeyler, 2006). For this reason, we decided to pursue with investigation of the expression of IL-10 and IL-4 even though microarray analysis showed no statistical indication that these genes were affected by lameness ($P > 0.10$).

Real-time RT-PCR on an Applied Biosystems 7000 DNA sequence detection system (Perkin Elmer Corp., Foster City, CA) was used for validation. Individual RNAs from Percoll purified PBMCs of the same cows used in the microarray experiment, were converted into first-strand cDNA as described in Madsen et al. (2004). Briefly, 2 µg of the RNA was combined with 10 mM oligo$(dT)_{12\text{–}18}$ primer and sterile water in a 10 µl volume that was incubated 5 min at 70°C followed by 5 min at 20°C. Master mix containing 4 µl of buffer (supplied by the RT manufacturer; final reagent concentrations of 50 mM Tris.HCl, pH 8.3, 75 mM KCl, and 3 mM $MgCl_2$), 200 U of SuperScript II RNase H^- reverse transcriptase (Invitrogen Life Technologies), and a final concentration of 10 mM DTT and 0.5 mM dNTP were added to achieve a final reaction volume of 20 µl. Reverse transcription was allowed to proceed at 42°C for 60 min, heated to 70°C for 15 min, and cooled to 37°C prior to the addition of 2 U of DNase-free RNase H (Invitrogen Life Technologies). Incubation at 37°C was continued for 20 min in the presence of RNase H to remove the original RNA template followed by enzyme

inactivation via addition of 0.5 μl of 0.5 M EDTA (pH 8.0). First-strand cDNAs were purified with QuickClean resin (Clontech) followed by precipitation with sodium acetate and ethanol. Purified cDNAs were suspended in DNase/RNase-free sterile water, quantified spectrophotometrically (NanoDrop Technologies, Wilmington, DE), diluted to a final concentration of 10 ng/μl, and stored at -20°C until use. Real-time RT-PCR was performed for 15 genes using the SYBR Green PCR Master Mix (PerkinElmer Applied Biosystems). Gene-specific primer pairs (**Table 4.2**) were designed using Primer Express Software (PerkinElmer Applied Biosystems) and synthesized at a commercial facility (Qiagen-Operon, Alameda, CA). Primers for β-actin were also made, and this gene was included in all real-time RT-PCR analyses for the purpose of data normalization (as in Madsen et al., 2002). The cDNA and primer concentrations used in the real-time RT-PCR assays for determination of each gene expression were optimized prior to the experiment (**Table 4.2**). Results were recorded as relative gene expression changes after normalizing for β-actin gene expression, computed using the $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen (2001). This method monitors relative gene expression changes across treatments (lameness groups – lame versus sound) based on differences in the PCR amplified target reaching a fixed threshold cycle (Ct) number for one treatment (lame) vs. a control treatment (sound). For our $2^{-\Delta\Delta Ct}$ analysis, the Ct for sound cows was the calibrator used to determine relative gene expression changes of lame cows for each β-actin-normalized test gene. Statistical analysis of these data was performed using the MIXED of SAS (SAS/STAT Software, SAS Institute) with a model that included lameness group as the fixed effect and cattle age (in days), weight (in kg), day in lactation (in days), and

pregnancy status (yes, no) as covariates when found to significantly affect gene expression at $P < 0.05$.

IV. RESULTS

A. Microarray experiment

Statistical analysis of resulting total spot fluorescence intensity revealed 31 genes that were putatively differentially expressed ($P \leq 0.05$) in lame versus sound cows (**Table 4.1**). Of these, BLASTn analysis and gene ontology information disclosed 28 genes with high similarity and/or homology to known human and/or rodent genes (**Table 4.1**). The false discovery rate (FDR) for the putatively differentially expressed genes was approximately 78 %.

B. Validation of differential expression

Real-time RT-PCR assessment of 13 of the 31 genes that were putatively altered by lameness ($P \leq 0.05$) in the microarray experiment confirmed the up regulation of 3 of these genes in lame compared to sound cows (**Figure 4.2**). These genes were: (i) IL-2: 12.68 ± 1.47 fold increase ($P = 0.03$); (ii) MMP-13: 10.44 ± 1.14 fold increase ($P = 0.01$); and (iii) CCR5: 5.26 ± 1.05 fold increase ($P = 0.03$). Furthermore, GM-CSF-R-alpha demonstrated a tendency ($P = 0.08$) for up regulation (2.30 ± 0.63 fold increase) in lame relative to sound cows (**Figure 4.3**).

Although microarray data suggested no effects of lameness on either IL-10 ($P = 0.24$) or IL-4 ($P = 0.28$), the expression of these two genes was investigated using real-time RT-PCR $2^{-\Delta\Delta Ct}$ analysis due to their recently reported role in mediating widespread

pain in humans (Üçeyler et al., 2004). Results revealed a significant up regulation (P = 0.03) of IL-10 (2.39 ± 0.55 fold increase) and a tendency for up regulation (P = 0.10) of IL-4 (2.06 ± 0.59 fold increase) in lame relative to sound cows (**Figure 4.3**).

V. DISCUSSION

The results of this study present PBMC up regulation of IL-2, MMP-13, CCR5, IL-10, IL-4 and GM-CSF-R-alpha as potential candidates to assist the diagnosis of inflammatory foot lesions in lame dairy cows. The small number of validated genes (3 out of 13) in this study supports the high false discovery rate (FDR) of 78 % found for the genes putatively differentially expressed ($P < 0.05$) in the microarray experiment. FDR is defined as the expected proportion of rejected null hypotheses that are false positives (Benjamini and Hochberg, 1995). Issues such as sampling cows with different lameness etiologies, severities and chronicities, the lack of control for the number and specific type of cells collected from each animal, and the small number of biological replicates, likely contributed to the high FDR and low statistical power and precision in this experiment (Churchill, 2002; Cui and Churchill, 2003). Despite these pitfalls, the microarray-based gene expression approach was effective in pointing to a small group of interesting candidate biomarkers that would not have been explored otherwise.

Overall, the PBMC gene expression pattern observed in this study indicates that lame cows have systemic changes consistent with immune system activation. For example, IL-2 is a growth factor for antigen-stimulated T lymphocytes that is responsible for T cell clonal expansion after antigen recognition. IL-2 is involved in several immunological events including the increase in monocyte-mediated natural cytotoxicity

against certain tumor cells *in vitro* and *in vivo* (Malkovsky et al., 1987), stimulation of IFN-γ production, and enhancement of B lymphocyte responses (Mosmann and Coffman, 1989; Abbas et al., 1996; Opal and DePalo, 2000). IL-2 also induces other cytokines that exert their effects largely through the cyclooxygenase pathway. Moreover, it has a unique role in activation-induced cell death and in the maintenance of peripheral regulatory T (Treg) cells, which makes it important in pathogenesis of autoimmune diseases (Fontenot et al., 2005; D'Cruz and Klein, 2005; Maloy and Powrie, 2005). The up regulation of IL-2 noted in the lame cows agrees with previous research dealing with other types of inflammatory and infectious conditions such as in vasculitis and meningitis (Zucker et al., 2006), and in tuberculosis (Guntupova et al., 2006; Turgut et al., 2006). Signals triggered by IL-2 initiate integrated host responses to infection and injury (Michie et al., 1988), and therefore, the up-regulation of this gene in lame cows indicates that in addition to the inflammatory lesions in the foot, lame cows have systemic changes that are amenable of objective measurement.

The disease fighting scenario painted by the PBMC gene expression profiles observed was re-enforced by the up regulation of MMP-13 and GM-CSF-R-alpha in lame cows. Matrix metalloproteinases (MMPs) were discovered in connection with their capacity to degrade extracellular matrix (ECM) proteins such as collagen and elastin. MMP-13 is the third member of the collagenase subfamily of MMPs and modulates ECM metabolism directly by degrading matrix molecules, as well as indirectly by activating other MMPs (e.g., MMP-2, MMP-9) (Leeman et al., 2002). It is expressed in chondrocytes, osteoblasts and periosteal cells, but also in PBMCs of humans with rheumatoid arthritis (Vázquez-Del Mercado et al., 1999). MMP-13 release is modulated

by proinflammatory cytokines and growth factors (Neidel et al., 1995) and it plays a major role in joint destruction (Firestein, 1996, Nagase and Woessner, 1999). Therefore, MMP-13 is of special interest for the pathogenesis of arthritic conditions such as rheumatoid arthritis (RA) and osteoarthritis (OA) in which its concentration is found to be up-regulated in both serum and synovial fluid (Itoh et al., 2002; Andereya et al., 2006). GM-CSF-R-alpha, a low affinity receptor for granulocyte-macrophage colony-stimulating factor (GM-CSF), transduces a signal that results in the proliferation, differentiation, and functional activation of hematopoietic cells. GM-CSF stimulates stem cells to produce granulocytes (neutrophils, eosinophils, and basophils) and macrophages. GM-CSF-R-alpha and its ligand are thus part of the immune/inflammatory cascade. Similar to IL-2, the up regulation of MMP-13 and GM-CSF-R-alpha in PBMCs of lame cows highlights the pro-inflammatory state of these animals and suggests the immune system as an important source of candidate biomarkers of inflammatory lameness in dairy cows.

Chemokine receptors (called CCR1 though CCR11) are expressed in leukocytes, with the greatest number of distinct chemokine receptors observed on T cells (Weber et al., 2000; Mueller and Strange, 2004). The increased chemokine receptor-5 (CCR5) expression in lame cows was an interesting finding of this study because previous *in vivo* studies confirm a direct role of chemokines in nociception. For example, when injected intradermally into a rat's paw, CCL5 (ligand for CCR1, CCR3 and CCR5) induces pain (Abbadie, 2005). In addition, chemokines are important for the extravasation of leukocytes from blood to the injury site, an essential step for the inflammatory process and subsequent wound healing (Johnson et al., 2005; Khan et al., 2006). CCR5 is one of

the major inflammatory chemokine receptors in immune cells and has been the focus of several studies because of its function as a co-receptor for entry of HIV into host cells (Murphy, 2001). Moreover, CCR5 is an important regulator of the analgesic activity of opioids in the brain (Szabo et al., 2002). CCR5 ligands apparently can exacerbate pain experience by inactivating normal neuronal signaling pathways involved in reducing the pain sensation (Scholtz and Woolf, 2002; Marchand et al., 2005). The up-regulation of molecules involved in pain modulation, such as CCR5, suggests the importance of pain within the lameness scenario (as in Rushen et al., 2006).

In addition to the importance of inflammation as means of immune defense and healing, strong inhibitory mechanisms to the inflammatory process are needed to avoid uncontrolled inflammation and consequent severe tissue damage. Endogenously, the "stop signals" for inflammation are mediated by several factors including lipoxins (block neutrophils influx to injury site) (Levy et al., 2001), cyclooxygenases, and anti-inflammatory cytokines produced by T_H2 class T cells, such as IL-10 and IL-4. IL-10 inhibits activated macrophages and dendritic cells and thus is involved in the control of innate immune inflammatory reactions and cell-mediated immunity (Abbas and Lichtman, 2003). IL-4 on the other hand, mediates adaptive humoral immunity. It is the signature cytokine of the T_H2 subset and functions as both the inducer and an effector of these cells (Okada et al., 2003), and the major stimulus for the production of Ig1 and IgE antibodies by B cells, and for the development of T_H2 cells from naïve $CD4^+$ T cells. IL-4 also antagonizes the macrophage-activating effects of IFN-γ and thus inhibits cell-mediated immune reactions (Abbas and Lichtman, 2003). Contrary to what was demonstrated in models of chronic widespread pain (Üçeyler et al, 2006), lame cows

demonstrated an increase in expression of both IL-10 and IL-4. These results suggest that lame cows have an active acquired anti-inflammatory response that is toning down inflammatory T_H1 type activity, perhaps in an attempt to limit further tissue damage and pain.

This study utilized a microarray-based gene expression approach to discover candidate biomarkers for inflammatory foot lesions in lame dairy cattle. Our results support the premise that PBMCs serve as a surrogate tissue for evaluation of disease-induced gene expression in cows suffering from inflammatory lameness. Considering the subjective and unreliable nature of lameness screening methods currently used in dairy herds, our findings foster the possibility of using objective measures to assist with lameness detection. However, before any genomic biomarkers are incorporated into clinical practice, several issues need to be addressed in order to generate the necessary levels of evidence to demonstrate analytical and clinical validity, and utility (Chuaqui et al., 2002). For example, in addition to validating array results at the mRNA level, it is equally important to evaluate expression levels of the corresponding protein products. Moreover, the universality of results (if the gene expression signatures are an essential feature of lameness) needs to be investigated. This can be addressed by evaluating a critical gene set in a larger and more extensive study group with different lameness types and severities. In addition to boosting diagnostic reliability, the prospect of developing such biomarkers of foot inflammation related-lameness could help identify animals in need of pain relief and provide appropriate molecular targets for the development and monitoring of novel lameness therapies.

Table 4.1 Genes detected in PBMC by cDNA microarray analysis as putatively altered in expression by lameness. * Genes selected for validation.

BOTL number	Gene name	P-value	TGR number	Annotation	Gene ontology and/or molecular function
0400264_PCR	RDC1	0.001	*TC230839	Chemokine orphan receptor 1	G-protein coupled receptor activity
0400209_PCR	Ephb4	0.002	TC252707	Mus musculus ES cells cDNA, RIKEN full-length enriched library, clone:C330025M20	Regulation of axonogenesis
0400053_PCR	MTHSP75	0.005	TC241061	Heat shock 70kD protein 9B	ATP binding, protein folding
0400319_PCR	JAK1	0.005	*TC290193	Janus kinase 1	Janus kinase activity
0100008_G08	ENOS	0.005	TC246252	ENOS interacting protein	Generates nitric oxide
0100003XD01R	Nudix type motif 3	0.006	TC225215	Diphosphoinositol polyphosphate phosphohydrolase	Cell signaling
0400351_PCR	p97-MAPK	0.008	*TC252815	Mitogen-activated protein kinase 6	Signal transduction
0100004XE06R	VDAC3	0.015	*TC277500	Voltage-dependent anion-selective channel protein 3	Voltage-dependent ion-selective channel activity
0400507_PCR	VDAC3	0.020	TC277873	Voltage-dependent anion-selective channel protein 3	Voltage-dependent ion-selective channel activity
0400445_PCR	KSras1	0.021	*TC248430	Kinase suppressor of ras-1	Signal transduction
0400278_PCR	IL-2	0.024	*TC230205	BOVIL2A interleukin 2 precursor	Immune response
0400004_PCR	CD34	0.025	*TC279896	CD34	Cell adhesion
0400231_PCR	FRAP2	0.028	TC226961	Rapamycin associated protein	Signal transduction

Table 4.1 Continuation.

0100010_G03	Tcerg1	0.028	*TC246171	Transcription elongation regulator 1	Transcription regulation
0100002XD04R	Rab5IP	0.028	TC229885	Sad1/unc-84-like protein 2	Unknown
0400524_PCR	Activin beta-A chain	0.030	TC265993	Inhibin beta A chain precursor	Cell signaling, cytokine activity
0100008_B04	IRF2BP2	0.031	*TC225446	Interferon regulatory factor-2 binding protein 2A	Cell growth control
0400601_PCR	LZF	0.037	TC243218	Leucine zipper domain protein	Positive regulation of cell proliferation, hematopoiesis
0400642_PCR	TGFR-2	0.038	*TC227688	Transforming growth factor beta type II receptor	Regulation of cell proliferation and growth
0100001XD03R	Limd2	0.038	TC289881	MGC10986 protein	Zinc ion binding
0100001XF05R	TBC1	0.040	TC250205	TBC1 domain family protein C20orf140	Unknown
0100006XC07R	Unknown	0.042	Unknown	Unknown	Unknown
0100006XD08R	Unknown	0.043	Unknown	Unknown	Unknown
0100011_F12	Unknown	**0.045**	Unknown	Unknown	Unknown
0100013_A11	TMSL8	0.045	TC247374	NB thymosin beta	Organization of the cytoskeleton
0400430_PCR	BNIP3	0.045	TC224465	BCL2/adenovirus E1B 19-kDa protein-interacting protein 3	Apoptosis, anti-apoptosis
0400254_PCR	GM-CSF-R-alpha	0.045	*TC226912	Granulocyte-macrophage colony-stimulating factor receptor alpha chain precursor	Cytokine receptor activity
0400069_PCR	Tuba6	0.046	TC274308	Tubulin alpha-6 chain	Microtubule-based process
0400561_PCR	PRBP	0.046	TC276507	Plasma retinol-binding protein precursor	Visual perception, retinol binding
0400358_PCR	MMP-13	0.048	*TC249148	Matrix metalloproteinase-13	Collagenase activity
0400373_PCR	CCR5	0.049	*TC234936	Chemokine C-C motif receptor 5	Cell signaling, chemotaxis

Table 4.2 Sequence of primers used for real-time RT-PCR measurement of gene expression and concentration of template PBMC cDNA used in the reactions. F = forward, R = reverse.

Gene name	Sequence (5' to 3')	[Primer] (μM)	[cDNA] (ng)
RDC1-F	ACCTACTGCCGGGCTTTCTAC	600	10
RDC1-R	GACCAGCTCCATGCTGATGA		
IL2-F	GGAGAAAGTTAAAAATCCTGAGAACCT	300	10
IL2-R	AACCTTGGGCGCGTAAAAG		
TGFRII-F	GCTTCGCCGAGGTCTACAAG	600	10
TGFRII-R	GGCCACGGTCTCGAACTG		
CCR5-F	GAGGCTCATCTTCGTGATCATG	300	40
CCR5-R	GGAAGGTGCTCAGGAGAAGGA		
MMP13-F	TTTTTCCCCCTTTAATCCTTCAT	600	30
MMP13-R	ACACAATGGTTCTTCCCTTCAAG		
CD34-F	TGGCTGAGCCTGGAACCA	600	30
CD34-R	CCAGATCCTCCAAACACACAGA		
Jak1-F	GACTCTGCATGAGCTGCTTACTTACT	300	10
Jak1-R	TGGGCCTATCATTTTCAGGAA		
MAPK-F	GCACTGGATTTCCTGGAACAA	300	30
MAPK-R	AGCGCTTCTTCTGCTGTCAAC		
Tcerg1-F	CACCTGTGCAAACCGTTCCT	300	10
Tcerg1-R	AGGCACTGAATGCGGAACA		
GM-CSF-Ralpha-F	CGGCTGCCCAAAACTTCTC	300	10
GM-CSF-Ralpha-R	GGCCCAGCTGCAGTTCAT		
IFNR2-F	CCTTCTGTTCCTTCGCACAAG	600	10
IFNR2-R	GCTCCCTGCTGTTTGATGCT		
KSras1-F	GCCCCTGCCTGTAGAATATCCT	300	10
KSras1-R	CGAGGGCACGGATTCTGT		
VDAC3-F	ACCAGAAGGTGAATGAGAAGATTGA	300	10
VDAC3-R	CCGAAGCGGGTGTTGTTACT		
IL-10-F	CCTTGTCGGAAATGATCCAGTTTT	300	10
IL-10-R	TCAGGCCCGTGGTTCTCA		
IL-4-F	GCCACACGTGCTTGAACAAA	300	10
IL-4-R	TGCTTGCCAAGCTGTTGAGA		

Figure 4.1 Microarray experimental design.

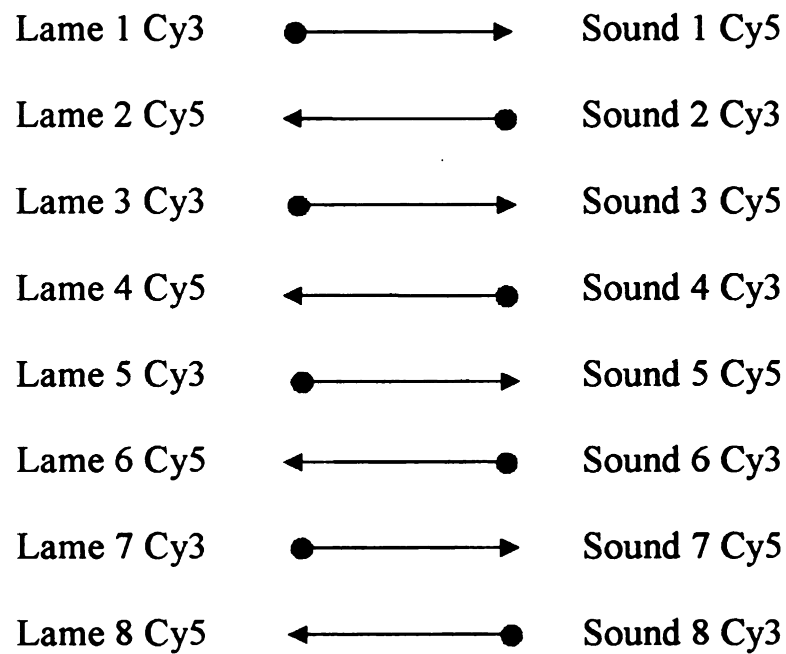


Figure 4.2 Real-time RT-PCR of lameness effects on PBMC expression of IL-2, CCR5 and MMP-13. The bars represent means of expression ratios between lame (n = 8) and sound (n = 8) cows derived using the $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen (2001), with β-actin as the control gene and sound cows as the calibrator. The asterisks (*) represent mean differences between lame and sound cows ($P \leq 0.05$).

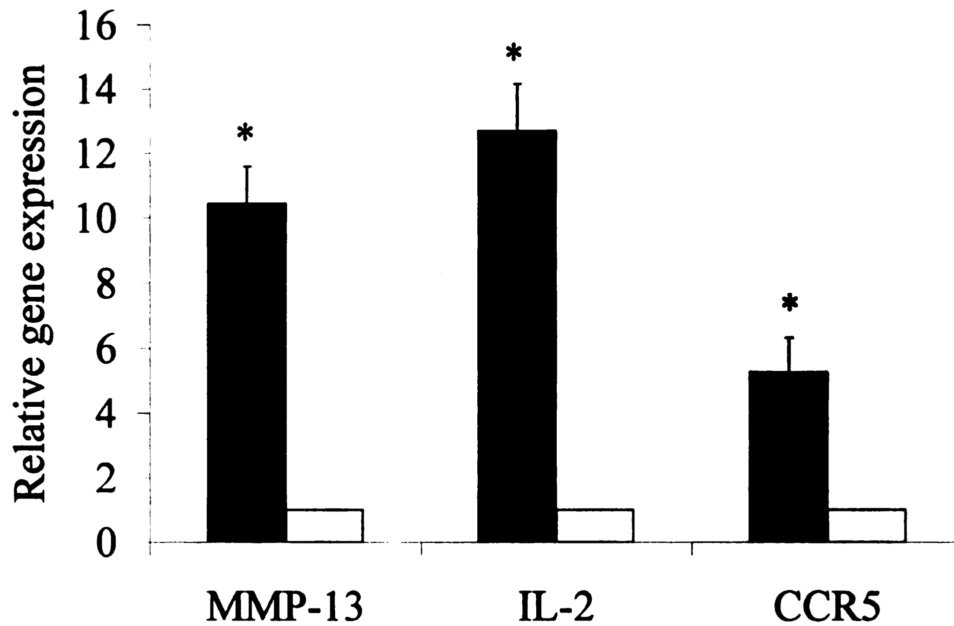
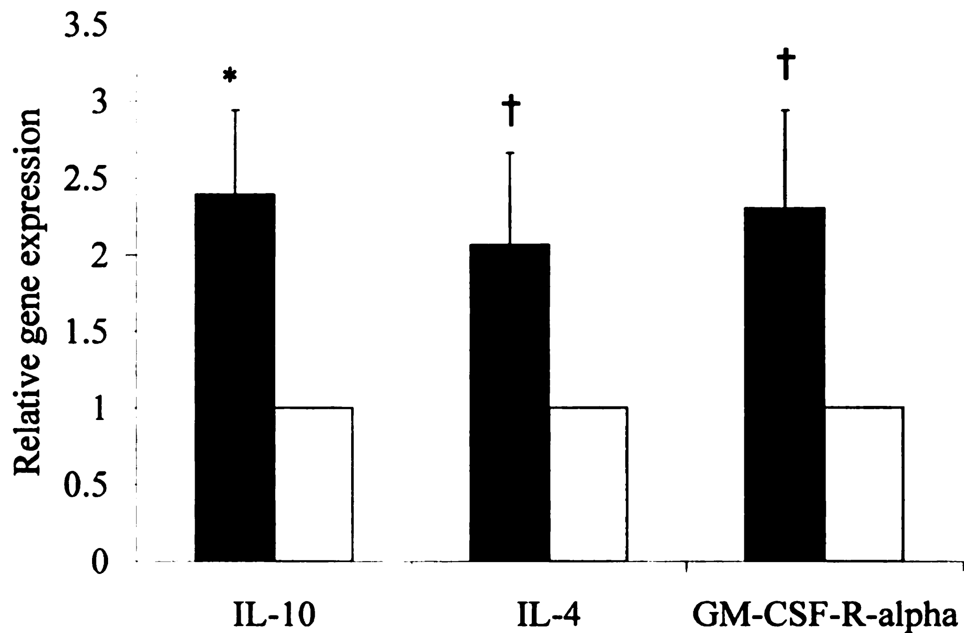


Figure 4.3 Real time RT-PCR of lameness effects on PBMC expression of the anti-inflammatory cytokines IL-10, IL-4, and pro-inflammatory GM-CSF-R-alpha. The bars represent means of expression ratios between lame (n = 8) and sound (n = 8) derived using the $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen (2001), with β-actin as the control gene and sound cows as the calibrator. The asterisk (*) represents mean differences ($P \leq 0.05$) and the crosses (†) represent a tendency for means differences ($0.05 \leq P \leq 0.10$) between lame and sound cows.



CHAPTER FIVE

General Discussion

Lameness is a significant economic factor for the dairy industry (Blowey, 1993), and an indicator of pain and compromised animal welfare (Whay et al., 1998; 2003). Several scoring systems that consider posture and gait have been proposed to allow categorization of lameness by severity. However, these methods are observer dependent (Whay et al., 2002) and thus lack reliability. The experiments described in Chapter Two through Chapter Four of this dissertation highlight several novel findings regarding the use of objective markers of locomotion abnormalities, and immune and neuroendocrine activation for detection of inflammatory foot lesions that cause pain and lameness in dairy cows.

The first prominent finding of these studies was that a pressure plate was sensitive in detecting the presence of *papillomatous digital dermatitis* (hairy heel wart) type lameness in heifers otherwise diagnosed as sound using the subjective lameness scoring system of Sprecher et al. (1997). Asymmetry in peak vertical ground reaction force (PVF) between the hindlimbs of heifers was shown to be a prominent marker for the diagnosis of hairy heel wart type lameness lesions. These results support previous findings in cows (Rajkondawar et al., 2002a; Scott 1989) and horses (e.g. Merkens and Schamhardt, 1988) and, importantly, highlight the potential of PVF for objective and early detection of lameness when compared to conventional subjective lameness scoring systems. If lameness is detected accurately and early, lameness prognosis and the overall welfare of the cows may be enhanced (Logue et al., 1998). However, to be considered an

auxiliary diagnostic tool, ground reaction force data collection must be practical, reliable, suit operating procedures in dairy farm settings, and interpreted easily. Furthermore, the magnitude of locomotor variability of dairy cattle at walk is unknown and needs to be explored. Locomotion variability dictates the number of trials necessary to have a representative profile of the animal's motion. Variability in locomotion can be minimized by using an average of multiple trials as a representative of the gait pattern, reducing inter-trial walking velocity variation, and (or) collecting data from both right and left limbs simultaneously. If multiple trials are indeed required to account for the locomotion variability at walk, then the feasibility of using PVF as an objective marker for lameness assessment 'at walk' at the farm level likely will be hindered. Therefore, measuring ground reaction forces with the cow standing (e.g., at the milking parlor or robot milking system) instead of walking might represent an alternative solution to this issue, as recently demonstrated by Pastell et al. (2006).

Although gait changes (e.g., PVF) are useful indicators of hoof discomfort associated with inflammatory and painful lesions (Neveux et al., 2006), these changes also might indicate conditions that are not animal welfare concerns. For example, factors such as physical constraints [e.g., distended udder; (Flower et al., 2006), conformational abnormalities, and rough flooring surfaces (Rushen and de Passille, 2006; Phillips and Morris, 2001)] are known to alter locomotion in otherwise sound cows. Therefore, gait changes alone will likely not provide accurate detection of lameness caused by painful inflammatory foot lesions. Accordingly, findings from the second and third studies of this dissertation present alternatives to the one-dimensionality of current lameness

assessments by providing new evidence that physiological markers (biomarkers) amenable of objective measurement may be useful in lameness diagnosis.

This alternative dimension of measures encompasses the changes within the immune and neuroendocrine systems in response to tissue injury and infection. For example, cows with behavioral alterations indicative of lameness from painful inflammatory foot lesions had lower serum DHEA concentrations and higher cortisol:DHEA ratios. These hormonal changes are characteristic of pituitary-dependent adrenal response stimulation, and, along with the sickness behaviors, they provide evidence to support the hypothesis that a sensitization of the central nervous system occurred in the subjects suffering from inflammatory painful foot lesions. To the author's knowledge, such observations have not been documented before in dairy cows. These findings characterize a potential dysregulation of the HPA axis and cortisol:DHEA which can cause immunological changes that predispose to pathologies by altering T_H1/T_H2 balance. DHEA favors a T_H1 immune response by increasing T cell production of IL-2 (Daynes et al., 1990; Suzuki et al., 1991), and decreases synthesis of proinflammatory cytokines by tissue macrophages and dendritic cells, such as IL-6 and TNF-α (Di Santo et al., 1996; Straub et al., 1998). On the other hand, cortisol favors a T_H2 response by suppressing macrophage production of IL-12 (Elenkov et al., 1996; Blotta et al., 1997). Thus, the steroid imbalance observed in the lame cows of the current study may be linked to a physiological coping strategy involving T_H1/T_H2 imbalance, such that T_H2 dependent humoral immune activity was favored over T_H1 dependent inflammatory activity. In light of the importance of discovering diagnostic biomarkers of lameness, this hypothesis is worthy of further testing.

The immune system is considered one of the most important indicators of organismal status because it is responsible not only for protecting the organism against pathogens but also for modulating different types of pain experiences (e.g., acute, neuropathic) (Moalem and Tracey, 2006) and sickness responses (Hart, 1988). In Chapter Four of this dissertation, activation of the immune system was characterized as an increased abundance of specific expressed genes in circulating PBMC in lame compared to sound cows. These data provide evidence that lame cows were systemically engaged in fighting infection and controlling inflammation. Specifically, PBMC co-regulation of IL-2, MMP-13, CCR5, IL-10, IL-4 and GM-CSF-R-alpha were presented as candidates' biomarkers for screening lame dairy cows with inflammatory foot lesions. Although not characterized in this study, the release of proinflammatory cytokines from damaged tissue and activated immune cells is assumed to be the main trigger for the sickness behaviors and immunological changes demonstrated by lame cows with painful inflammatory painful foot lesions (Buckingham, 1994). Thus, these up regulated PBMC genes may have contributed to the sickness behaviors and endocrine changes in lame cows that were documented in Chapter Three of this dissertation.

During injury and infection, activation of T cells by antigens and co-stimulators induces transcription of the IL-2 gene (13 fold increase in lame compared to sound cows) and synthesis and secretion of the IL-2 protein, which initiates integrated host responses to infection and injury (Michie et al., 1988). The secretion of IL-2 leads to T-cell proliferation and other activities that are crucial for regulation of the immune response, including immune cell survival (activation of Bcl-2), stimulation of adjacent T cells, proliferation and differentiation of B cells and NK cells, and increase in production of

other cytokines such as IFN-γ by T_H1 cells and IL-4 by T_H2 cells. Therefore, the up regulation of IL-2 observed in lame cows suggests that these animals are actively responding to microbial infection at the site of tissue injury.

Similarly, the approximate 2-fold up regulation of IL-4 observed in lame cows supports the hypothesis that the animals are trying to cope with inflammation by expressing a cytokine that suppresses inflammatory responses via inhibition of IFN-γ macrophage-dependent reactions. If true, there would be an imbalance in the T_H1/T_H2 ratio in lame cows such that T_H2 cell responses and subsequent antibody production would be favored. One way to test this would be to measure serum concentration of antibodies that are induced by T_H2 type cells cytokines, such as IgE and IgG1.

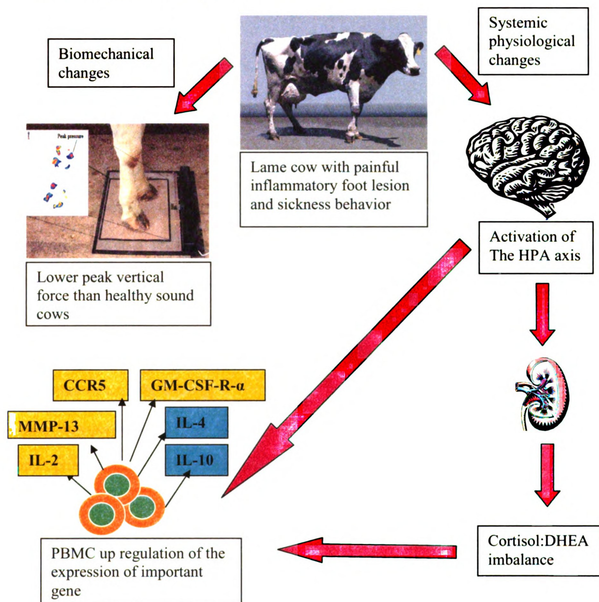
Likewise, IL-10 (up regulated 2.4 fold in lame cows) also participates in the down regulation of inflammatory immune responses by hampering activation of macrophages and dendritic cells and thus inhibiting T_H1 responses. The mechanism by which this occurs is similar to that of IL-4. That is, IL-10 is an inhibitor of IFN-γ and also of IL-12, which provide the main stimuli for T_H1 cell differentiation and effector functions. IL-10 is produced by activated macrophages (Abbas and Litchman, 2003). Because most macrophages are settled within tissues and not in the circulating PBMC population, the increased abundance of IL-10 gene observed in PBMC of the lame cows of this study might have derived from T lymphocytes, which also can produce IL-10 (Abbas and Litchman, 2003). Overall, the up regulation of IL-4 and IL-10 in PBMC of lame cows may indicate an attempt by the animal's immune system to squelch ongoing inflammation in the affected foot (**Figure 5.1**). Importantly, this physiological change

was readily detectable and quantifiable in blood PBMC and thus may be amenable to objective measurement as a potential biomarker of lameness.

However, contrary to the putative anti-inflammatory defense scenario suggested by the PBMC IL-4 and IL-10 changes in lame cows, the increased expression of MMP-13, CCR5 and GM-CSF-R-alpha indicated that an active inflammatory and algesic state was also present. For example, MMP-13 plays a major role in joint destruction (Firestein, 1996, Nagase and Woessner, 1999) and its release is modulated by proinflammatory cytokines and growth factors (Neidel et al., 1995). Thus MMP-13 is actively involved in the pathogenesis of arthritic conditions such as rheumatoid arthritis and osteoarthritis (Itoh et al., 2002; Andereya et al., 2006). Similarly, the observed up regulation of GM-CSF-R-alpha and chemokine receptor CCR5 in PBMCs of lame cows of this study suggests the presence of an active immune response. GM-CSF is important for the production of granulocytes and macrophages, and chemokines are essential to attract and direct circulating leukocytes into peripheral sites of inflammation and infection (Foxman et al., 1997). Furthermore, CCR5 also is involved in exacerbating pain experience because its ligands (e.g., RANTES, macrophage inflammatory protein [MIP]-α, and MIP-β) inactivate normal neuronal signaling pathways involved in reducing pain sensation (Scholtz and Woolf, 2002; Marchand et al., 2005) (**Figure 5.1**). Pain is a major element of inflammatory type lameness (Whay et al., 1998) and is one of the reasons why cows alter their locomotion (Rushen et al., 2006). Overall, the results of the studies described in this dissertation begin to portray a picture of key physiological changes that occur in the immune and neuroendocrine systems as lame animals try to cope with their condition. These physiological indicators, together, may provide a signature that could be used for

diagnosis of painful inflammatory foot lesions and thus should be confirmed in future field studies.

Figure 5.1 Tentative model to summarize the changes observed in the lame cows of the studies of this dissertation. Genes in orange and blue boxes are involved in pro- and anti-inflammatory activities, respectively.



It is clear that lameness is a complex disease with multiple etiologies. As such, its study using genome level technologies is appropriate. However, such studies must be designed very carefully, especially in terms of statistical power. For example, the high false discovery rate observed in the presented microarray study suggests that there were several drawbacks in the study design. This might have included too few animals studied, poor choice of cells for study, lack of purifying one cell type from another prior to microarray analysis, lack of control for lameness types, or chronicities, and so on. Statistical power and precision of the microarray experiment described in Chapter Four, and potentially the candidate gene approach described in Chapter Three, could have been better if a specific cell type within the PBMC population (e.g., monocytes), or if a cell type with a greater presence in blood with known sensitivity to changes in the serum cortisol:DHEA ratio (e.g. neutrophils) were used instead. Furthermore, an alternative tissue sample, such as from the lesion area could also have enhanced the experimental outcome. However, although pitfalls in the experimental design must be considered, the feasibility of actually isolating a pure cell population under the conditions in which the study was performed (at the farm level) would not have been possible due to lack of appropriate laboratorial instrumentation at the farm site. Furthermore, the practicability of harvesting a tissue sample from the lesion area (foot) would likely need to involve considerable restraining of the cow, similar to what is performed during trimming, and a thoroughly cleansing of the foot area prior to tissue collection. Once again, the feasibility of this procedure would have been constrained by issues with practicality and the stress associated with restraining of the animal, which might compromise the validity of results. The current need for 'practical' and 'clinically applicable at the farm level' biomarkers

for inflammatory lameness lesions detection supports the choice of blood samples and PBMCs as a surrogate tissue for evaluation of disease-induced gene expression in cows suffering from inflammatory lameness. Overall, systemic physiological changes detected in blood provide a novel spectrum to be explored in future lameness studies and foster the possibility of having objective measures available for diagnosis of important diseases that debilitate the welfare of animals, such as lameness detection.

However, the preliminary nature of the findings described in this dissertation needs to be acknowledged. The translation of biomarkers to clinical practice will depend on several factors, including the accuracy of testing methodologies, strength of correlation with clinical phenotype or outcome, and utility of information. They may be discovered at the bench, but getting them to the clinic is another story altogether. That is, finding a useful biomarker is necessary, but hardly sufficient, for getting it validated and eventually approved by regulators for clinical practice (Chuaqui et al., 2002). This can be addressed by evaluating the candidate biomarker genes in alternative and more extensive group of cows, by exploring different lameness types and severities, and by including different diseases in the study design. Moreover, in addition to validating array results at the mRNA level, connecting function of the genes to the actual disease pathogenesis would require an evaluation of expression levels of the corresponding protein products.

As a final comment, the multifactorial nature of lameness, and inappropriate level of understanding on the pathophysiology and etiology of different lameness types impairs the progress with objective biomarkers discovery for this condition and consequently, slows down the development of effective therapies and prevention strategies for lameness. In addition to boosting diagnostic reliability, the prospect of developing

reliable biomarkers of painful inflammatory lameness-related foot lesions could help identify animals in need of pain relief and provide appropriate targets for the development and monitoring of novel lameness therapies.

APPENDIX ONE

Table A.1 Real-time RT-PCR on PBMC expression of genes with no lameness effect from **Chapter Four**. Fold change represents means of expression ratios between lame (n = 8) and sound (n = 8) derived using the $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen (2001), with β-actin as the control gene and sound cows as a calibrator. The *P*-values represent the main effect of lameness.

Gene	Fold change	*P*-value
RDC1	1.73 ± 0.49	0.13
CD34	1.62 ± 0.43	0.13
MAPK	1.47 ± 0.36	0.15
IFNR2	2.27 ± 0.88	0.19
TGFR2	0.71 ± 0.39	0.22
VDAC3	1.63 ± 0.70	0.33
JAK1	1.17 ± 0.28	0.42
Tcerg	1.44 ± 0.71	0.47
KSras1	1.44 ± 0.35	0.54

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