BEHAVIORAL AND PHYSIOLOGICAL RESPONSES TO LIPOPOLYSACCHARIDE INDUCED CLINICAL MASTITIS

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By
Jennifer Laura Zimov, B.S., B.A.
Animal Sciences Graduate Program
The Ohio State University
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Masters Examination Committee:

Dr. Joseph S. Hogan, Advisor
Dr. William P. Weiss
Dr. Steven J. Moeller
ABSTRACT

The behavioral and physiological effects of lipopolysaccharide induced mastitis were examined in lactating Holstein cows. Twenty cows were assigned to five blocks of four cows grouped by parity and stage of lactation. The experimental design for this study was a 2 X 2 factorial arrangement. Cows within the blocks were randomly assigned to one of four treatments: 1) intramammary infusion with 25 µg lipopolysaccharide and intravenous flunixin meglumine; 2) intramammary infusion with 25 µg lipopolysaccharide and intravenous PBS; 3) intramammary infusion with PBS and intravenous flunixin meglumine; and 4) intramammary infusion PBS and intravenous with PBS. Uninfected mammary quarters were infused 3 h post milking. Intravenous treatments were given 7 h post milking. Experimental cows were under continuous video monitoring during the study. Cows receiving the lipopolysaccharide treatment had higher mean peak quarter milk somatic cell counts, rectal temperatures, concentrations of milk amyloid and serum cortisol during the first 12 h after infusion compared with intramammary saline treated cows. Lipopolysaccharide treated cows spent a reduced percentage of the first 24 h after challenge eating and cud chewing compared with saline infused cows. Rumen contractions were reduced in lipopolysaccharide infused cows compared with saline infused cows at sample times corresponding with peak rectal temperatures. Flunixin treatment 4 h after intramammary challenge mitigated the clinical
systemic responses of increased rectal temperature and decreased rumen activity. Cows receiving LPS infusion and treatment with flunixin spent more time eating the first 12 h after challenge than animals receiving infusion of LPS and treatment of PBS. Flunixin treatment increased time spent cud chewing in all cows. Acute clinical mastitis changed physiological and behavioral parameters in lactating dairy cows. The administration of flunixin meglumine mitigated the adverse systemic affects associated with LPS induced mastitis.
Dedicated to my parents, Dave and Carol for their love and encouragement to capture all of my dreams.
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VITA

March 6, 1982. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . Born – Cincinnati, Ohio

2007. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . B.S., Department of Animal Sciences, The Ohio State University, Columbus, Ohio

2007. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . B.A., Department of Psychology, The Ohio State University, Columbus, Ohio

2007- present . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . Department of Animal Sciences, The Ohio State University, Ohio Agriculture Research and Development Center, Wooster, Ohio

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# TABLE OF CONTENTS

ABSTRACT ................................................................. ii

DEDICATION .......................................................... iv

ACKNOWLEDGEMENTS ............................................... v

VITA ................................................................. vii

LIST OF FIGURES .................................................... x

INTRODUCTION ......................................................... 1

REVIEW OF LITERATURE ............................................... 3

MASTITIS ............................................................. 3

ENDOTOXIN-INDUCED MASTITIS ....................................... 5

NEUTROPHILS .......................................................... 6

ACUTE PHASE PROTEINS ............................................... 8

AMYLOID A ............................................................ 9

CORTISOL ............................................................ 10

FLUNIXIN MEGLUMINE .................................................. 10

COSTS ................................................................. 11

BENEFITS TO EARLY DETECTION OF MASTITIS ............. 12

MASTITIS BEHAVIOR .................................................. 13

THESIS STATEMENT .................................................. 18

MATERIALS AND METHOD ............................................. 19

EXPERIMENTAL COWS ............................................... 19

EXPERIMENTAL DESIGN .............................................. 19

INTRAMAMMARY CHALLENGE ....................................... 20

FLUNIXIN TREATMENT ............................................... 20

SAMPLE COLLECTION ................................................ 20

MILK AMYLOID ........................................................ 21

CORTISOL ............................................................. 21

DRY MATTER INTAKE AND MILK PRODUCTION .................. 21

RECORDING BEHAVIORS ............................................. 22
LIST OF FIGURES

FIGURE PAGE
1. Mean SCC following intramammary challenge with either 25 µg lipopolysaccharide (LPS) or sterile saline at 0 h and intravenous injection with either 50 mg/45kg BW flunixin meglumine or sterile saline at 4 h after intramammary challenge. Dispersion bars represents standard error of the means. .................................................. 32

2. Mean temperature following intramammary challenge with either 25 µg lipopolysaccharide (LPS) or sterile saline at 0 h and intravenous injection with either 50 mg/45kg BW flunixin meglumine or sterile saline at 4 h after intramammary challenge. Dispersion bars represents standard error of the means. .................................................. 33

3. Mean gut sounds following intramammary challenge with either 25 µg lipopolysaccharide (LPS) or sterile saline at 0 h and intravenous injection with either 50 mg/45kg BW flunixin meglumine or sterile saline at 4 h after intramammary challenge. Dispersion bars represents standard error of the means. .................................................. 34

4. Mean serum cortisol following intramammary challenge with either 25 µg lipopolysaccharide (LPS) or sterile saline at 0 h and intravenous injection with either 50 mg/45kg BW flunixin meglumine or sterile saline at 4 h after intramammary challenge. Dispersion bars represents standard error of the means. .................................................. 35

5. Mean amyloid following intramammary challenge with either 25 µg lipopolysaccharide (LPS) or sterile saline at 0 h and intravenous injection with either 50 mg/45kg BW flunixin meglumine or sterile saline at 4 h after intramammary challenge. Dispersion bars represents standard error of the means. .................................................. 36
6. Mean milk production following intramammary challenge with either 25 µg lipopolysaccharide (LPS) or sterile saline at 0 h and intravenous injection with either 50 mg/45kg BW flunixin meglumine or sterile saline at 4 h after intramammary challenge. Daily values are percentages of the average milk production during the 7 d prior to intravenous challenge. Dispersion bars represents standard error of the means. .............................................. 37

7. Mean dry matter intake following intramammary challenge with either 25 µg lipopolysaccharide (LPS) or sterile saline at 0 h and intravenous injection with either 50 mg/45kg BW flunixin meglumine or sterile saline at 4 h after intramammary challenge. Daily values are percentages of the average DMI during the 7 d prior to intravenous challenge. Dispersion bars represents standard error of the means. ......................................................... 38

8. Mean clinical score following intramammary challenge with either 25 µg lipopolysaccharide (LPS) or sterile saline at 0 h and intravenous injection with either 50 mg/45kg BW flunixin meglumine or sterile saline at 4 h after intramammary challenge. Dispersion bars represents standard error of the means. ................................................................. 39

9. Mean time spent cud chewing following intramammary challenge with either 25µg lipopolysaccharide (LPS) or sterile saline at 0 h and intravenous injection with either 50 mg/45kg BW flunixin meglumine or sterile saline at 4 h after intramammary challenge. Dispersion bars represents standard error of the means. ................................................................. 40

10. Mean time spent eating following intramammary challenge with either 25 µg lipopolysaccharide (LPS) or sterile saline at 0 h and intravenous injection with either 50 mg/45kg BW flunixin meglumine or sterile saline at 4 h after intramammary challenge. Dispersion bars represents standard error of the means. ................................................................. 41

11. Mean time spent laying following intramammary challenge with either 25 µg lipopolysaccharide (LPS) or sterile saline at 0 h and intravenous injection with either 50 mg/45kg BW flunixin meglumine or sterile saline at 4 h after intramammary challenge. Dispersion bars represents standard error of the means. ................................................................. 42

12. Mean time spent cud chewing following intramammary challenge with either 25µg lipopolysaccharide (LPS) or sterile saline at 0 h and intravenous injection with either 50 mg/45kg BW flunixin meglumine or sterile saline at 4 h after intramammary challenge. Dispersion bars represents standard error of the means. ................................................................. 43
13. Mean time spent eating following intramammary challenge with either 25 µg lipopolysaccharide (LPS) or sterile saline at 0 h and intravenous injection with either 50 mg/45kg BW flunixin meglumine or sterile saline at 4 h after intramammary challenge. Dispersion bars represents standard error of the means.

14. Mean time spent laying following intramammary challenge with either 25 µg lipopolysaccharide (LPS) or sterile saline at 0 h and intravenous injection with either 50 mg/45kg BW flunixin meglumine or sterile saline at 4 h after intramammary challenge. Dispersion bars represents standard error of the means.
INTRODUCTION

Mastitis is the inflammation of the mammary gland typically caused by an infectious agent (NMC, 2004). When experiencing this type of infection, the host reacts through a combination of physiological and behavioral changes. In order to modify the environments and administer treatments when necessary, researchers and producers need to be able to recognize symptoms of pain, injury and infection in dairy cows.

Common causative agents of bovine mastitis are Gram-negative bacteria (Bramley and Dodd, 1984). The lipopolysaccharide (LPS) portion of a Gram-negative bacteria cell wall is the main virulence factor for Gram-negative bacteria and gives the bacteria the capability of causing tissue damage in the host. At the time of cell death, LPS is released by the bacteria into the animal’s system. This causes an inflammatory response in the host (Hogan et al., 2003). During the intramammary infusion of sterile LPS, local and systemic clinic signs representative of natural infections are expected (Perkins et al., 2002). These signs are increased respiration, increased heart rate, increased somatic cell count, fever, decreased rumen motility and a decrease in peripheral or blood leukocyte counts (Perkins et al., 2002). The ability of LPS to cause an inflammatory response in the body with minimal tissue damage and only temporary animal discomfort makes it a valuable tool in understanding the inflammatory response.
Understanding dairy cow behavior will improve our understanding of overall welfare and ways to improve it during times of pain and illness. Therefore, the purposes of the current trial were to use LPS challenge model to determine: 1) changes in behavioral variables during acute mastitis; and 2) the effects of flunixin meglumine on physiological and behavioral responses to acute clinical mastitis.
REVIEW OF LITERATURE

Mastitis

Mastitis is the term given for the inflammation of the mammary gland (Kehrli et al., 1994). Many times the inflammation is a result of an infection in the mammary gland caused by the entrance and multiplication of microorganisms. Many different types of microorganisms have the ability to cause infection inside the mammary gland, but some are more prevalent than others in dairy cow herds. These microorganisms are classified as either contagious or environmental based on habitat for survival and multiplication.

Fox et al. (1993) defined contagious mastitis as caused by microorganisms that are transmitted from cow to cow causing an intramammary infection. Examples of contagious organisms are *Mycoplasma spp.*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Corynebacterium bovis* (Bramley et al., 1984). These microorganisms are easily transmitted to uninfected animals during the milking process. Exposure to the milking unit, milkers’ hands and contaminated udder wash clothes all have the ability to spread contaminants and expose healthy glands to contagious microorganisms (Fox et al., 1993). Culling animals that are infected, applying total dry cow therapy, teat dipping after milking, maintaining and properly using milking equipment and treating clinical cases of mastitis will lower the exposure load of contagious microorganisms present in a herd (Bramley et al., 1984). Lowering
the amount of contagious mastitis present will also decrease the source of bacteria for new intramammary infection in a herd. Continuing to lower infection rates will make the exposure load of microorganisms in the herd decrease to low or nonexistent levels (Smith et al., 1993).

Environmental mastitis is caused by mammary pathogens primarily residing in the animals’ environment (Smith et al., 1993). Examples of these pathogens are the Gram-negative bacteria such as *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., *Serratia* spp., *Pseudomonas* spp., *Proteus* spp. and species of streptococci other than *Streptococcus agalactiae* (Smith et al., 1983; Bramley et al., 1984; Smith et al., 1985; Bramley et al., 1990; Smith et al., 1993). Rates of new environmental pathogen infections are affected by parity, housing and management, season of the year and stage of lactation. The only way to control environmental mastitis is to increase the resistance of the cow to intramammary infection or decrease the exposure of teat ends to environmental mastitis pathogens. Non-specific mammary resistance will be maximized by feeding diets sufficient in selenium and vitamin E, providing a stress free environment and minimizing teat end injuries. Exposure to mastitis pathogens can be minimized by providing the animals cool, dry and clean environments (Smith et al., 1993).

Just as the types of microorganisms causing the infection are classified into two groups, the severity of infection is also classified into two groups. Depending on the severity the infection, it will either be labeled clinical or subclinical. Clinical mastitis is apparent by visible changes in the mammary gland, appearance of the animal, or the milk appearance and composition. Subclinical mastitis is not as easily observed. Although no
obvious changes to the animal are apparent, milk from the infected quarters may have an elevated somatic cell count and other compositional changes (McDougall et al., 2009).

**Endotoxin-induced Mastitis**

The lipopolysaccharide portion of a Gram-negative bacteria cell wall is referred to as an endotoxin. Endotoxin is the main virulence factor for Gram-negative bacteria and gives the bacteria the capability of causing tissue damage in the host. At the time of cell death, endotoxin is released by the bacteria into the animals system. This causes an inflammatory response in the host (Hogan et al., 2003).

During endotoxin intramammary infusion, local and systemic clinic signs are expected (Perkins et al., 2002). These signs are increased respiration, increased heart rate, increased somatic cell count, fever, decreased rumen motility and a decrease in peripheral or blood leukocyte counts (Perkins et al., 2002). After intramammary infusion with endotoxin, Jain et al., (1978) cows displayed signs of gross mastitis within 1 h and these symptoms continued to elevate over the next 2 to 3 h reaching a peak at 4 to 8 h. Recovery occurred within 16 to 20 h post infusion. Neutrophils found in the milk increased from $10^4 \log_{10}/\text{ml}$ (0 h) to $20 \times 10^6 \log_{10}/\text{ml}$ (by the 6 h.) These amounts continued to increase until 12 h post inoculation reaching a level of $13.9 \times 10^7 \log_{10}/\text{ml}$. This suggests that neutrophils respond to endotoxin infusion in a similar way as they respond to pathogens entering the mammary gland. Eight hours after infusion, signs of mastitis (swelling of teats) declined and were unobservable 3 to 6 d later (Jain et al., 1978). The ability of endotoxin to cause an inflammatory response in the body with
minimal tissue damage and only temporary animal discomfort makes it a valuable tool in understanding the inflammatory response.

**Neutrophils**

One of the first steps the host takes to prevent the multiplication and escape of pathogens is the infiltration of neutrophils; this is also considered the first step of the inflammatory reaction. Polymorphonuclear neutrophil leukocytes (PMN) possess multiple cytoplasmic granules that help kill bacteria, a segmented nucleus, a complex surface used for phagocytosis of bacteria and stored glycogen for energy (Paape et al., 2003). The multi sectioned nucleus is important because it allows rapid migration between endothelial cells by lining up the nuclear lobes in a straight line. This ability allows the neutrophils to be the first white blood cell to reach the site of infection (Paape et al., 2003).

Many receptors are present on the outside of a neutrophil and some of them are used for detection of chemoattractants which allow the cell to respond to areas of inflammation (Paape et al., 2003). The neutrophils must arrive in the lumen of the udder quickly so that they can quickly begin sequestering and killing pathogens (Burvenich et al., 2003). The cell is activated when immunoglobulins and complement components bind to receptors on the neutrophil and oxidative bursts are initiated (Paape et al., 2003). If all of the bacteria are destroyed, only a mild inflammation response will occur because neutrophils will stop being sent to the site of infection (Kehrli et al., 1994).

Specific and non-specific molecules such as (poly) peptides and systemic and tissue hormones make up the rapid inflammatory reaction. So that the health of the host is not
put into jeopardy, it is important for the inflammation process to be short lasting, fast and regulated (Burvenich et al., 2003). Nonspecific molecules help clear infection, but some chemical mediators from the neutrophils can cause cell damage and destroy mammary tissue (Paape et al., 2002). Reactive oxygen metabolite (ROM) generation and granular enzyme release both increase cell damage and result in a decreased milk yield. Damage from the mammary epithelium may result from the phagocytic function of the PMN (Capuco et al., 1986). If ROM are not safely removed, oxidative stress may damage lipid and macromolecules. The ROM may also change metabolic pathways which can lead to illness or disease (Miller et al., 1993). The final stage of the inflammation process is the curing stage. This stage is only successful if minimal tissue damage has occurred (Burvenich et al., 2003). Polymorphonuclear neutrophil leukocytes that have aged will undergo programmed cell death before their removal by macrophages. This is done so that harmful agents aren’t released into the tissues. The process of recognition, ingestion and degradation by the macrophage happens very quickly (Savill et al., 1989). Macrophages will then remove and take the place of neutrophils in the tissue (Paape et al., 2003).

Successful intramammary defense is dependent on an increase of PMN arriving at the site of infection. Fast response of many PMN to the site of infection and high levels of phagocytosis will decrease the chance of mastitis. After the infection is eradicated, rapid PMN apoptosis is necessary to limit the amount of damage done to mammary tissue by the neutrophils. The resolution stage is dependent on the disappearance of these neutrophils and therefore returning the tissues to their normally functioning levels (Paape et al., 2003).
Acute Phase Proteins

After the occurrence of trauma, infection or injury of tissue the host respond by releasing a series of reactions aimed at preventing continuous tissue damage by destroying all infectious agents and beginning the repair process. The first set of reactions that occur is the acute phase response (APR) (Baumann et al., 1994). The proteins released during this response are called acute phase proteins and are liver derived proteins whose concentrations change as a response to an infection or injury (Horadagoda et al., 1999).

Proteins called cytokines initiate the acute phase response. These proteins act as messengers between the hepatocytes that synthesis the acute phase proteins and the point of infection or injury (Petersen et al., 2004). Cytokines are intercellular signaling polypeptides and most have multiple functions, multiple targets and multiple sources. Multiple cell types can produce these peptides or molecules but monocytes and macrophages can produce them at sites of inflammation (Gabay et al., 1999). The cytokines interleukin 1 and tumor necrosis factor are considered ‘alarm’ cytokines because they are important in triggering the secondary phase of cytokines. This secondary phase signals the cellular and cytokine cascades that are part of the APR (Baumann et al., 1994). This triggered host defense helps activate tissue repair and isolate and destroy microbial pathogens (Suffredini et al., 1999).

Infection, surgery, trauma, burns, tissue infarction and advanced cancer will all lead to large changes in the plasma concentrations of acute phase proteins (Gabay et al., 1999). Subjects with the same illness may not have the same uniform change in acute phase proteins. Variations suggest that the patterns of production of specific cytokines or the
modulators are individually regulated in the acute phase response (Gabay et al., 1999). Within 24-48 h of onset the APR subsides and the host returns to a normal homeostatic and protective nature. The reaction can be prolonged in cases where there is a disruption of normal control mechanisms or a persistence of stimulation. Instead of normal APR, this is considered chronic inflammation (Baumann et al., 1994).

Some acute proteins such as leptin and CRP have anti-inflammatory effects while serum amyloid A affects leukocyte activation and trafficking (Suffredini et al., 1999). In cattle, haptoglobin and serum amyloid A are both recognized as major acute phase proteins. Individual APR responses can vary. As shown in a study done by Horadagoda et al., (1999) 100% of animals that were diagnosed with acute inflammation had an increase in serum amyloid A but only 68% of these animals showed an increase in haptoglobin levels.

**Amyloid A**

Proteins made by the liver are released during an acute phase reaction (Pyörälä., 2003). In cows, amyloid A is considered a major acute phase protein (Horadagoda et al., 1999). During inflammation amyloid A greatly increases in the host (Boosman et al., 1989).

In a study done by Boosman et al., (1989) cows were administered endotoxin intravenously. Two to four h after the injection the cows showed signs of discomfort including inhibition of ruminal motility, cold extremities, increased rectal temperature and labored respiration. Within 1 h of administration, severe neutrophilic leukopenia was displayed, however pack cell volumes did not change. Concentrations of serum amyloid
A increased 5 h after the challenge and reached peaked amounts between 17 and 20 h post challenge. Serum Zn, serum Fe, white blood cell counts and amyloid A concentrations were all measured but only amyloid A showed an increased measure 12 h post challenge. This suggests that measuring amyloid A levels is reliable and helpful especially if time of injury or inflammation is not known.

Cortisol

Glucocorticoids such as corticosterone or cortisol interact with growth, development, neuronal function and metabolism (Borski, 2000). They are released in the host minutes after an initial stressor. Glucocorticoids are able to alter neuronal excitability, cell morphology, carbohydrate metabolism and hormone secretion (Borski, 2000).

Systemic responses during mastitis cause an increase in serum cortisol levels (Shuster et al., 1991). After midlactation Holstein and Jersey cows were intramammary infused with endotoxin, a 5- to 10-fold increase was recorded in serum cortisol levels. The high concentration was temporary and happened soon after the infusion of endotoxin (Shuster et al., 1991). During endotoxin mastitis, high serum cortisol levels are not necessary for a decrease in milk production. An increase in cortisol levels alone is not capable of suppressing milk production. This suggests that other pathophysiological mechanisms are responsible for this sign of mastitis (Shuster et al., 1992).

Flunixin Meglumine

The only nonsteroidal anti-inflammatory drug (NSAID) labeled for use in The United States for beef and dairy cows is flunixin meglumine (Smith et al., 2008). Flunixin
meglumine is labeled for the control of inflammation connected to endotoxemia and pyrexia caused by bovine respiratory tract disease and mastitis. This drug is only labeled for use via intravenous route and giving the drug extravascular (intramuscular or subcutaneous) is considered extra-label use (Smith et al., 2008).

Flunixin is a weak acid. Milk has a lower pH than blood which makes it difficult for flunixin to pass from the blood into the milk. Flunixin concentrations found in milk are low because of this (Rantala et al., 2002). Flunixin use is considered safe in food animals if the appropriate dosage is used (which is 1.1 to 2.2 mg/kg) and administered as an intravenous injection. The meat withdrawal time for this drug is 4 d and the milk withdrawal time for this drug is 36 h.

Animals intramammarily infused with endotoxin received an injection of flunixin 1 hour after showing signs of clinical mastitis (approximately 5.3 h post challenge) (Wagner et al., 2004). Flunixin treated animals had significantly higher frequencies of rumen contractions for 14 h post challenge time than untreated controls. Animals given the flunixin treatment also had a lower heart rate and rectal temperatures when compared with the control group.

**Costs**

The exact costs associated with a dairy cow developing mastitis is variable depending on genetic factors of the animal, age of the animal, stage of lactation, type of agents causing the illness and the speed and extent to which treatment is administered. Therefore studies attempting to determine costs related to the development of mastitis are merely estimates. Bar et al., (2008) used information from five large dairy herd in New
York and estimated that the overall cost for an animal developing clinical mastitis was $179. The costs included in this estimate were from potential milk lost, treatments used and the increased chance of mortality. McInerney et al., (1992) explained the total loss from mastitis should be considered from two distinct areas. The first is the amount of output loss due to the disease. Output is milk production lost during the illness from discarded milk and a decrease in milk production. Lower fertility rates and changes in body conditions should also be considered as output losses. The second area is the costs to treat the disease and prevent mastitis occurrence in the future. Any money spent on medications, veterinary visits, lab cultures, additional labor and preventative measures should be included. This calculation gives a more complete analysis of the ways in which mastitis affects overall profitability of the cow (McInerney et al., 1992).

Individual cows should be assessed before a treatment regiment is begun. Cow factors such as age, fertility rates and milk yield should be considered when deciding the benefits of treating the animal. Bar et al., (2008) reported that high treatment costs can be justified for animals with great milking potential. Little money should be spent on animals that will be culled in the near future. Culling these animals earlier than expected and then replacing them with younger healthier animals is more profitable than treatment of potential cull cows.

**Benefits to early detection of mastitis**

In a study by Bascom et al., (1998), mastitis was listed as the second most common primary reason for culling. Fifteen percent of the animals in this study were culled as a result of mastitis. The information does not specify whether this was because of lower
milk production or the animals’ inability to recover from the infection. Ways to prevent and decrease mammary infection would lower culling rates and allow farmers to keep high producing animals longer.

The ability to quickly recognize signs of mastitis and appropriately treat the infections will decrease the impact on milk production. A model was developed and tested by Lescourret et al. (1994) to predict the effects of mastitis on milk production during different stages of lactation, seasons and before or after peak yield. Data was collected from three different experimental farms and included 3851 lactations by 1179 cows. The model compared the differences between control curves and individual production curves affected by mastitis. Mastitis had a short term effect and only accounted for 10-40% of total loss for more than half of the animals studied in mid to late lactation and more than one third of the cases observed in the beginning of lactation. Mastitis cases were detected early and medical treatment was systematic. This may be the reason animals in this study that developed mastitis had low impact on production amounts.

Careful observations of animals each day and workers understanding of the microorganisms will help eliminate and reduce mastitis infections. Rapid treatment will provide faster recovery times and decrease the financial loss from the illness.

**Mastitis Behavior**

In order to modify the environments and administer treatments when necessary, researchers and producers need to be able to recognize symptoms of pain, injury and infection in their animals. Understanding the intensity and duration of pain in a prey animal can be an extreme challenge because such animals have evolved method of
enduring and concealing painful symptoms though time. Morton and Griffith (1985) showed that animals demonstrate different behaviors when experiencing painful situations. If a pain killer or an analgesic is given to the animal the behaviors will abate. However, the disappearance of these behaviors in no way predicts the cause or magnitude of the pain source. Flower et al., (2008) found not all painful behaviors were eliminated by the dispensing of analgesics. Cows may show the same signs (i.e. the reluctance to bear weight) for a variety of reasons. This helps clarify the reasoning for a variety of responses to pain medication. Analgesics may eliminate slight discomfort or pain in animals, but larger amounts of pain possibly caused by damaged muscles, broken bones or severe injuries will need more time for healing before painful behaviors cease.

Behaviors that differ in pattern, frequency or context from what is shown by the majority of animals of the same species when allowed a full range of behavior are considered abnormal or aberrant behaviors (Fraser and Broom, 1990). Abnormal behaviors may be expressed more often during times of stress and pain. Understanding and recognizing these behaviors would be helpful from an industry and animal welfare point of view. Knowing the ways in which different species act in response to pain would increase the likelihood of animals receiving the care and treatment they need to survive and meet peak production abilities.

Sanford and others, (1986) came up with species specific guidelines for detection of pain in research animals. The guidelines suggested that cattle dealing with pain tend to be depressed and dull, lose their appetite and may lose weight. A drop in milk yield is typical and animals tend to show little to no interest in their surroundings. Severe pain may result in more drastic behaviors such as getting up and laying down frequently,
grinding their teeth, grunting and rapid, shallow respiration. In cases of abdominal pain, animals may become very rigid in posture or kick and bite the abdomen.

An ailment that commonly affects dairy cows is mastitis. Mastitis is the inflammation of the mammary gland and can be caused by a variety of agents. When experiencing this type of infection the animal reacts though a combination of physiological and behavioral changes. One behavior that is observed in infected animals is a decreased amount of time spent laying down. Time spent idling (a period of time where the cow is standing and not engaged in any other behavior) was used by Chaplin et al., (2000) as a measurement of possible cow comfort. The more time spent idling, the less comfortable the cows were deemed to be. Niss et al., (2009) reported heifers made uncomfortable from an oligofructose overload spent more time with pre-laying intentional movements and had difficulties laying down. These actions suggest that typical laying behaviors were disrupted from the discomfort of the illness.

The distance between hocks has been measured as a possible indicator to the presence of mastitis by Kemp et al., (2008). When mastitis was present the distance between the hocks of the infected cows increased. Kemp et al., (2008) reported that the severity of the mastitis had no additional effect on the resulting hock distances. Hock distance did not differ among cows with mastitis of varying severity.

A study done by Waldron and others (2006) showed dairy cows decreased feed intake after being challenged with a lipopolysaccharide (LPS) solution. Feed intake decreased 49% on the day of the challenge and was still 19% lower than the control animals the day after the challenge. This study agrees with similar studies done with calves (Borderas et al., 2008) and pigs (Wright et al., 2000). Johnson and others (1994) showed that small
amounts of LPS given to pigs will cause an initial decrease in feeding behavior which
hours later is followed by a compensatory increase in food intake. This information
suggests that the LPS’s negative effect on eating behavior is temporary and that animals
will increase feeding behaviors to make up for the time in which they were too ill to eat.

Multiple studies have also shown a significant decrease in time spent ruminating
directly following an LPS challenge. A study by Borderas and others (2008) showed
calves given the LPS challenge had decrease frequency and duration of rumination for
several hours after infusion. Goats infused with LPS (Takeuchi et al., 1995) had a
decrease in rumen contractions when compared to self responses to an injection of saline.
This may have been attributed to a lack of ruminal movement or rumen stasis caused by
the LPS depressing the appetite. A study by Chaiyotwittayakun et al., (2002) suggests
that cows treated with ascorbic acid therapy after LPS induced mastitis have a quicker
recovery from rumen stasis and also had a faster rate of milk production recovery.

Understanding dairy cow behavior will improve our understanding of overall welfare
and ways to improve it during times of pain and illness. Determining the welfare of an
animal is dependent on multiple factors. Fraser and Broom (1990) reported the
importance of considering all aspects of an animals life when considering how their pain
systems are functioning. Animal that are prey species, display pain very differently when
surrounded by a possible predator (even humans), than the way it is displayed in
isolation. Age may also affect the way animals react to pain or stress. Young animals
will often vocalize to get the attention of their mother whereas older animals may hide
symptoms of distress. The ability to recognize and understand different behaviors
presented by animals is not an easy task. However, in order to improve overall welfare of
animals used in the industry this is a necessary duty. Further research on farm animal
behaviors will hopefully improve productivity of our animals by improving their comfort
and care.
Thesis Statement

Cow comfort is an important aspect to recovery from mastitis. Some physiological signs shown to occur during clinical intramammary infections are pyrexia, increased somatic cell count, increased cortisol levels, increased amyloid A levels and decreased rumen activity. Behavioral signs that occur during mastitis are decreased time spent eating, decreased time spent cud chewing and decreased time spent laying. Antipyretics such as flunixin meglumine are marketed to prevent or lower fever in dairy cows. One of the most debilitating results of endotoxin mastitis is the onset of fever. The hypothesis is that the administration of antipyretics such as flunixin meglumine will decrease the affects of pyrexia allowing for less physiological and behavioral changes in animals with endotoxin mastitis, thus increasing cow comfort.
Materials and Methods

Experimental Cows

Twenty cows in the herd at the Ohio Agricultural Research and Development Center dairy were used for this study. Cows were assigned to five blocks of 4 cows by parity and DIM. One block was composed of primiparous cows and the other four blocks were made up of multiparous cows. Days in milk ranged from 65 to 110. All cows were housed in tie-stalls for the duration of the experiment. Stalls were 170 x 150 cm concrete base with inlaid rubber mats. The stalls were bedded twice daily with 10kg of dried sawdust. The animals remained in the tie-stalls except for when they were moved to the milking parlor at 03:00h and 15:00h. Cows were fed a TMR each day at 03:30h.

Experimental Design

The experimental design for this study was a 2 x 2 factorial arrangement. Cows within the blocks were randomly assigned to one of four treatments: 1) intramammary infusion with 25 µg LPS and IV flunixin; 2) intramammary infusion with 25 µg LPS and IV PBS; 3) intramammary infusion with PBS and IV flunixin; and 4) intramammary infusion with PBS and IV PBS. Cows averaged 40.5 ± 5.0 (SD) kg milk production the 7 d prior to challenge.
**Intramammary Challenge**

Cows within blocks were challenged the same day with an intramammary infusion via teat canal using a 33.78 mm sterile teat infusion cannula (Jorgensen Laboratories, Incorporation Loveland, CO). Animals were challenged in a front quarter 3 hours after milking (06:00 h). Quarter samples were taken 7, 5 and 3 d prior to challenge to determine IMI status of glands (NMC, 2004). No infected quarters were challenged.

The concentrated LPS used was *Escherichia coli* 026:B6 (Sigma Chemical Co., St. Louis, MO). This LPS was diluted in sterile PBS (pH 7.2) and filtered-sterilized (0.2µm) (Gelman Laboratory, Ann Arbor MI). The challenge inoculum was 25µg LPS in 10 ml PBS. Control cows were infused with 10 ml filter-sterilized PBS.

**Flunixin Treatment**

Flunixin meglumine (Banamine, Schering-Plough Animal Health Corp., Union NJ) was injected via jugular puncture 4 h after challenge (10:00 h) at the dose of 50 mg/45 kg BW. Control cows were given sterile PBS via a jugular puncture administered 4 h after challenge (10:00 h) at the dose of 1 ml/45 kg BW.

**Sample Collection**

Quarter-fore milk and blood samples were collected immediately prior to challenge and then at 3, 6, 9, 12, and 24 hours after challenge. Approximately 10 ml of quarter foremilk was collected by aseptic techniques (NMC, 2004). Two milliliters of milk were frozen until analyzed for amyloid A. The remainder of the milk samples were used for bacteriological analyses and SCC (Hogan et al., 1995). Blood samples (5 ml) were
collected via coccygeal vein puncture using 20 gauge needles. Serum collected was stored at -20C. Measurements taken at sample collection times were rumen contractions, rectal temperatures and clinical mastitis scores. Rumen contractions were measured by auscultation (Perkins et al, 2002). Clinical mastitis scores were measured on a five point score where 5 = systemic signs of infection, swollen quarter and abnormal milk; 4 = a swollen quarter and abnormal milk; 3 = normal quarter but abnormal milk; 2 = normal quarter but questionable milk and 1 = normal quarter and normal milk (Hogan et al., 1995).

**Milk amyloid**

Milk amyloid was measured in whole milk (Mast ID RANGE: Tridelta Development, Maynooth, Ireland) with a working range of 0.438 to 7.5 µg/ml and a sensitivity of 0.1 µg/ml.

**Cortisol**

Cortisol was measured in serum (ACTIVE Cortisol EIA; Diagnostic Systems Laboratories, Inc., Webster, TX) with a working range of 4.1 to 30.16 µg/dL and a sensitivity of 0.1 µg/dL.

**Dry Matter Intake and Milk Production**

Cows were milked twice daily at 12 h intervals throughout the experiment. Milk production was electronically measured at these times (DeLaval, Kansas City, MO). Dry matter intake was recorded throughout the experimental period. Post challenge daily
milk production and dry matter intake were expressed as percentage of means for the 7 d prior to challenge [(b/a) X 100, where a = mean value for the 7 d prior to challenge, and b = daily value post challenge] (Hogan, et al. 1995).

**Recording behaviors**

Cows were filmed for the duration of the study using Geovision GV-1000 software (USA vision systems inc, Irvine, CA.) Two cameras were placed on each cow. One camera was located directly above the animal and one camera was positioned to record the front of the animal. The following activities were counted and timed for the first 24 h of the study; 1) laying in stall, 2) eating and, 3) cud chewing. The activities were timed in increments of minutes. The 24 h after challenge were stratified into 3 h segments in order to correlate behavioral activities with physiological parameters.

**Statistics**

Treatment differences between SCC, temperature, milk amyloid concentrations, serum cortisol concentrations, rumination sounds, time spent cud chewing, time spent eating, time spent laying, DMI and milk production were all compared using the Proc Mixed of SAS (2009). The equation used was Y = mean + block + MG + RX + MG*RX + error. MG represents mammary gland treatment which could be 1) infusion of LPS or 2) infusion of sterile saline. RX represents the type of treatment the animal received 1) flunixin injection or 2) sterile saline injection.

22
Results

Physiological Measurements

SCC

Cows challenged with the LPS infusion showed a significant increase (P < 0.001) in SCC at 3 h through 24 h post challenge when compared with control cows which were challenged with a PBS infusion (Figure 1). At 9 h after the challenge LPS cows showed a peak SCC of 6.75 log_{10}/ml compared to PBS challenged cows that showed a SCC of 4.22 log_{10}/ml. Overall, no effects were measured post-challenge for the flunixin treatment (P > 0.05) or interaction effects between LPS and flunixin (P > 0.15) measured post-challenge.

Rectal Temperature

Rectal temperatures of LPS challenged cows significantly (P < 0.01) increased at 3, 6 and 9 h post challenge when compared with rectal temperatures of cows challenged with PBS (Figure 2). The treatment of flunixin had no main effects (P > 0.05) on rectal temperatures through the first 24 h post-challenge.

Significant interactions were measured at 6 h (P < 0.05), 9 h (P=0.07), and 12h (P < 0.01) post challenge between LPS and flunixin. Animals that were challenged with the
LPS infusion and treated with flunixin had a lower rectal temperature than cows infused with LPS and treated with PBS.

**Rumen Sounds**

At 6, 9 and 12 h post challenge, cows infused with LPS show a significant (P < 0.05) (Figure 3) reduction in gut sounds compared with cows infused with the PBS challenge. Rumen sounds from animals receiving the flunixin treatment did not differ (P > 0.05) compared with cows receiving IV PBS.

The LPS challenged cows with the flunixin treatment showed a trend for interactions at 6 (P = 0.06), 9 (P= 0.13) and 12 h (P= 0.10) post-challenge. These animals had 2-fold more rumen sounds than animals receiving the LPS challenge and receiving a treatment of PBS during these three time periods.

**Cortisol**

Animals receiving the LPS challenge showed elevated serum cortisol concentrations at 3, 6 and 12 h post challenge when compared with cows that were challenged with PBS (Figure 4). Mean (X ± S.E.) cortisol concentrations of LPS challenged cows increased quickly, peaking at 26.8µg/dL ±3.3µg/dL compared with PBS infused cows which were at 10.9 µg/dL ± 2.9 µg/dL 3 h after challenge. The 24h post-challenge samples showed no difference in cortisol levels for both challenge groups. No measureable effects of flunixin (P > 0.05) or interaction effects (P > 0.15) between LPS and flunixin were measured following challenge.
**Milk Amyloid**

Animals challenged by the LPS infusion showed significantly ($P < 0.01$) greater concentration of milk amyloid at 9 and 24 h post challenge than animals infused with the PBS solution (Figure 5). LPS challenged quarters had milk amyloid levels 3- to 7- fold greater at 9 h and 24 h after challenge than in PBS challenged cows. No measurable effects of flunixin ($P > 0.05$) or interaction effects ($P > 0.15$) between LPS and flunixin were detected.

**Milk Production**

Milk production for LPS challenged cows significantly ($P < 0.05$) (Figure 6) decreased 24 and 48 h post-challenge compared with animals given the PBS challenge. The LPS challenge was responsible for a 25% decrease of milk production the day of challenge and 7% decrease the day after challenge when comparing production with 7 d pre-challenge average. No measurable effects from flunixin treatment ($P > 0.05$) or interaction effects ($P > 0.15$) between LPS and flunixin were found post challenge.

**DMI**

Dry matter intake was not affected ($P > 0.05$) (Figure 7) by the LPS challenge or the flunixin treatment ($P > 0.05$) administered after challenge. Interaction effects were not measured ($P > 0.15$) between LPS and flunixin following the challenge. However, cows that were challenged with LPS and then given the treatment of flunixin had a 3% decrease in dry matter intake compared to a 11% decrease in dry matter intake for cows that were given the LPS challenge and administered a treatment of PBS.
Clinical Score

During the first 24 h after the time of challenge, LPS challenged cows had a significantly (P < 0.05) (Figure 8) greater clinical mastitis score than cows that were challenged with the PBS. Each quarter challenged with LPS resulted in clinical mastitis scores ≥ 3 by 3 hours post challenge. Quarters infused with PBS did not have clinical mastitis during the 24 h after challenge. No effects of flunixin (P > 0.05) or interaction effects (P > 0.15) between LPS and flunixin were measured for a clinical score after challenge.

Behavioral

Cud Chewing

The amount of the time LPS challenged cows spent cud chewing was less (P < 0.001) at 3 to 6 and 6 to 9 h post challenge than cows challenged with PBS (Figure 9). In fact, cows challenged with PBS spent approximately twice as much time cud chewing at these two time periods than animals given the LPS challenge. Types of IV treatment also had effects on time spent cud chewing. Animals given the flunixin treatment spent a greater amount of time (P < 0.05) at 3 to 6 and 6 to 9 h post challenge cud chewing than animals treated with PBS. At 6 to 9 h post challenge an interaction between the LPS challenge and flunixin treatment was suggested (P = 0.13). Animals challenged with the LPS infusion followed by a treatment of flunixin spent 2-fold time more time cud chewing than animals given the LPS infusion followed by a treatment of PBS during this time period.
Eating

Time spent eating differed between cows challenged with LPS and cows challenged with PBS (Figure 10). Animals receiving the LPS challenge spent less time eating at 0 to 3 h ($P < 0.05$), 3 to 6 h ($P < 0.01$), and 6 to 9 h ($P < 0.05$) post challenge than the animals that received the PBS challenge. Animals that received the flunixin treatment after time of challenge averaged more time eating ($P = 0.05$) at 9 to 12 h post challenge than the animals that received the treatment of PBS. Animals receiving the challenge of LPS followed by a treatment of flunixin showed a trend for an interaction effect between LPS and flunixin at 3 to 6 h ($P = 0.15$) and 6 to 9 h ($P = 0.11$) post challenge. Cows challenged with LPS and treated with flunixin spent more time eating at these times than animals challenged with LPS and treated with PBS.

Laying

Time the animals spent laying down was not affected by the LPS challenge ($P > 0.05$) or the treatment of flunixin ($P > 0.05$) (Figure 11). No measurable interaction effects ($P > 0.15$) between LPS and flunixin were found after the time of challenge.

Accumulative chewing

Animals challenged with the LPS showed a decreased ($P < 0.05$) accumulative time spent cud chewing in the first 12 h after challenge time than animals challenged with the PBS (Figure 12). Cows receiving the treatment of flunixin showed an increased ($P < 0.05$) amount of time spent cud chewing during the first 9 h after time of challenge than cows that received a treatment of PBS. Lipopolysaccharide and flunixin had no
measurable interaction effects on accumulative cud chewing (P > 0.15) following the
time of challenge.

**Accumulative eating**

Time spent eating during the first 12 h after challenge time differed for LPS and PBS
challenged groups. Animals receiving the LPS challenge spent a reduced (P < 0.05)
amount of time eating during the first 12 h after challenge than animals given the PBS
challenge. Flunixin treatment showed no main effects (P > 0.05) following the challenge.
During the first 12 h post challenge, a significant (P < 0.01) interaction between LPS and
flunixin was measured. Animals receiving the LPS infusion and treatment of flunixin
spent more time eating the first 12 h post challenge than animals receiving the infusion of
LPS and treatment of PBS.

**Accumulative laying**

Amount of time spent laying down was not affected by the LPS (P > 0.05) challenge,
or flunixin treatment (P > 0.05). No interaction effects (P > 0.15) between the LPS
challenge and flunixin treatment were measured following the time of challenge.
DISCUSSION

The overall purpose of the current trial was to test a model for investigating the physiological and behavioral signs of acute clinical mastitis. Acute clinical mastitis changed physiological and behavioral variables in lactating dairy cows. The administration of flunixin meglumine mitigated some of the adverse affects associated with LPS induced mastitis.

The intramammary infusion of LPS is an established experimental model to investigate the local and systemic inflammatory responses of lactating dairy cows (Jain et al., 1978). The results of the current study agreed with previous reports (Perkins et al., 2002 and Shuster et al., 1991) that LPS intramammary challenge caused increased rectal temperature, increased serum cortisol concentrations, and decreased rumen activity. In the current study, changes in each of these variables peaked rapidly during the first 12 h after challenge and returned to measures comparable with negative control cows by 24 h after challenge. Perkins et al. (2002) reported that cows with endotoxin induced mastitis developed a fever and had reduced rumen activity within the first 24 h post-challenge. Intramammary infusion of Holstein and Jersey cows with LPS caused mastitis and a 5- to 10-fold increase in serum cortisol levels after time of challenge (Shuster et al., 1991).

Flunixin treatment 4 h after intramammary challenge mitigated the systemic responses of increased rectal temperature and decreased rumen activity in the current trial. Flunixin
is an antipyretic (Smith et al., 2008) drug capable of reducing the febrile response to LPS. Results of the current trial agree with those of Wagner et al. (2004) indicating the use of flunixin in cattle challenged with LPS had greater frequency of rumen contractions compared with untreated cattle challenged with LPS. The administration of flunixin to cows with experimental *Escherichia coli* mastitis reduced the deleterious effects of the disease on rumen activity (Lohuis et al., 1989). The effects of flunixin on rumen contractions lead Lohuis et al. (1989) to speculate the decrease in rumen motility during *E. coli* mastitis is at least partly due to a mechanism involving prostaglandin. Serum cortisol was not responsive to flunixin treatment in the current trial. The lack of flunixin response after LPS challenge may have been due to the timing of the treatment. Flunixin was administered 4 h after challenge and the peak cortisol response in cows was measured 1 h before treatment.

Milk production was significantly reduced following LPS challenge. Reduction in milk production following intramammary LPS infusion was consistent with previous reports (Lehtolainen et al., 2003). The decrease in milk production during LPS release into the gland appears to be mediated at the local secretory tissue and systemically by reducing appetite (Hogan et al., 2003). In contrast to previous experimental mastitis trials (Waldron et al., 2006), LPS infusion did not significantly alter DMI in the current study. Waldron et al. (2006) reported cows receiving an LPS challenge decreased feed intake on the day of the challenge and were still 19% lower than the control animals the day after challenge. Although not statistically significant, in the current trial LPS challenged cows did have an 11% decrease in DMI compared 3% decrease in controls the day of challenge. The relatively small number of animal per treatment made DMI comparisons
tenuous among experimental groups in the current trial. Flunixin treatment was not a factor in either milk production or DMI.

The intramammary infusion of LPS created local changes in the mammary gland. In the current study, SCC and milk amyloid increased within hours after the intramammary infusion and remained elevated at 24 h after challenge. These results support previous reports (Boosman et al., 1989) of LPS infusion creating a rapid influx of neutrophils into the gland and a subsequent increase in milk amyloid concentrations. In contrast to systemic signs of clinical mastitis, these local factors of inflammation were not altered by flunixin treatment. The initial influx of SCC into challenged mammary glands preceded the treatment with flunixin, therefore a change in rate of SCC was not expected due to flunixin. Flunixin treatment of clinical mastitis failed to influence local mammary responses in previous trials (Anderson et al., 1986b; Dascanio, et al., 1995).

Infusion of LPS created differences in behaviors in animals. Amount of time spent cud chewing and eating were lower in cows receiving the LPS challenge when compared with cows receiving the PBS challenge. The reduced time spent eating and cud chewing during the first 12 h after LPS challenge corresponded to the peak in systemic clinical signs at 3 to 9 hours after infusion. Therefore, the cumulative time spent eating and cud chewing were reduced the first 12 h after challenge. However, the cumulative minutes spent eating and cud chewing the 24 h after challenge did not differ between cows with acute mastitis and controls receiving PBS intramammary infusion. These data agree with those of Johnson et al. (1994) investigating the effects of LPS in swine. Pigs injected with LPS showed an initial decrease in feeding behavior which hours later is followed by a compensatory increase in food intake (Johnson et al., 1994). These trials suggest the
negative effect of LPS on eating behavior is temporary and that animals will increase feeding behaviors to make up for the time in which they were too ill to eat.

Flunixin treatment reduced the negative effects of LPS induced clinical mastitis on time spent eating and cud chewing. In fact, flunixin treatment increased time spent eating and cud chewing in both cows challenged with LPS and in PBS infused controls during the 5 to 9 h after treatment. Results of the current trial suggest treatment with flunixin had a stimulatory effect on eating behavior and rumen activity typically reduced by acute mastitis. In contrast, Shwartz et al. (2008) reported postparturient cows treated with flunixin had decreased DMI. A possible explanation for this discrepancy was the difference in energy balance in peripartueint cows (Shwartz et al., 2008) and cows from 65 to 110 DIM used in the current trial.

Accumulative time spent laying did not differ between experimental challenge groups nor did flunixin treatment affect time laying in stalls. These data differ from a Finnish study (Hänninen et al., 2007) in which mastitis cows spent more time resting from 0 to 2 h post-challenge and less time resting for 3 to 11 h post-challenge when compared to times spent laying prior to challenge. The time of challenge post-milking and feeding may have influenced the time spent laying in the current study. Both LPS and saline challenged cows spent a considerable greater amount of time laying in stalls for 12 to 24 h after challenge compared with the first 12 h after challenge when acute signs of mastitis were present in LPS challenged cows.
SUMMARY

Understanding species specific pain guidelines (Sanford et al., 1986) will help researchers and producers improve living conditions and cow comfort. The pain mastitis causes in dairy cows affects them both behaviorally and physiologically. Flunixin alters arachadonic acid metabolism and has been credited with analgesic activity (Anderson et al., 1986a; Van Donkersgoed et al., 2009). Results of the current study: 1) confirmed the use of the LPS challenge model to investigate physiological and behavioral changes during acute mastitis; 2) the behavioral traits of time spent eating and cud chewing were measurable parameters altered by acute mastitis; and 3) administration of flunixin mitigated the adverse effects of acute mastitis on both systemic physiological and behavioral parameters.
Figure 1. Mean SCC following intramammary challenge with either 25 µg lipopolysaccharide (LPS) or sterile saline at 0 h and intravenous injection with either 50 mg/45 kg BW flunixin meglumine or sterile saline at 4 h after intramammary challenge. Dispersion bars represents standard error of the means.
Figure 2. Mean rectal temperature following intramammary challenge with either 25 µg lipopolysaccharide (LPS) or sterile saline at 0 h and intravenous injection with either 50 mg/45 kg BW flunixin meglumine or sterile saline at 4 h after intramammary challenge. Dispersion bars represent standard error of the means.
**Figure 3.** Mean rumen sounds/min following intramammary challenge with either 25 µg lipopolysaccharide (LPS) or sterile saline at 0 h and intravenous injection with either 50 mg/45 kg BW flunixin meglumine or sterile saline at 4 h after intramammary challenge. Dispersion bars represents standard error of the means.
Figure 4. Mean serum cortisol following intramammary challenge with either 25 µg lipopolysaccharide (LPS) or sterile saline at 0 h and intravenous injection with either 50 mg/45 kg BW flunixin meglumine or sterile saline at 4 h after intramammary challenge. Dispersion bars represents standard error of the means.
Figure 5. Mean milk amyloid following intramammary challenge with either 25 µg lipopolysaccharide (LPS) or sterile saline at 0 h and intravenous injection with either 50 mg/45 kg BW flunixin meglumine or sterile saline at 4 h after intramammary challenge. Dispersion bars represents standard error of the means.
Figure 6. Mean milk production following intramammary challenge with either 25 µg lipopolysaccharide (LPS) or sterile saline at 0 h and intravenous injection with either 50 mg/45 kg BW flunixin meglumine or sterile saline at 4 h after intramammary challenge. Daily values are percentages of the average milk production during the 7 d prior to intravenous challenge. Dispersion bars represents standard error of the means.
Figure 7. Mean dry matter intake following intramammary challenge with either 25 µg lipopolysaccharide (LPS) or sterile saline at 0 h and intravenous injection with either 50 mg/45 kg BW flunixin meglumine or sterile saline at 4 h after intramammary challenge. Daily values are percentages of the average DMI during the 7 d prior to intravenous challenge. Dispersion bars represents standard error of the means.
Figure 8. Mean clinical score following intramammary challenge with either 25 µg lipopolysaccharide (LPS) or sterile saline at 0 h and intravenous injection with either 50 mg/45 kg BW flunixin meglumine or sterile saline at 4 h after intramammary challenge. Dispersion bars represents standard error of the means.
Figure 9. Mean time spent cud chewing following intramammary challenge with either 25 µg lipopolysaccharide (LPS) or sterile saline at 0 h and intravenous injection with either 50 mg/45 kg BW flunixin meglumine or sterile saline at 4 h after intramammary challenge. Dispersion bars represents standard error of the means.
Figure 10. Mean time spent eating following intramammary challenge with either 25 µg lipopolysaccharide (LPS) or sterile saline at 0 h and intravenous injection with either 50 mg/45 kg BW flunixin meglumine or sterile saline at 4 h after intramammary challenge. Dispersion bars represents standard error of the means.
Figure 11. Mean time spent laying following intramammary challenge with either 25 µg lipopolysaccharide (LPS) or sterile saline at 0 h and intravenous injection with either 50 mg/45 kg BW flunixin meglumine or sterile saline at 4 h after intramammary challenge. Dispersion bars represents standard error of the means.
Figure 12. Mean time spent cud chewing following intramammary challenge with either 25 µg lipopolysaccharide (LPS) or sterile saline at 0 h and intravenous injection with either 50 mg/45 kg BW flunixin meglumine or sterile saline at 4 h after intramammary challenge. Dispersion bars represents standard error of the means.
Figure 13. Mean time spent eating following intramammary challenge with either 25 µg lipopolysaccharide (LPS) or sterile saline at 0 h and intravenous injection with either 50 mg/45 kg BW flunixin meglumine or sterile saline at 4 h after intramammary challenge. Dispersion bars represents standard error of the means.
Figure 14. Mean time spent laying following intramammary challenge with either 25 µg lipopolysaccharide (LPS) or sterile saline at 0 h and intravenous injection with either 50 mg/45 kg BW flunixin meglumine or sterile saline at 4 h after intramammary challenge. Dispersion bars represents standard error of the means.
List of References


