To the Graduate Council:

I am submitting herewith a dissertation written by Mitchell E. Hockett entitled "Influence of Experimentally-induced Clinical Mastitis on Reproductive Function of Dairy Cows." I have examined the final paper copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a concentration in Animal Science.

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Influence of Experimentally-induced Clinical Mastitis On Reproductive Function of Dairy Cows

A Dissertation

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Abstract

The objectives of the current studies were to determine if experimentally-induced clinical mastitis has an impact on reproductive function of lactating dairy cows. Preliminary data have indicated that clinical and subclinical mastitis are associated with increased number of days to first service, increased services per conception, and increased days open. Others have speculated that clinical mastitis may result in early luteal regression. To date, no direct effects of clinical mastitis have been documented.

The initial study was to determine if alterations occurred in endocrine status of cows or alterations in stress hormone profiles. Hormones of particular interest were luteinizing hormone, prostaglandin $F_{2\alpha}$, and cortisol. These data indicated that clinical mastitis did alter physiology of the cow by increasing concentrations of cortisol and increasing prostaglandin metabolite following oxytocin challenge.

The second study was aimed to determine if clinical mastitis during the immediate preovulatory period or postovulatory period altered reproductive function. Data collected from these experiments allowed the conclusions to be made that mastitis during the immediate preovulatory period altered expression of estrus. Meanwhile, clinical mastitis during the early postovulatory period was of little consequence to normal reproductive function and pregnancy.

The final study was to determine if clinical mastitis, which resulted in alterations in estrous expression during the preovulatory period altered endocrine and follicular function. Data from the experiment indicated that alterations did

occur in the hypothalamo-pituitary-ovarian axis such that normal endocrine function is diminished.

Collectively, data collected in these studies indicated that clinical mastitis resulted in alterations in normal endocrine function, especially during the preovulatory time period. These changes in endocrine and follicular function resulted in decreased expression of estrus, decreased ovulation, and increased days to service opportunity.

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Chapter 1

Introduction of Problem and Review of the Literature

Introduction

Mastitis is the most economically-impacting disease facing dairy producers, costing an estimated $2 billion annually (Degraves and Fetrow, 1993). While great efforts have been made by producers to adopt management practices, treatments and technologies developed by scientists to overcome mastitis, clinical infections still occur in 20 to 28% of cows (Judge et al., 1997; Barkema et al., 1998; Sargeant et al , 1998). Furthermore, a study consisting of 108,000 dairy cows in New York and Pennsylvania reported that mammary infections, defined by positive milk cultures, were present in 49% of cows (Wilson et al., 1997) This study indicated that subclinical and clinical infections are abundant on dairy farms and while management practices have improved, the disease is still prevalent.

Production losses that were once calculated to be caused by mastitis may have been grossly underestimated, as mastitis has recently been linked to decreased reproductive performance Previously, economic losses associated with mastitis were attributed primarily to decreased milk production and loss of potential production due to damaged mammary glands. Such costs are in addition to enormous cost of antibiotic treatment and associated milk discard More recently, mastitis has been implicated as a factor for also decreasing reproductive efficiency (Cullor, 1990; Moore et al., 1991; Moore and O'Connor,

1993; Barker et al., 1998; Loeffler et al , 1999; Schrick et al., 2001). Increases in days to first service and services per conception have been associated with mastitis infections during different stages of lactation with the end result being increased number of days open during lactation. While different investigators have reported similar associations, a true cause and effect relationship has not been well established. Furthermore, mechanisms by which mastitis exerts a negative influence on reproductive function have not been delineated. The primary goal of this dissertation is to "fill in" the gap of knowledge that exists between mastitis and decreased reproductive performance

Data from our laboratory (Barker et al., 1998; Schrick et al , 2001) have demonstrated that mastitis resulting from either Gram-positive or Gram-negative pathogens early in lactation and before first insemination was associated with increased days to first service Likewise, mastitis after insemination was associated with increased services per conception. These effects, both before and after insemination, result in increased days open and extended lactation required to achieve pregnancy What is not well understood is how mastitis during these periods impact reproductive efficiency. The overall question addressed in this dissertation is "*by what mechanism(s) does mastitis alter reproductive efficiency?*" A *Streptococcus uberis* challenge model has been developed and is currently used by Dr. Stephen Oliver at the University of Tennessee to induce clinical mastitis during early lactation in dairy cows. UT888, a strain of *S. uberis* isolated from a cow at the Dairy Experiment Station (DES) in

Lewisburg, TN, is utilized in the challenge model. The selection of *S. uberis* as the challenge organism was for multiple reasons First of all, *S uberis* is prevalent at the DES causing as much as 15% of clinical cases of mastitis (Barker et al., 1998) and around 8% of the total clinical and subclinical cases (Schrick et al , 2001). Secondly, the challenge protocol utilized a Gram-positive organism, which is successful in inducing clinical mastitis without resulting in death of animals Utilizing this challenge model to experimentally induce clinical mastitis, the primary questions to be tested include: 1) *does clinical mastitis alter endocrine profiles of cows during early lactation*, 2) *what impact does clinical mastitis during the preovulatory period have on estrous expression, follicular development, ovulation, pregnancy, and associated endocrine changes*, and 3) *does clinical mastitis following artificial insemination have an effect on development of the early embryo, corpora lutea function and lifespan, and pregnancy* Completing these objectives should increase understanding of events following mastitis that lead to delays in first service and increased services required per conception. Additionally, results may lead to treatment solutions to help decrease or avoid additional loss of production potential and income due to negative impacts of mastitis on reproductive function

Rationale and Significance

Reproductive efficiency is of great concern for dairy producers Etherington et al. (1993) observed that 6 8% of culling was due to mastitis, 23.7% due to low

production, and 20.6% due to reproductive problems. However, much of the culling for production and reproduction may be secondary effects of mastitis Mastitis has been reported to cost producers $2 billion annually (Degraves and Fetrow, 1993) Between 20 and 49% of cows are reported to experience clinical mastitis (Judge et al., 1997; Wilson et al., 1997; Barkema et al., 1998; Sargeant et al., 1998). Furthermore, subclinical infections occur at a rate of 15 to 40 per one case of clinical mastitis (Nickerson et al., 1989). Mastitis markedly decreases profitability and often results in culling of superior animals.

The most profitable cows are those that spend the greatest amount of their productive life in peak milk production. For this to occur, cows must reproduce regularly. Long, extended lactations due to inability of cows to establish pregnancy or long anestrous periods lead to cows spending large amounts of their productive life later in lactation when production is greatly reduced. Each day an animal is not pregnant over 90 days has been associated with a great decrease in monetary gain. Costs between $0.50 to over $4.00 have been reported per day open over 90 days (Louca, 1968; Britt, 1974; Fetrow and Blanchard, 1987). If a medium value of $2.00 is assumed per day a cow is not pregnant over 90 days and applied to the data of Schrick et al. (2001), a loss of $107 was experienced by cows with clinical mastitis after first service and $32 per cow with clinical mastitis before first service for that lactation These costs are in addition to the realized costs of mastitis associated with decreased milk production, increased involuntary culling rate, and mastitis treatment.

Additionally, genetic improvement is slowed due to an increase in the generation interval and culling of animals that are genetically superior due to decreased production and reproductive performance These costs do not reflect added semen costs or opportunity costs for lost genetics However, they are obviously of great enough magnitude to understand that the problem is very real and should be investigated

Mastitis defined

Mastitis is an inflammation of the udder that affects a high proportion of dairy cows Many bacteria are able to invade the mammary gland, multiply, and produce harmful substances that result in an inflammatory response. Signs of clinical mastitis include decreased milk production, alterations in milk composition and appearance, elevated body temperature, and swelling, redness, or elevated temperature in infected quarters. Subclinical infections also decrease milk production but often do not result in clinical signs that may be easily detected. Mastitis is difficult to control because several different bacteria have the ability to infect the udder Microorganisms that most frequently cause mastitis can be divided into two categories: *contagious pathogens* that are spread from cow to cow primarily during the milking process, and *environmental pathogens* that are found throughout the environment

Intramammary infections can be further separated by bacteria type· Gram-negative or Gram-positive (Sears et al., 1993) Gram-negative bacteria have a

lipopolysaccharide (LPS) component, also known as endotoxin, inside the cell wall (Lopez-Diaz and Bosu, 1992). Liberation of endotoxin from the cell wall results in an immune response. In severe cases, cows infected with Gram-negative bacteria may experience endotoxemia. No endotoxins are associated with Gram-positive bacteria; however, the outer cell wall is composed of many layers of peptidoglycan, which is found to a lesser degree in the cell wall of Gram-negative bacteria (Saylers and Whitt, 1994). Peptidoglycan has been shown to illicit a pyretic response similar to that elicited by endotoxin (Rotta, 1975).

Immune response associated with mastitis

Mastitis is recognized initially as abnormal milk and alterations in the mammary gland. Associated with this abnormal appearance of milk comes an increase in somatic cells. Neutrophil recruitment into milk is the primary cellular response to inflammation (reviewed by Paape et al., 1979, Sordillo et al., 1999). During the initial processes, several components exist that may trigger an acute inflammatory response such as platelet activating factor, prostaglandins, and nitric oxide (Anderson et al., 1985; Persson et al., 1993; Bouchard et al., 1999). These components may be released from endothelial cells as well as somatic cells and inflammatory cells in the milk Also released during the initial stages of response are chemotactic agents Some of these have been identified in order of their potency to cause neutrophil migration into the mammary gland during

Gram-negative infections: interleukin-1β > Tumor Necrosis Factor-α (TNF-α) > platelet activating factor > bovine C5a (Persson et al., 1993).

Once in the mammary gland, leukocytes recognize pathogen-associated molecular patterns through pattern recognition receptors (PRRs; as described by Medzhitov and Janeway, 2000) Among these receptors are the *Toll* receptors, which recognize lipopolysaccharide and peptidoglycan Toll receptor activation results in recruitment of the MyD88 adaptor protein consisting of an amino-terminal death domain and a carboxy-terminal TIR domain The TIR domain of MyD88 interacts with the TIR domain of *Toll* while the death domain of MyD88 interacts with the death domain of the serine/threonine kinase, IRAK 9 (Figure 1-1). The IRAK complex is then autophosphorylated and associates with the adaptor protein TRAF6, which associates with a MAP-kinase-kinase-kinase (TAK-1). Ultimately, the activation of TAK-1 leads to phosphorylation of IκB kinases, IKK, which phosphorylate IκB, the inhibitor of NF-κB The inhibitor is then degraded and NF-κB translocates to the nucleus. Once in the nucleus, NF-κB may activate transcription and induce expression of inflammatory cytokines and chemokines (as reviewed by Medzhitov and Janeway, 2000). As a result, acute phase components are released into the mammary gland.

The release of cytokines and chemokines results in the development of a chemotactic gradient, which is most concentrated at the site of infection and spreads to the post-capillary venules of the mammary gland. In the post-capillary venules, released products affect expression of adhesion molecules and ligands,

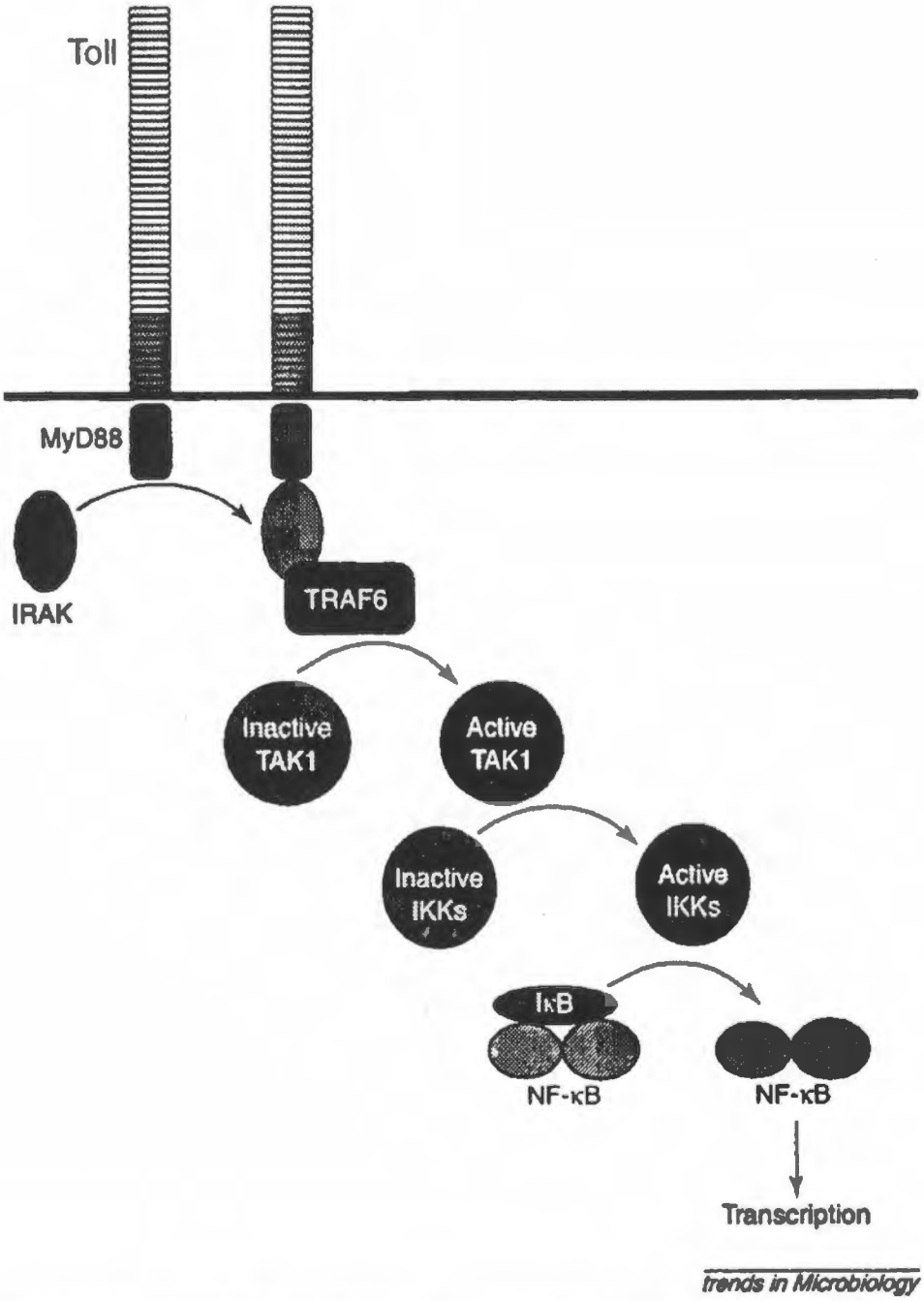


Figure 1-1. Model for *Toll* receptor signaling proposed by Medzhitov and Janeway, Jr. (2000). The *Toll* receptor is important in inducing NF-êB signaling pathway and plays a crucial role in adaptive immune response to determine what type of effector response is appropriate for the specific pathogen.

allowing migration of neutrophils, monocytes and lymphocytes into the mammary gland (as reviewed by Kehrli and Harp, 2001). Lymphocytes may travel in blood and migrate into tissues due to expression of integrins and selectins and their respective ligands expressed on the surface of endothelial cells (Butcher and Picker, 1996). Naïve cells may travel into the mammary gland where they may be exposed to antigen. Once exposed to antigen, lymphocytes may "home" preferentially to specific tissues or sites of infection/inflammation (Butcher, 1986) The ability for cells to "remember" and return to the location where infection first occurred allows for quicker response to infectious agents. In the bovine, mammary lymphocytes are recirculated to the mammary gland, in part, by the homing receptor CD62L (Bosworth et al., 1993).

Cytokines that are involved in the immune response are determined by the pathogen causing the infection and the severity of infection Those present in greatest concentration during severe infection include TNF-α and several members of the interleukin (IL) family, namely IL-1, IL-6, and IL-8 (Shuster et al , 1993) Cytokine gene expression in cows with *Escherichia coli* mastitis is first detected in tissue around the sinus of the mammary gland and then spreads to the mammary parenchyma The major cells initially producing cytokines are macrophages and endothelial cells of the lactiferous and teat sinuses. Neutrophils make up the greatest population of cells during mastitis and have the ability to produce cytokines (Scapini and Lapinet-Vera, 2000)

Association of mastitis to reproductive efficiency

The role mastitis plays in altering reproductive function remains unclear (Cullor, 1990; Moore et al , 1991; Moore and O'Connor, 1993; Barker et al., 1998, Loeffler et al., 1999, Schrick et al., 2001) Moore et al. (1991) reported some of the first observations of reproductive deficiencies when observing altered interestrous intervals and decreased lengths of luteal phases in cows with clinical mastitis caused by Gram-negative pathogens Moore and O'Connor (1993) hypothesized that Gram-negative bacteria stimulated production of prostaglandins, which subsequently caused luteal regression. Supporting these ideas, Cullor (1990) suggested that endotoxin may induce luteolysis and influence conception and early embryonic survival by release of inflammatory mediators.

Preliminary data from our laboratory (Barker et al., 1998) indicated that clinical mastitis had a negative effect on reproductive efficiency in dairy cows. Cows with clinical mastitis during the first 150 d of lactation were evaluated Groups were balanced according to lactation number and days in milk and subdivided as follows. group 1, clinical mastitis before first insemination, group 2, clinical mastitis between first insemination and pregnancy, group 3, clinical mastitis after confirmed pregnancy, and group 4, control cows with no clinical mastitis No differences in reproductive performance were detected due to milk production or if mastitis was caused by a Gram-positive versus a Gram-negative pathogen. Days to first service were significantly increased for cows with clinical

mastitis before first insemination (93.6 d) than for all other groups (71.0 d) Services per conception were significantly higher for cows with clinical mastitis after first service (2.9) than for cows with clinical mastitis before first service (1.6) and for cows with no clinical mastitis or cows with clinical mastitis after confirmed pregnancy (1.7) Days to conception for cows with clinical mastitis after first insemination (136 6 d) were significantly increased over those for control cows or cows that developed clinical mastitis after confirmed pregnancy (92 1 d) Results from this study clearly demonstrate that clinical mastitis during early lactation was associated with a marked reduction in reproductive performance. As hypothesized by Moore and O'Connor (1993), these data suggest that luteolysis and subsequent loss of embryos in cows with clinical mastitis may be a possible mechanism for reduced reproductive efficiency. Another significant observation by Barker et al. (1998) was that Gram-positive bacteria and Gram-negative bacteria were not different in their negative effects on days to first service, services per conception, or days to conception

In a subsequent study, Schrick and co-workers (2001) reported that subclinical mastitis had similar negative effects on reproductive performance as clinical mastitis. Reproductive data were analyzed retrospectively on 758 Jersey cows at The University of Tennessee Dairy Experiment Station research herd from 1986 to 1997. Quarter milk samples were collected every 4 to 8 weeks from all lactating cows. Cows were subjected to a voluntary waiting period of 60 d before first insemination Cows were first separated by type of mastitis.

Subclinical mastitis was defined as the presence of the same pathogen in at least two consecutive samples during lactation. Cows were further separated based on the time of clinical or subclinical mastitis as follows: before first service, between first insemination and pregnancy, and after confirmed pregnancy or uninfected. Milk production (305 d Mature Equivalent), lactation number (range 1 - 10), season of year, and bacterial type were included in the model Milk production did not differ between groups and no differences in reproductive performance were observed between mastitis caused by Gram-negative or Gram-positive pathogens. Season of calving negatively affected days to first service and days open, whereas, lactation number negatively impacted services per conception Cows with mastitis before first service had extended number of days to first service (75.7 vs. 67.8), days open (106.2 vs. 85.4), and services per conception (2 0 vs. 1.6) compared to cows with mastitis after establishment of pregnancy or uninfected cows, respectively Cows with mastitis between first insemination and pregnancy had days open (143.5) and services per conception (3.1) elevated above both other groups Clinical and subclinical mastitis reduced reproductive parameters similarly. These data further support negative effects of mastitis reported by Barker et al. (1998). However, Schrick et al. (2001) also reported that cows with mastitis before first insemination had an elevation in services per conception compared to cows with mastitis after pregnancy or uninfected cows

Loeffler and coworkers (1999) reported in an observational study of 4,382 cows that occurrence of mastitis during the 21-d period after artificial

insemination decreased pregnancy rates (29%) compared to cows without mastitis (45%) In this same study, cows with mastitis 20-40 d prior to artificial insemination had no decrease in pregnancy rates while cows with mastitis less than 20 d prior to insemination tended to have lower pregnancy rates (36%, P=0.08). Mastitis was also correlated with retained fetal membranes, displaced abomasum and lameness

Based upon preliminary data collected in our laboratory and data reported from other studies, the working hypothesis of this dissertation is that mastitis may impact normal reproduction function at these three critical points: 1) endocrine function and follicular development, 2) oocyte, and 3) the embryo or uterus.

What is the effect of mastitis on endocrine profiles and follicular development?

Mastitis may have a negative impact on normal endocrine function and follicular development Data support cortisol as having a negative impact on normal follicular growth and endocrine function (Echternkamp, 1984; Peter at al., 1989; Lopez-Diaz and Bosu, 1992; Battaglia et al., 1997). Stressful conditions associated with mastitis may result in increased concentrations of cortisol. Battaglia et al. (1997) treated ewes intravenously with endotoxin and sampled hypophyseal portal blood simultaneous with jugular blood at 10 min intervals to observe gonadatropin releasing hormone (GnRH), luteinizing hormone (LH), cortisol and progesterone Ewes treated with endotoxin had significantly lower GnRH pulse amplitude, lower concentrations of GnRH and LH, elevated

concentrations of cortisol and progesterone, and a higher body temperature compared to control animals. Peter et al. (1989) reported that cycling heifers receiving intrauterine infusion of endotoxin had elevated cortisol concentrations as well as smaller follicles on day 12 of the estrous cycle Peter et al (1989) also observed that follicles that failed to ovulate from $PGF_{2\alpha}$-induced luteal regression resulted in cysts, which persisted for 7 to 21 d. The formation of follicular cysts on the ovaries could be a result of cortisol suppression of the LH surge (Lopez-Diaz and Bosu, 1992).

Echternkamp (1984) further documented the negative correlation of elevated cortisol to suppressed LH in cows experiencing "handling" stress compared to cows that had been previously acclimated. Cortisol infusion in ewes decreased follicular development and the ovulatory surge of LH was absent (Macfarlane et al., 2000). When cortisol infusion ceased, follicular development returned to normal and the LH surge did occur. The impact of cortisol infusion was much more profound during the early follicular phase than during the luteal phase (Macfarlane et al., 2000). In a similar study, Daley and coworkers (1999) reported that infusion of cortisol in sheep blocked the LH surge in 54% of sheep and estrus either did not occur or was delayed. Furthermore, administering pulses of GnRH during cortisol infusion increased concentrations of estrogen and presence of the LH surge as in controls.

Women are also susceptible to the negative impacts of endotoxin or ACTH infusion. Infusion of each resulted in premature LH surge and interference in

follicular maturation and ovulation related to an increase in production of adrenal progesterone (Puder et al., 2000). Ovarian cysts in rats have been associated with an increase in norepinephrine release from the ovary and increased sympathetic activity (Paredes, 1998).

Many "stressful" conditions result in elevated concentrations of cortisol, which has been associated with altered endocrine function. Handling stress in rainbow trout (Contreras-Sanchez et al , 1998) resulted in altered timing of ovulation and the impact was determined to be downstream of gonadatropin release (Pankhurst et al., 2000) The stress of increased production in dairy cows has also been associated with alterations in normal follicle physiology Cows with high first test milk production and increased fat.protein ratio are more likely to have ovarian cysts and decreased reproductive performance (Heuer et al , 1999) Alternatively, stress in pigs caused by electric shock pulses did not have any impact on ovulation or embryo numbers (Turner et al., 1998).

Heat stress has been recognized to decrease reproductive performance of dairy cows by decreasing expression of estrus, follicular development, oocyte competence and embryonic development (as reviewed by Hansen et al., 2001) Wilson et al. (1998) applied elevated temperatures to cows to mimic effects of heat stress Cows in thermoneutral conditions were more likely to ovulate the second wave follicle than cows in the heat stress condition (91 vs 18%, respectively). Furthermore, luteolysis was delayed by as many as 9 d in heat stressed cows. Similarly, luteolysis occurred later in dairy heifers experiencing

heat stress and a higher percentage of heifers had three follicular waves (Wilson et al., 1998b). Roth et al (2000) reported decreased inhibin and elevated FSH concentrations during and following heat stress in cows. Alterations in FSH resulted in increased number of large follicles and a slower rate of decrease of the first wave follicles. Stress during experimental procedures also impacts pregnancy rates in dairy cows. Acclimatization of cows to experimental procedures resulted in increased pregnancy rates (86%) compared to cows that were not acclimated to the experimental procedures (27%; Mann, 2001) Cows in this study that were not acclimated also had impaired ovulation under the "stress of experimentation."

Inflammatory or infectious diseases often result in stimulation of an immune response that interferes with LH function by releasing cytokines such as TNF or IL. Darbon et al. (1989) reported that TNF inhibited the stimulating action of FSH on LH receptor formation in cultured rat granulosa cells and had an inhibitory effect on FSH-induced cAMP production. Additionally, TNF-α inhibited FSH-induced LH receptor formation indicating that TNF-α acts on LH receptors both through and downstream of the FSH receptor and cAMP formation. Darbon et al. (1989) hypothesized that TNF released during infections may reduce ability of granulosa cells to differentiate upon FSH stimulation and to respond to LH because of alterations in LH receptors during follicle growth and maturation. Granulosa cells are important for production of estrogens and secondarily impact LH production via estrogen concentrations.

Interleukins are key mediators in the effects of Gram-negative bacteria and endotoxins (Nathan, 1987). McCann et al (1997) reported interleukin-1 (IL-1), normally released during infection with endotoxin, blocks pulsatile secretion of LH, but not FSH, through alterations in nitric oxide production to inhibit GnRH Furthermore, IL-1 disrupts normal estrous cyclicity, GnRH gene expression, and LH and FSH secretion (Rivest et al., 1993) Interleukin-6 is a cytokine produced by macrophages during an immune response and is a potent inhibitor of proliferation and FSH-induced E_2 production from small and large follicles *in vitro* (Alpizar and Spicer, 1994).

While many of the previous studies have reported specific impacts of mastitis/endotoxin/or cytokine on estrous activities or normal endocrine function, few have actually centered on the mechanism by which these events occur. Utilizing sheep as a model, Michigan State has reported an extensive amount of data delineating the mechanisms by which endotoxin negatively impacts estrus (Battaglia et al., 1997, Battaglia et al., 1998; Battaglia et al., 1999; Battaglia et al , 2000, Harris et al., 2000; Battaglia et al , 2001). Studies have determined that endotoxin administration interrupted the preovulatory rise of estradiol and also delayed or completely blocked the preovulatory LH surge and expression of estrus. Endotoxin has different impacts on LH frequency in different animals, causing decreased LH pulsatility in some and not in others However, the LH surge was suppressed or inhibited in all animals (Battaglia et al , 2000).

A subsequent study (Battaglia et al., 2000) reported that low doses of endotoxin were sufficient to slightly decrease GnRH release Corresponding with the slight decrease in GnRH release was a significantly large decrease in LH pulsatility Luteinizing hormone pulses were completely inhibited in some animals while GnRH pulses still occurred. These data led to the hypothesis that endotoxin not only decreased GnRH secretion, but also separately decreased ability of the pituitary to respond to GnRH. In support of this hypothesis, Battaglia and coworkers (2001) tested effects of administering exogenous GnRH while blocking endogenous GnRH secretion during endotoxin infusion Endotoxin-challenged sheep had decreased amplitude of LH pulses in response to exogenous GnRH administration. Collectively, these data indicate that endotoxin acts at the level of the hypothalamus and pituitary to impair reproductive function.

Earlier work by Battaglia and coworkers (1999) indicated that negative effects of endotoxin were expressed by disrupting the "early activating effects of the estradiol signal" before the LH surge and did not actually impact GnRH and LH directly. Research from this group has proven each of these areas to combine to inhibit estrus and ovulation. Also, a key observation was the coupling between corticotropin-releasing hormone (CRH) release, increases in cortisol, and the suppression of GnRH/LH with timing of endotoxin infusion (Battaglia et al., 1998). One proposed mechanism for suppression of GnRH was that prostaglandins might mediate the action of endotoxin at the level of the hypothalamus and/or pituitary. To test this hypothesis, flurbiprofen was utilized as an inhibitor of

prostaglandin synthesis. Flurbiprofen was successful in preventing decreased LH pulsatility, stimulation of fever, elevated cortisol and progesterone, and inhibition of GnRH release, all typically induced by endotoxin infusion (Harris et al , 2000)

What is the effect of mastitis on the oocyte?

For pregnancy to occur, several events must unfold including maturation of the oocyte, ovulation, corpus luteum function and proper development of the embryo. Luteinizing hormone is responsible for the initiation of oocyte maturation (Wise et al., 1994). Oocyte maturation is accompanied by an expansion of the cumulus cell layer mediated by FSH and LH (Lawrence et al., 1980; Younis et al , 1989, Saeki et al , 1990; Zuelke and Brackett, 1994) Furthermore, LH increased glucose, pyruvate oxidation, and glutamine reduction in cumulus cells in order to nourish the oocyte (Zuelke and Brackett, 1993, 1994) During times of stress, LH may be suppressed and such reductions could interfere with proper expansion of the cumulus and oocyte maturation.

Another symptom of clinical mastitis and immune response is elevated body temperatures Experimentally-induced clinical mastitis by a Gram-positive pathogen resulted in elevated body temperatures (Hockett et al., 2000). Thatcher and Hansen (1993) documented that cows exposed to heat stress experience increased embryonic mortality. Furthermore, it has been reported that hyperthermia has its greatest effects when experienced between the onset of estrus and insemination (Putney et al., 1989) or during the early embryonic

development (Ealy et al., 1993). Edwards and Hansen (1996) reported that *in vitro* exposure of oocytes at 39°C compared to 41°C for 12 h resulted in decreased blastocyst formation (30% vs. 10%, respectively).

A third mechanism by which mastitis may negatively affect the oocyte is a secondary response to changes in endocrine status. Suppressed LH surge (delayed ovulation) or elevation of $PGF_{2\alpha}$ (decreased progesterone) may result in persistent follicles (Savio et al., 1993; Ahmad et al., 1995; Ahmad et al., 1996) Pregnancy rates were greater in animals that ovulated the first-wave dominant follicle (54%) compared to persistent follicles (14%; Ahmad et al , 1996). Ahmad et al. (1995) reported decreased recovery of oocytes (70%) in cows with persistent follicles than in cows with growing follicles (90%) Furthermore, fewer embryos had reached 16-cell or morula stage of development from persistent follicles (14% and 14%, respectively) than from growing follicles (86% and 73%, respectively). Peter et al. (1989) reported that *intrauterine* infusion of endotoxin suppressed LH and resulted in follicles that did not ovulate. Therefore, cows with mastitis or other infections may have increased services per conception and days open due to formation of persistent follicles or follicular cysts that result in decreased pregnancy rates. Suppression of the preovulatory LH surge would inhibit the ability of oocytes to be ovulated from follicles and subsequent maturation of oocytes. Thus, decreased LH would lead to persistent or cystic follicles

What is the effect of mastitis on the embryo or uterus?

In order for pregnancy to be achieved, the embryo must develop properly and find the uterine environment hospitable to continued development One component normally elevated in Gram-negative infections is $PGF_{2\alpha}$ (Gilbert et al , 1990; Peter et al., 1990; Kindahl et al , 1996, Janosi et al , 1998) Gilbert et al (1990) reported that *intrauterine* infusion of *E. coli* resulted in shortened estrous cycles in Holstein heifers, implying that prostaglandins were stimulated and luteolysis occurred Prostaglandins may decrease reproductive efficiency by decreasing luteal life span or by decreasing embryo quality and development Gir et al (1990) reported endotoxin exposure caused a dose-dependent increase in plasma $PGF_{2\alpha}$ level with a subsequent drop in progesterone concentrations suggesting occurrence of luteal regression

Schrick et al. (1993) reported a negative correlation between embryo quality and uterine luminal concentrations of $PGF_{2\alpha}$ Prostaglandins have been shown to decrease embryonic development *in vitro* in other species (Stormshak and Casida, 1965; Harper and Skarnes, 1972, Breuel et al., 1993). Seals et al (1998) reported administration of $PGF_{2\alpha}$ 5 to 8 d following mating to progestogen-supplemented cows resulted in decreased pregnancy rates versus cows administered saline. Furthermore, transfer of good quality, day-6 embryos to progestogen-supplemented recipients administered $PGF_{2\alpha}$ resulted in decreased pregnancy rates compared to recipients administered saline (Hernandez-Fonseka et al., 1997). Administration of $PGF_{2\alpha}$ to progestogen-supplemented

cows resulted in a decreased percentage of embryos that developed beyond the morula stage and a decrease in embryo quality scores (Hockett et al., 1998). The elevation of $PGF_{2\alpha}$ associated with mastitis may cause a decrease in embryonic development and ultimately result in an increased number of services required per conception and number of days open. Risco and others (1999) observed 2087 cows that were diagnosed pregnant and used logistical regression to determine association of mastitis to abortion Cows that had clinical mastitis during the initial 45 d of pregnancy were at 2.7 times higher risk to abort within the following 90 days than cows that were not diagnosed with clinical mastitis.

Mastitis may also influence the uterine environment by affecting the nutritional status of animals. Cows with a negative energy balance have reduced fertility and increased period of anestrus following parturition in near 30% of cows in lactation (Staples et al., 1990). Additionally, changes in protein intake and metabolism may lead to elevated concentrations of plasma urea nitrogen levels which are associated with changes in uterine pH (Butler et al., 2000). Elevated urea also results in increased secretion of $PGF_{2\alpha}$ from endometrial cultures (Butler et al., 1998) Decreased feed intake of animals during clinical infection may lead to negative energy balance and subsequent decreased reproductive performance through changes in uterine environment and $PGF_{2\alpha}$ release

Summary

Mastitis is a very costly disease that impacts a high percentage of dairy cows during lactation. Costs of mastitis may be much greater than originally recognized due to lack of knowledge of impacts that mastitis may have upon reproduction. Activation of the immune response results in release of cytokines and inflammatory mediators. Effects of these are to elicit immune response, however, negative impacts may also occur upon reproduction Cortisol, interleukins, TNF_{α}, and $PGF_{2\alpha}$ are likely factors impacting reproduction during mastitis (Figure 1-2) Effects of cortisol and $PGF_{2\alpha}$ are of primary interest Cortisol may impact the reproductive system through alterations in the hypothalamo-pituitary-ovarian axis. The suppressive effects of cortisol upon release of GnRH from the hypothalamus, responsiveness of the pituitary to GnRH signals, release of LH from the pituitary, and the ability of LH to bind receptors and initiate signaling pathways are of consequence. Furthermore, $PGF_{2\alpha}$ has been implicated to cause early luteal regression, decrease embryonic development and quality, and aid to elicit hyperthermic conditions as well as alter hypothalamus-pituitary communication Observed associations of mastitis with shortened estrous cycles, increased services required for conception, increased days to first service and increased days open lead to the hypothesis that mastitis has a direct effect upon normal reproductive physiology and may alter function through inflammatory mediators associated with the disease The overall objective was to examine effects of clinical mastitis on reproductive function.

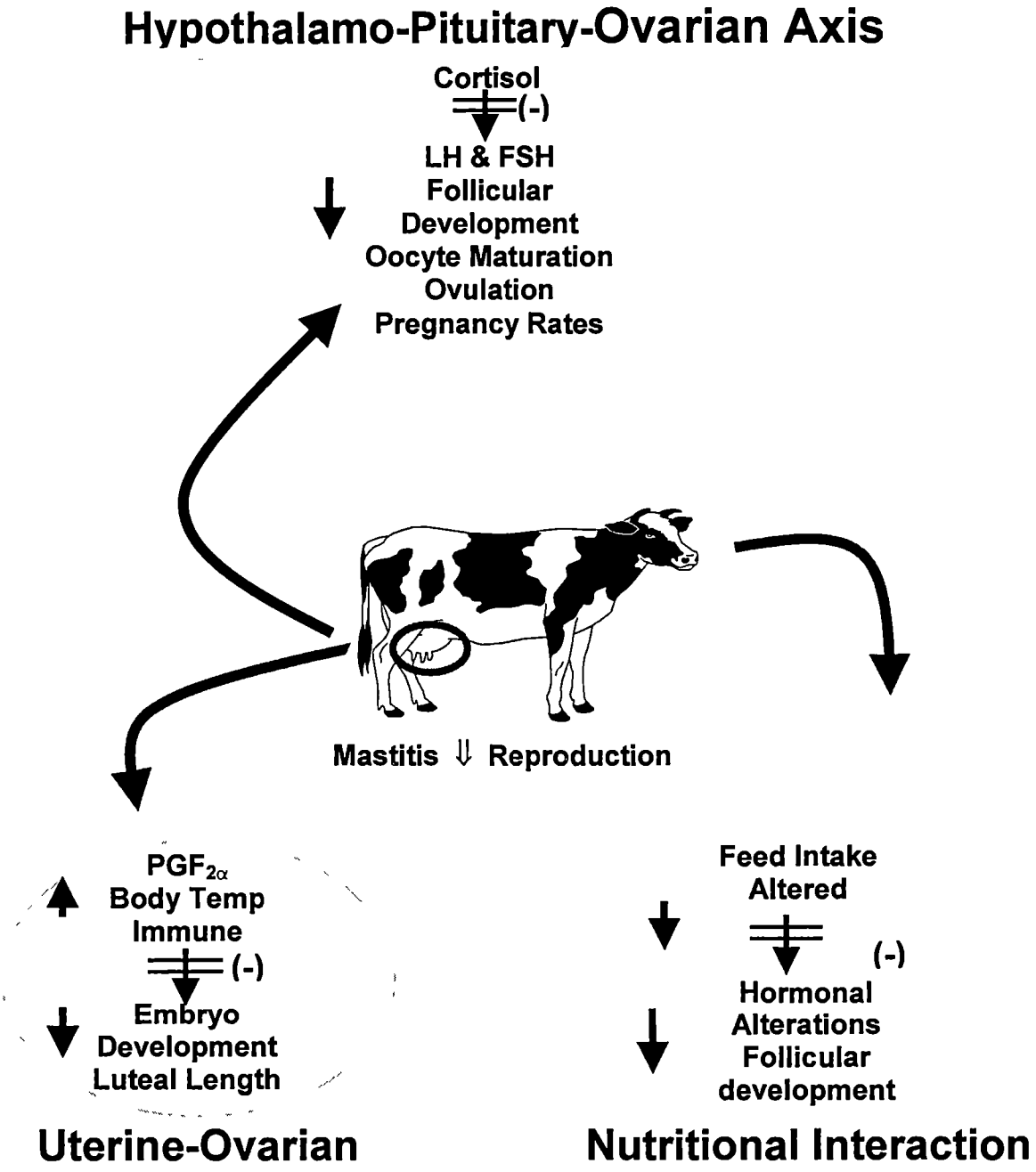


Figure 1-2 Proposed model for how mastitis may interact with reproductive function Author proposes that mastitis may decrease reproductive efficiency through alterations in the hypothalamo-pituitary-ovarian axis, uterine-ovarian axis, and/or via nutritional interactions.

Chapter 2

Endocrine Profiles of Dairy Cows Following Experimentally-Induced Clinical Mastitis During Early Lactation

This chapter is a lightly revised version of a paper by the same name published in the journal Animal Reproduction Science in 2000 by Mitchell Hockett, Fred Hopkins, Mark Lewis, Arnold Saxton, Henry Dowlen, Stephen Oliver, and Neal Schrick

Hockett, M E , F.M Hopkins, M.J. Lewis, A.M. Saxton, H H Dowlen, S.P Oliver and F.N. Schrick. 2000. Endocrine profiles of dairy cows following experimentally-induced clinical mastitis during early lactation. Anim Reprod Sci 58. 241-251

My use of "the authors" in this chapter refers to my co-authors and myself My primary contributions to this paper include: (1) selection of the topic and development of experimental design, (2) sampling and analysis of samples, (3) gathering and interpretation of the literature, (4) data analysis, (5) interpretation of the data, and (6) most of the writing.

Abstract

Concentrations of LH, cortisol, estradiol-17β, prolactin and 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ were determined in cows with experimentally-induced clinical mastitis during early lactation. Cows free of intramammary infection (IMI) and in the luteal phase of the estrous cycle were balanced by lactation number and days in milk and assigned to either control (n = 5) or treatment (n = 5) groups. Treated cows were infected experimentally (day 0) in two mammary quarters with *Streptococcus uberis* and developed clinical mastitis within 60 h after inoculation as evidenced by increased mastitis scores, elevated rectal

temperatures, mammary swelling and isolation of *S.uberis* pathogen. Four days following bacterial challenge, blood samples were collected every 20 min for 8 h for determination of 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ (PGFM) and LH following administration of oxytocin and GnRH, respectively Blood samples were also collected on d 0, 4 and 7 of the experiment to determine concentrations of estradiol-17β, prolactin and cortisol Four days after bacterial challenge, concentrations of cortisol were higher (P = 0 04) in experimentally-infected cows than controls. Experimentally-infected cows had increased (P = 0.02) concentrations of cortisol on d 4 and 7 compared with d 0. Control cows had no significant increase in blood cortisol during the experimental period. Baseline concentrations of PGFM did not differ between groups; however, peak concentrations of PGFM following oxytocin challenge were elevated (P = 0.006) in cows with clinical mastitis compared with control animals. Prolactin, estradiol-17β and LH did not differ between cows with clinical mastitis or controls Experimentally-induced mastitis during early lactation elevated concentrations of cortisol during the luteal phase of the estrous cycle Furthermore, mastitic cows demonstrated an increased PGFM response following oxytocin administration. Altered reproductive efficiency in cows with clinical mastitis caused by Gram-positive pathogens may be the result of increased uterine sensitivity to prostaglandin $F_{2\alpha}$.

Key words: cortisol, luteinizing hormone, mastitis, prostaglandin

Introduction

Mastitis is a costly disease to the dairy industry through several factors including decreased milk production, increased health care costs and milk discard, and premature culling of genetically superior animals. Etherington et al (1996) reported that 6.8% of culling was due to mastitis Significant culling was also attributed to low production and poor reproductive efficiency Given that mastitis reduces both milk production (Fetrow et al., 1991) and reproductive performance (Cullor, 1990; Moore and O'Connor, 1993; Barker et al , 1998; Schrick et al., 2001) in lactating cows, actual culling rates due to mastitis may be higher than previously realized.

Data from our laboratory (Barker et al., 1998) demonstrated that cows with clinical mastitis during early lactation exhibited a prolonged interval until first service (94 d) compared with animals with no clinical mastitis (71 d). Additionally, cows with clinical mastitis between first service and establishment of pregnancy had increased number of days open and a two-fold increase in services per conception.

Previous studies reported that clinical mastitis caused by Gram-negative bacteria altered interestrus intervals and increased number of days to first service (Cullor, 1990; Moore and O'Connor, 1993) Barker et al. (1998) observed that Gram-positive and Gram-negative bacterial pathogens had similar effects on increasing days to first service, days open and services per conception. The delay to first service in cows exhibiting clinical mastitis may be related to

ovulation failure resulting from insufficient concentrations of LH and/or estradiol-17β (E_2) or altered interestrus intervals resulting from elevated concentrations of $PGF_{2\alpha}$. The objective of the present study was to evaluate endocrine profiles *during the luteal phase* of Jersey cows with experimentally-induced clinical mastitis.

Materials And Methods

Ten lactating Jersey cows in early lactation (< 30 days in milk, DIM) and free from major pathogen intramammary infection (IMI) were utilized for the current experiment. Intramammary infection status was based on microbiological evaluation of quarter foremilk samples collected on two occasions approximately 7 and 14 days after parturition. Before sample collection, teats were washed thoroughly and dried with individual disposable paper towels Teat ends were sanitized with cotton swabs containing 70% isopropyl alcohol Samples (10 µl) of foremilk from each mammary gland were plated onto one quadrant of a trypticase soy agar plate supplemented with 5% defibrinated sheep blood (Becton Dickinson Microbiology Systems, Sparks, MD). Plates were incubated at 37°C, and bacterial growth was observed and recorded daily for 3 days. Gram stain reaction, colony morphologic features, hemolytic characteristics and catalase test were performed to identify bacteria on primary culture medium. Cows in the control group remained unchallenged to avoid incidental contamination of bacteria into mammary gland The treatment of the designed

study was clinical mastitis, therefore, no sham infusion occurred in control animals to increase ability to maintain mammary glands free of major pathogens.

Experimental Bacterial Challenge

An encapsulated strain of *Streptococcus uberis* isolated initially from a cow with clinical mastitis was used for induction of IMI *Streptococcus uberis* was grown in Todd Hewitt broth (Difco Laboratories, Detroit, MI) for 7 h at 37°C Colony counts on solid agar were performed to determine the number of colony forming units/ml in serial dilutions. Dilutions necessary to achieve the desired bacterial concentration for intramammary inoculation were determined before experimentation. One front and one rear quarter of each cow in the treated (TRT; n = 5) group was infused with 5 ml of a suspension of *S. uberis* containing 5.05 x 10^3 colony forming units/ml into the teat cistern following disinfection of teat ends with ethanol. All inoculations were conducted within 0.5 h after milking on d 0.

Quarter foremilk samples for microbiological evaluation were obtained aseptically from all cows in the TRT group at the milking before inoculation, daily for 7 d and at weekly intervals for 7 weeks. Cows in the non-challenged control (CON; n = 5) group were sampled at the same intervals as TRT cows, except no daily samples were collected during the first week Isolates identified presumptively as streptococci were evaluated initially for growth in 6.5% NaCl, hydrolysis of esculin and sodium hippurate, and CAMP reaction Upon first

isolation, streptococcal organisms were identified to the species level using the API 20 Strep system (bioMerieux Vitek, Inc , Hazelwood, MO).

Clinical Observations

Qualified personnel uninformed of treatment performed clinical assessment of TRT and CON cows at each milking during the experimental period Quarters of experimentally-challenged cows were considered subclinically infected if *S uberis* was isolated on two occasions within 7 days of challenge or clinical when clinical mastitis was detected accompanied by positive isolation of *S uberis*. In addition, rectal temperatures were taken twice daily on TRT cows for one week after challenge. Clinical status (mastitis scores) of all mammary glands and appearance of milk were evaluated by qualified personnel using the following scheme: 1 = normal mammary gland and normal milk, 2 = normal mammary gland and slight alterations in milk (a few flakes), 3 = abnormal mammary gland and abnormal milk (clots, clumps, changes in milk color), and 4 = swollen mammary gland, abnormal milk and systemic signs (hyperthermia, depression, dullness) of infection. Cows that developed mild cases of clinical mastitis (number 2 or 3 in the scheme described above) were monitored closely. Experimentally-infected cows were not treated for mastitis until d 5-6 following bacterial challenge.

Blood Sample Collection

Blood samples were obtained from CON and TRT cows every 20 min for 8 h beginning 4 d after intramammary inoculation of *S. uberis* to allow for onset of clinical mastitis in TRT cows Blood (10 ml) was collected via jugular catheter (Tygon microbore tubing; i.d. 1.27mm; o.d. 2.29 mm; Fisher Scientific, Pittsburgh, PA) into chilled tubes containing 200 µl of saturated sodium citrate solution (25 mg/ml, Baxter Scientific Products, McGaw Park, IL) Samples were collected every 20 min for 2 h to determine baseline values for 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ (PGFM) after which an intramuscular injection of 100 U of oxytocin (Oxoject, Anthony Products Co , Arcadia, CA) was administered Following oxytocin administration, samples were collected every 20 min for 6 h to evaluate PGFM release.

Since a previous study (Wathes et al , 1992) indicated that oxytocin administration did not influence LH secretion, blood collection for LH analysis was performed simultaneously with PGFM. After 4 h of sample collection (used for baseline calculation of LH), an intramuscular injection of 100 µg of GnRH (Cystorelin, Rhone Merieux Inc , Athens, GA) was administered and blood samples collected every 20 min for an additional 4 h. Plasma samples collected on d 0, 7, and samples obtained every two hours on d 4 were analyzed for cortisol. Single blood samples collected on d 0, 4 and 7 were evaluated for concentrations of E_2 and prolactin. Following collection, blood samples were

placed on ice, centrifuged (2500 x g), and plasma was harvested and stored at -20°C until radioimmunoassays were performed.

Radioimmunoassays and Statistical Analyses

All blood samples collected on day 4 were analyzed for LH (Moura and Erickson, 1997; sensitivity of 0.3 ng/mL) and PGFM (Seals et al., 1998; sensitivity of 5 pg/mL) Concentrations of cortisol were determined utilizing a solid phase radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA) as described by Bossuyt et al. (1997) and validated in our laboratory. Sensitivity of the assay was 2 ng/mL. Concentrations of prolactin and E_2 were determined as described by Moura and Erickson (1997). Assay sensitivities were 0.5 ng/mL for prolactin and 0 15 pg/mL for E_2. Weekly blood samples were analyzed for progesterone to monitor luteal activity and to determine stage of the estrous cycle at time of clinical mastitis in association with thrice daily estrous detection. Concentrations of progesterone were determined using a solid phase radioimmunoassay (Diagnostic Products Corp.) as described by Seals et al. (1998). Sensitivity of the assay was 0 02 ng/mL. All individual hormones were determined in single assays and intra-assay coefficients of variation were < 6%.

Statistical analyses were performed using MIXED analysis of variance (SAS, 1997). The statistical model included days postpartum, treatment group, experimental day, and interactions amongst these. Log transformation was used to normalize data for E_2, cortisol, PGFM and prolactin. Least square means were

back-transformed for reporting data. Data from time of oxytocin administration and four hours following were analyzed for area under the curve for PGFM. Log_{10} transformation was utilized for reporting SCC data Data were interpreted as significantly different at $P < 0.05$, P values of 0.06 to 0 10 were suggestive of a trend towards significance, and P values greater than 0 10 were concluded as not different.

Results

All experimentally-infected cows exhibited clinical mastitis in at least one challenged quarter by d 3 after bacterial challenge as evidenced by an elevation in mastitis scores (P = 0 0001; Table 2-1) Furthermore, *S. uberis* was isolated from two consecutive milk samples from all challenged quarters within 72 h after bacterial challenge (data not shown). The mean number of *S. uberis* colony forming units recovered from challenged quarters per milliliter of milk at d 3 and d 7 after bacterial challenge was 4.97 log_{10} ± 1 2 and 4 24 log_{10} ± 0.85, respectively Somatic cell counts were increased (P = 0.0001) in challenged quarters within 24 h (Table 2-1). No CON cows exhibited signs of clinical mastitis during the experimental period Daily milk production for TRT cows was significantly reduced (P = 0.03) an average of 5 6 kg per cow from day of challenge (d 0) to day of intensive bleed (d 4, Table 2-1) Milk production of CON cows did not change during the experimental period (data not shown). Furthermore, mastitic cows experienced an increase (P = 0.003) in rectal

temperature from 38.1°C on d 0 to a maximum of 39 2°C on d 3, with rectal temperatures remaining elevated through d 5 after challenge (Table 2-1)

All cows were determined to be in the luteal phase of the estrous cycle on d 4 after experimental challenge as verified by concentrations of progesterone (3.4 ± 1.2 and 4.7 ± 1.2 ng/mL for CON and TRT, respectively) and observed estrous dates. Baseline concentrations of PGFM did not differ between treatment groups However, peak PGFM concentrations following oxytocin challenge were significantly higher for cows with mastitis (418.7 ± 86.4 pg/mL) than CON cows (138 7 ± 86 4 pg/mL, P = 0.006, Figure 2-1). Area under the curve tended to be higher for cows with clinical mastitis (660.2 ± 195 6) than for CON (136.9 ± 195.6, P < 0 10).

Concentrations of cortisol did not differ on day of challenge (d 0) between TRT and CON (Figure 2-2). However, concentrations of cortisol were higher in mastitic cows than CON cows (P = 0 02) on d 4 post-challenge. Cows that developed clinical mastitis had elevated (P = 0.02) concentrations of cortisol on days 4 and 7 compared with day 0. However, cortisol did not differ by day in control cows. Although cortisol concentrations remained elevated on day 7 in treated cows, concentrations on that day did not differ significantly from that of controls cows.

Table 2-1 Clinical observations in cows with experimentally-induced mastitis

	Day[1]							
Variable	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Quarter 1[2]	1 0^{a}	1 2^{a}	1 1^{a}	2 1^{b}	2 0^{b}	2 6^{c}	2 9^{c}	3 1^{c}
Quarter 2[2]	1 0^{a}	1 0^{a}	1 0^{a}	1 9^{b}	1 6^{b}	2 4^{c}	2 6^{c}	2 6^{c}
Rectal Temp[3]	38 1^{a}	38 7^{abc}	38 3^{ab}	39 2^{c}	39 1^{c}	39 0^{cb}	38 5^{abc}	38 9^{abc}
Milk[4]	26 2^{a}	24 6^{ab}	25 3^{ab}	18.3^{bc}	20 6^{abc}	16 2^{c}	20 0^{abc}	15 4^{c}
SCC[5]	4 4^{a}	6 4^{b}	6 8^{c}	6 9^{c}	7 0^{c}	-	-	6.5^{bc}

[1] Day of sample collection (day 0 = day of bacterial challenge)
[2] Average mastitis score for challenge quarter, ^{a,b,c} Mastitis scores with different superscripts differed (P = 0 0001) SEM = 0 2
[3] Rectal temperature (°C), ^{a,b,c} Rectal temperatures with different superscripts differed (P < 0 05) SEM = 0 3
[4] Average daily milk production (kg/d), ^{a,b,c} Daily milk production with different superscripts differed (P = 0 03) SEM = 2 6
[5] Average daily somatic cell count of challenged quarters (log_{10} cells/ml), ^{a,b,c,d} Somatic cell counts differed (P = 0.0001) SEM = 0 1
"-" SCC not determined on these days

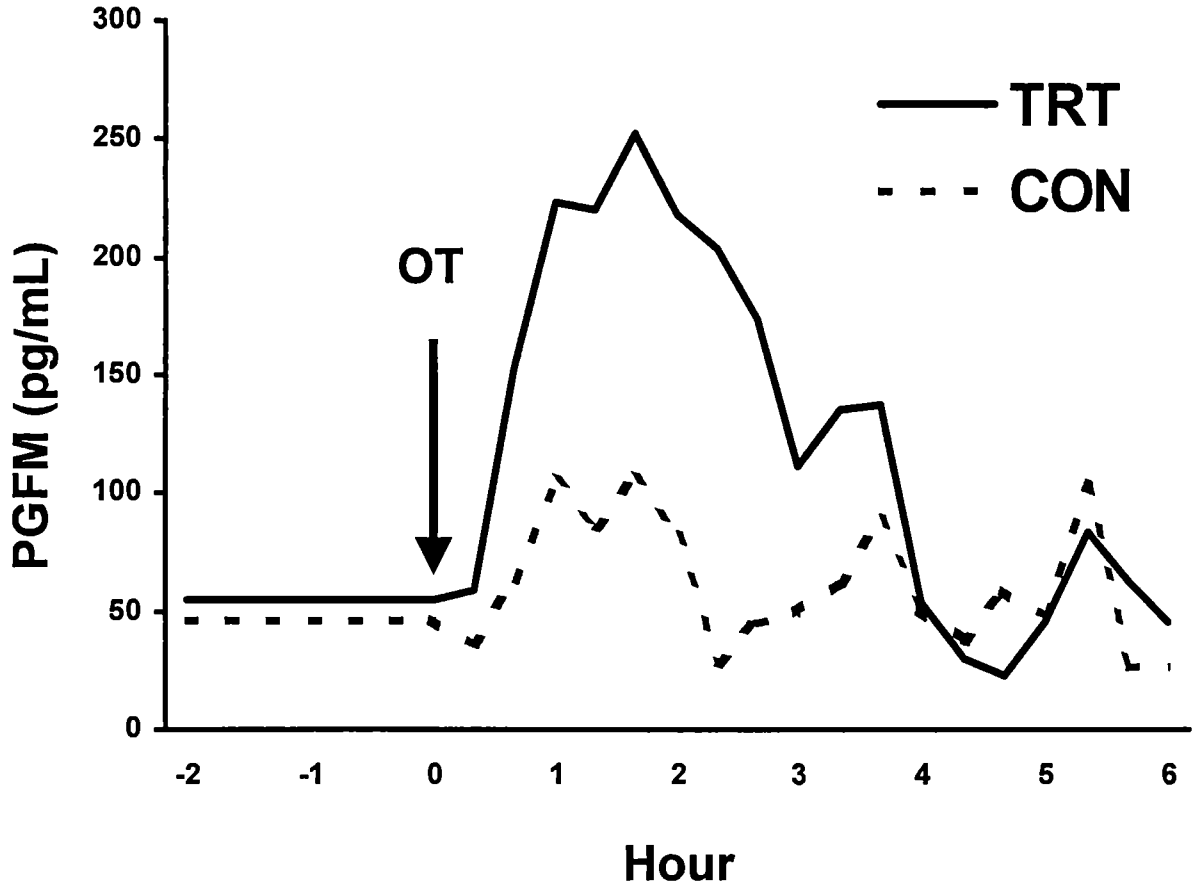


Figure 2-1. Concentrations of PGFM on day 4 following experimentally-induced clinical mastitis Baseline concentrations were determined during the initial 2 hours followed by administration of oxytocin (OT, arrow, 100 U administered intramuscularly) and subsequent sampling for 6 hours. Peak concentrations of PGFM differed between treatments following administration of oxytocin (P = 0.006) Pooled SEM = 33 5 pg/mL Area under the curve during the first 4 h after oxytocin administration treatment tended to be higher for the TRT cows than CON cows (P < 0.10).

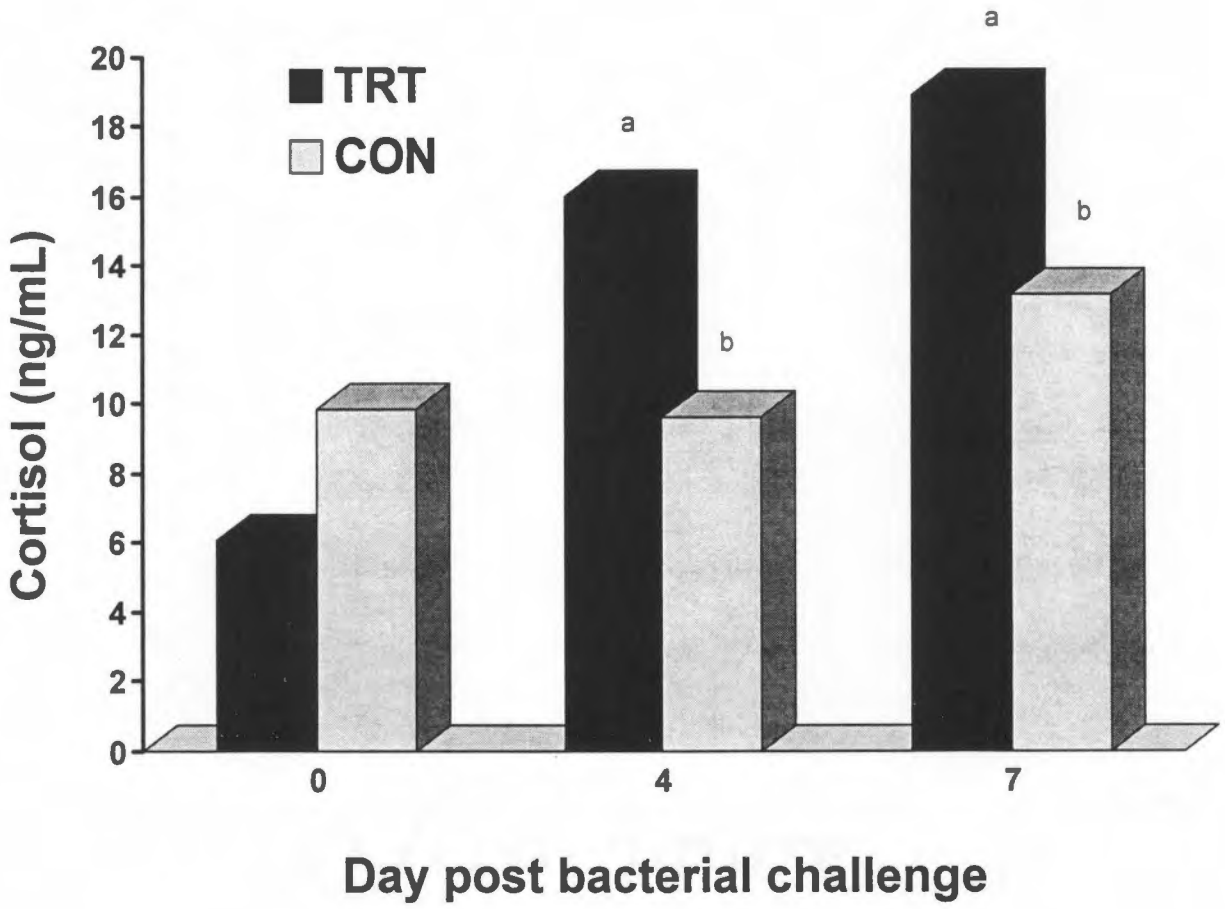


Figure 2-2. Concentrations of cortisol differed after bacterial challenge with *S. uberis* in two mammary quarters on day 0. [a,b] Bars (treatments) within day with different superscripts differed (P = 0.04). Within treatment, cows that developed clinical mastitis had elevated (P = 0.02) concentrations of cortisol on days 4 and 7 compared with day 0. Cortisol did not differ by day during the experimental period in control cows. Pooled SEM = 2.2 ng/mL.

Concentrations of E_2 and prolactin on d 0, 4 or 7 post-challenge did not differ between CON and mastitic cows (Table 2-2). Concentrations of LH were not different between cows that developed clinical mastitis and CON animals for values related to baseline, peak, or change from baseline to peak (Figure 2-3). Furthermore, area under the curve and time from GnRH until peak did not differ for concentrations of LH between CON and mastitic cows (Figure 2-3).

Discussion

All experimentally-challenged cows developed clinical mastitis within 60 h after *intramammary* infusion of *S. uberis*. Symptoms following *intramammary* bacterial challenge included decreased milk production, flakes and/or clots in milk from infected quarters, swelling of challenged quarters, increased somatic cell count and elevated rectal temperatures. Also associated with onset of clinical mastitis were elevated concentrations of PGFM following oxytocin administration, indicating increased sensitivity to release $PGF_{2\alpha}$. One possible source of $PGF_{2\alpha}$ release is from the uterus Data from our laboratory indicated that $PGF_{2\alpha}$ might have detrimental effects on embryonic development outside the effects on the corpus luteum. Seals et al. (1998) reported that $PGF_{2\alpha}$ administration to progestogen-supplemented beef cows resulted in decreased pregnancy rates. Likewise, Hockett et al. (1998) demonstrated that $PGF_{2\alpha}$ administration caused decreased embryonic development and quality in progestogen-supplemented beef cattle.

Table 2-2 Concentrations of estradiol-17β and prolactin on days 0, 4 and 7 of bacterial challenge

Hormone[a]	Day[b]	CON	TRT
Estradiol-17β[c] (pg/mL)	0	4 3 ± 1 4	4 3 ± 1 4
	4	2 5 ± 1 4	4 3 ± 1 4
	7	1 7 ± 1 4	2 4 ± 1 4
Prolactin[d] (ng/mL)	0	5 4 ± 1 5	4 4 ± 1 5
	4	6.2 ± 1 5	4 6 ± 1 5
	7	5.3 ± 1 5	3 1 ± 1 7

[a] Concentrations are reported as least square means ± standard error
[b] Day 0 = day of experimental challenge and day 4 = day of intensive sample collection following initiation of mastitic infection
[c] Concentrations of estradiol-17β did not differ (P > 0 05) between CON and TRT on day 0, 4 or 7
[d] Concentrations of prolactin did not differ (P > 0 05) between CON and TRT on day 0, 4 or 7

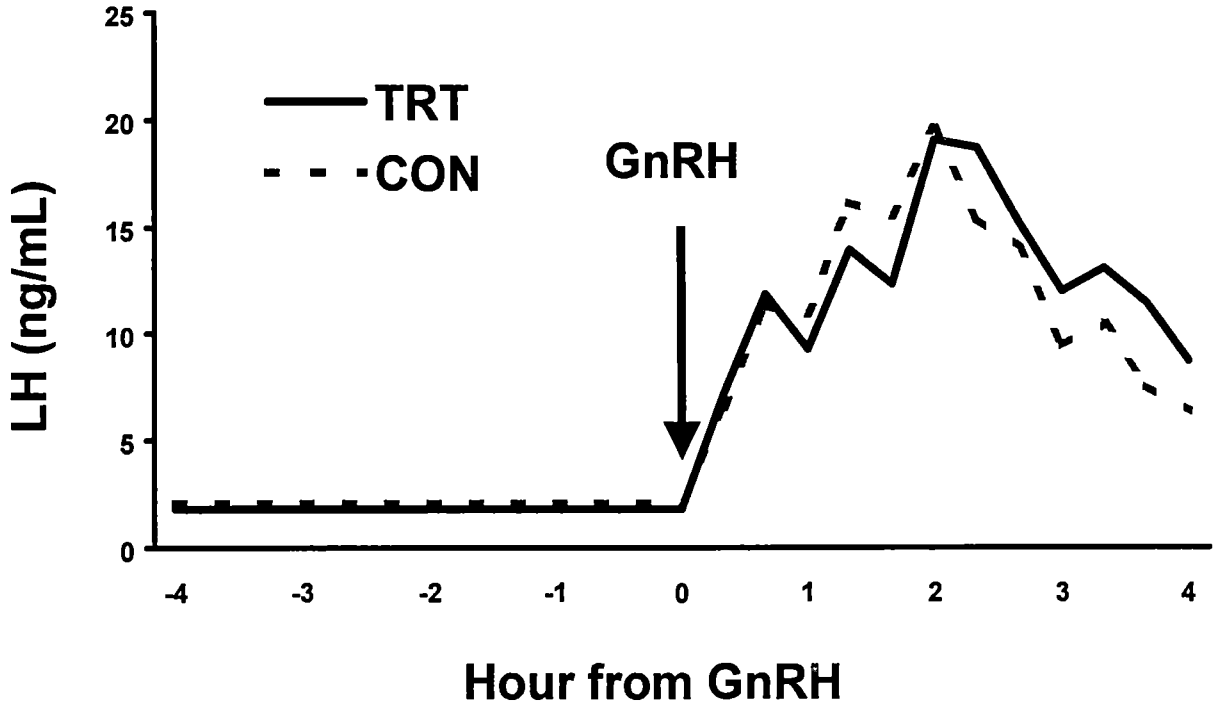


Figure 2-3 Influence of experimentally-induced clinical mastitis on concentrations of LH on day 4 after bacterial challenge Baseline concentrations were evaluated during the initial four hours followed by intramuscular administration of GnRH (arrow, 100 ug) and subsequent sample collection for four hours Pooled SEM = 2 7 ng/mL

Elevated concentrations of $PGF_{2\alpha}$ in dairy cows with clinical mastitis may cause decreased embryonic development and quality and ultimately result in increased services per conception and days open Barker et al (1998) noted that cows that developed clinical mastitis after first service, but before establishment of pregnancy, had increased services per conception and days open. This phenomenon could result from increased $PGF_{2\alpha}$ synthesis and subsequent luteal regression and (or) embryonic death Prostaglandin $F_{2\alpha}$ is synthesized in the bovine mammary gland (Manns, 1975; Hansel et al , 1976; Lopez-Diaz and Bosu, 1992) and infusion of *Escherichia coli* endotoxin into the mammary gland resulted in increased concentrations of $PGF_{2\alpha}$ above that normally seen in milk (Giri et al., 1984). Gilbert et al. (1990) reported that *intrauterine* infusion of *E coli* resulted in shortened estrous cycles as a result of luteal regression

Shuster et al (1995) reported that infusion of *E. coli*, a Gram-negative pathogen, into the mammary gland of dairy cows resulted in swelling of infected quarters. High concentrations of tumor necrosis factor and interleukin-1 (IL-1) were detected in milk of infected glands and increases in these cytokines coincided with mammary gland inflammation Luteal cells cultured with IL-1 resulted in increased $PGF_{2\alpha}$ production. Interleukin-1 stimulated phospholipase A_2 enzyme and IL-1 was hypothesized to stimulate prostaglandin H synthase (Townson and Pate, 1994). Phospholipase A_2 cleaves arachidonic acid, a precursor to prostaglandin synthesis, directly from phospholipids. Prostaglandin H synthase is one enzyme essential in conversion of arachidonic acid to $PGF_{2\alpha}$

Swelling of infected mammary glands was observed in all experimentally-infected cows in the present study Interleukins were not measured in the current experiment; however, an inflammatory response was observed in all cows, which potentially could result in IL-1 production and an increase in $PGF_{2\alpha}$ Increased synthesis of $PGF_{2\alpha}$ may result in luteal regression and subsequent loss of the embryo. Day et al (1982) observed that cortisol reduced estrogen-induced phospholipase A2 activity in rat uterine tissue This enzyme provides free arachidonic acid for the production of prostaglandins. Therefore, baseline PGFM concentrations may have been suppressed due to elevated cortisol reducing the activity of phospholipase A2.

Other studies demonstrated that increased cortisol concentrations resulted in suppressed LH in cows (Echternkamp, 1984, Peter et al., 1990; Stahringer et al., 1994) and ewes (Battaglia et al , 1997), and may result in decreased development of follicles (Peter et al , 1990) and anovulation (Lopez-Diaz and Bosu, 1992, Peter et al., 1990). In the present study, cortisol concentrations were elevated; however, LH concentrations were not different between CON and mastitic cows Cows in the current study were in the luteal phase of the estrous cycle where suppression of LH may not be as apparent. Peter et al. (1989) also reported suppression of estrogen following endotoxin infusion in heifers, which ultimately suppressed the LH surge. Intramammary infections caused by *S. uberis* may act through similar mechanisms in cows during the follicular phase of

the estrous cycle, but no suppression of estradiol-17β or LH was observed during the luteal phase in the present experiment

Conclusion

Clinical mastitis occurs frequently in dairy cows during early lactation Changes in $PGF_{2\alpha}$ release or sensitivity to oxytocin in cows with clinical mastitis as seen in this study may relate to impaired reproduction reported in other studies. Elevated concentrations of $PGF_{2\alpha}$ could lead to decreased embryonic development and quality or luteal regression Current results further substantiate the proposed mechanism of Cullor (1990) and Moore and O'Connor (1993) for interactions of mastitis from Gram-negative bacterial origin and decreased reproductive performance. Additionally, present data may explain results of Barker et al. (1998) who reported that cows with clinical mastitis after first service but before establishment of pregnancy had increased services required per conception, number of days open, and days to first service Additional experiments are in progress to determine if mastitis interrupts the hypothalamus-pituitary-ovarian axis during periods of the estrous cycle more critical to development of the ovulatory follicle, ovulation, and oocyte maturation.

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Chapter 3

Effects of Experimentally-Induced Clinical Mastitis during Early Lactation on Estrous Behavior, Luteal Function, and Establishment of Pregnancy

Abstract

Objectives of the current studies were: 1) to determine if experimentally-induced clinical mastitis **prior to ovulation** resulted in altered estrous expression or decreased pregnancy rates, and 2) to determine if clinical mastitis **during early embryonic development** altered function and/or lifespan of the corpus luteum and reduced pregnancy rates. Estrus was synchronized in Jersey and Holstein cows during early lactation by two injections of prostaglandin $F_{2\alpha}$. Cows were challenged with *Streptococcus uberis* in two mammary quarters In Objective 1, challenged cows were inoculated with *S uberis* 4 d **prior to** the second injection of prostaglandin Cows in Objective 2 were inoculated with *S uberis* 5 d **following** the second prostaglandin injection Control cows were not challenged. Cows from all groups were monitored for estrous behavior and artificially inseminated Blood samples and rectal temperatures were obtained every 2 d for 20 d beginning immediately prior to bacterial inoculation Serum was analyzed for concentrations of progesterone, blood urea nitrogen (BUN), nonesterified fatty acids (NEFA), insulin, and cortisol Pregnancy status was determined by ultrasonography 30 d following insemination Cows with mastitis infections during the preovulatory period (Objective 1) had decreased expression

of estrus (12.5 vs. 66.7%; P < 0.02) and increased days until estrus (27.1 ± 5.2 vs 7 0 ± 4.4 d, P < 0.06) compared to controls. Cortisol was higher (P = 0 01) following bacterial challenge compared to unchallenged control animals. Pregnancy rate did not differ on first service opportunity Challenged cows in Objective 2 that displayed estrus and developed clinical mastitis postovulatory (n = 15) had increased mammary scores and concentrations of cortisol following bacterial challenge, but pregnancy rates and progesterone concentrations were similar to controls (CON; n = 14) and cows that were challenged that did not develop clinical mastitis (NOMAS; n = 4). These results suggest that clinical mastitis prior to onset of estrus interfered with expression of estrus possibly via associations with cortisol. Induction of clinical mastitis during the first 5 days following estrus did not alter luteal development, function, or establishment of pregnancy.

Key words cortisol, dairy, mastitis, pregnancy, prostaglandin, uterus

Introduction

Mastitis is an inflammation of the udder that affects a high proportion of dairy cows (Judge et al., 1997; Barkema et al , 1998; Sargeant et al , 1998) Signs of clinical mastitis include alterations in milk composition and appearance, decreased milk production, elevated body temperature, and swelling, redness, or heat in infected mammary glands Mastitis has been described as the most economically-imposing disease facing dairy producers in the United States,

costing an estimated $2 billion annually (DeGraves and Fetrow, 1993). Aside from the decrease in milk production and alterations in milk composition, mastitis has recently been implicated as a causative agent for decreased reproductive performance

Recent studies from our laboratory have shown an association between reproductive failure and clinical mastitis in lactating dairy cows. Cows with clinical or subclinical mastitis prior to first service had increased number of days to first service, increased days open and elevated services required per conception Additionally, clinical mastitis after first service was associated with increased services per conception and days open (Barker et al , 1998; Schrick et al., 2001). Hockett et al. (2000) reported that experimental induction of mastitis in cows in the luteal phase of the estrous cycle resulted in increased blood cortisol during the experimental period Baseline concentrations of PGFM (stable metabolite of $PGF_{2\alpha}$) did not differ between groups, however, peak concentrations of PGFM following oxytocin challenge were elevated in cows with clinical mastitis compared with control animals

One component normally elevated in milk during Gram-negative infections is $PGF_{2\alpha}$ (Gilbert et al., 1990, Peter et al., 1990, Kindahl et al., 1996; Janosi et al , 1998). Cullor (1990) suggested that endotoxin might induce luteolysis and influence conception and early embryonic survival by release of inflammatory mediators. Moore and O'Connor (1993) hypothesized that luteolysis and subsequent loss of embryos in cows with clinical mastitis due to Gram-negative

pathogens may be a viable mechanism of action for reduced reproductive efficiency. In support of this hypothesis are numerous studies that have utilized either *Escherichia coli* or endotoxin, a component of the cell wall of Gram-negative bacteria, as a model of infection. Gilbert et al (1990) reported that intrauterine infusion of *E. coli* resulted in shortened estrous cycles in Holstein heifers, implying that prostaglandins were stimulated and luteolysis occurred Giri et al (1990) reported endotoxin exposure caused a dose-dependent increase in plasma $PGF_{2\alpha}$ with a subsequent decline in progesterone suggesting occurrence of luteal regression. Risco et al. (1999) reported on 2,087 cows that were diagnosed pregnant and used a logistical regression to determine the association of mastitis with abortion. Cows that had clinical mastitis during the initial 45 d of pregnancy were at 2 7 times higher risk to abort within the following 90 days than cows that were not diagnosed with clinical mastitis.

Schrick et al (2001) reported that cows with clinical or subclinical mastitis prior to first service experienced an increase in days to first service and services per conception. Furthermore, this study also reported that mastitis following insemination but prior to pregnancy determination also was associated with increased services required per conception and days open The physiological basis for these associations is not well understood The timing of clinical mastitis and impacts of mastitis on endocrine function of the animal as well as pregnancy should be studied to delineate mechanisms by which mastitis results in decreased reproductive performance

Objectives of the current study were 1) to determine if experimentally-induced clinical mastitis **during the preovulatory time period** altered expression of estrus and/or pregnancy rate, and 2) to determine if experimentally-induced clinical mastitis **during early embryonic development** interferes in luteal and uterine function thus compromising pregnancy

Materials and Methods

Cows (n = 81) were allocated randomly by stage of lactation (60-90 days in milk, DIM, for Objective 1; 60-140 DIM for Objective 2), lactation number (range 1-4) and milk production prior to initiation of treatment All cows tested negative for the presence of major mastitis pathogens on two occasions one week apart before allotment to experimental groups Estrus was synchronized with two intramuscular injections of 25 mg $PGF_{2\alpha}$ (Lutalyse, Pharmacia Animal Health, Kalamazoo, MI) administered at 14-d intervals. Cows were milked twice daily at 12-h intervals in a Herringbone trigon parlor. Milk production was recorded electronically at each milking

Description of Bacterial Inoculum Preparation

Streptococcus uberis strain 888 was revived from storage at -80°C by thawing in a water bath at 37°C The thawed bacterial suspension (20 μL) was streaked onto blood agar plates and incubated overnight at 37°C. After incubation, 3 colonies were picked using a sterile loop and inoculated into 20 mL of Todd-

Hewitt broth and incubated for 6 h at 37 ± 1.0°C in portable incubator in transit to experiment station Following incubation, broth culture was diluted in sterile phosphate-buffered saline to provide an inoculum of 600 - 1000 colony forming units (CFU)/mL

Description of Challenge Inoculum Administration

Within 0 5 h after the PM milking (four days prior to the second injection of $PGF_{2\alpha}$, Figure 3-1), 5 mL of inoculum containing a total of 3000 to 5000 CFU of *S. uberis* in sterile phosphate-buffered saline were infused into two uninfected quarters of each cow in the PRE (n = 15) and POST (n = 29) challenge groups. Initiation of challenge four days prior to the second injection of $PGF_{2\alpha}$ was performed for PRE with the planned result of cows displaying clinical mastitis on the day of, or day following, $PGF_{2\alpha}$ administration (approximately two days prior to estrus) This time point was chosen to allow cortisol to reach peak values prior to the preovulatory rise in E_2 and LH as well as exposing the developing oocyte to inflammatory mediators prior to ovulation. Challenge occurred five days following $PGF_{2\alpha}$ administration (approximately one-two days after estrus) for POST. Initiation of challenge soon after estrus was performed with the planned result of cows displaying clinical mastitis on days 5 or 6 of embryo development (Figure 3-2). Embryos at this time point have been reported to be susceptible to elevated levels of $PGF_{2\alpha}$ (Hockett et al., 1998; Seals et al., 1998). The number of bacteria was determined by average of viable plate count on blood agar before

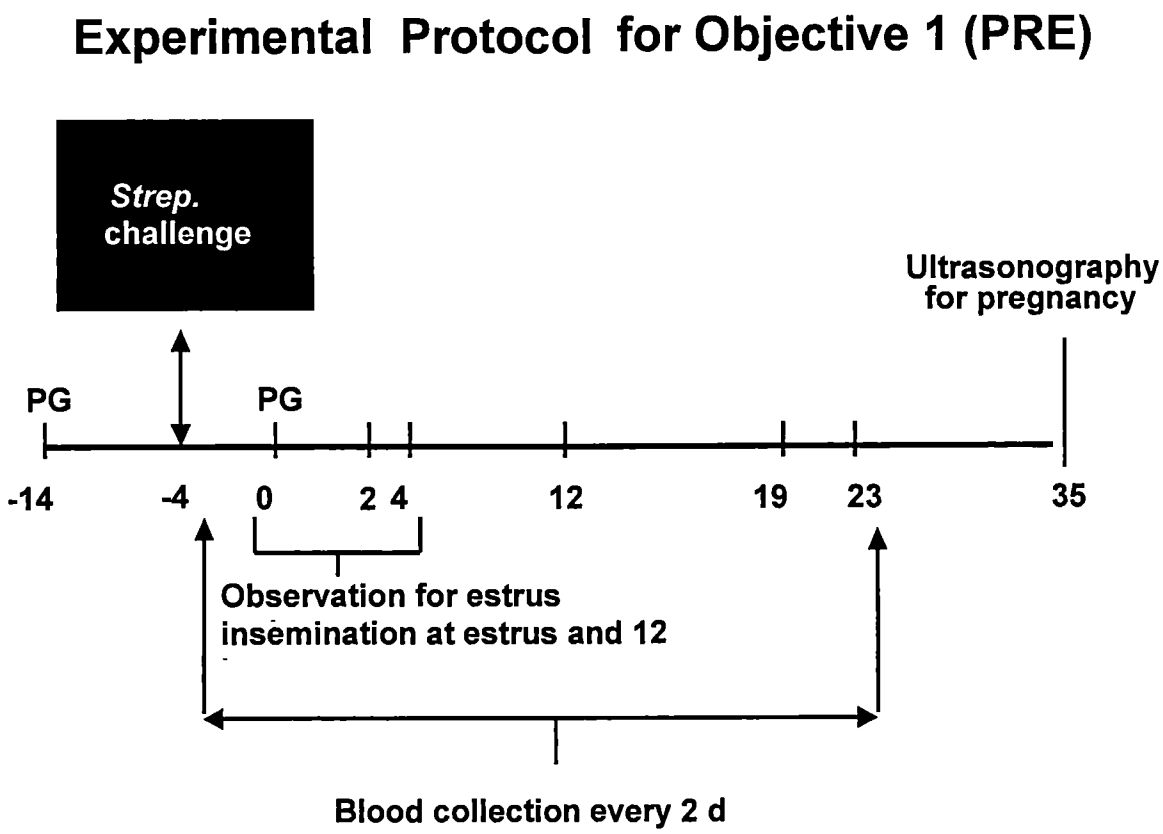


Figure 3-1. Schematic representation of experimental design for all PRE challenge animals.

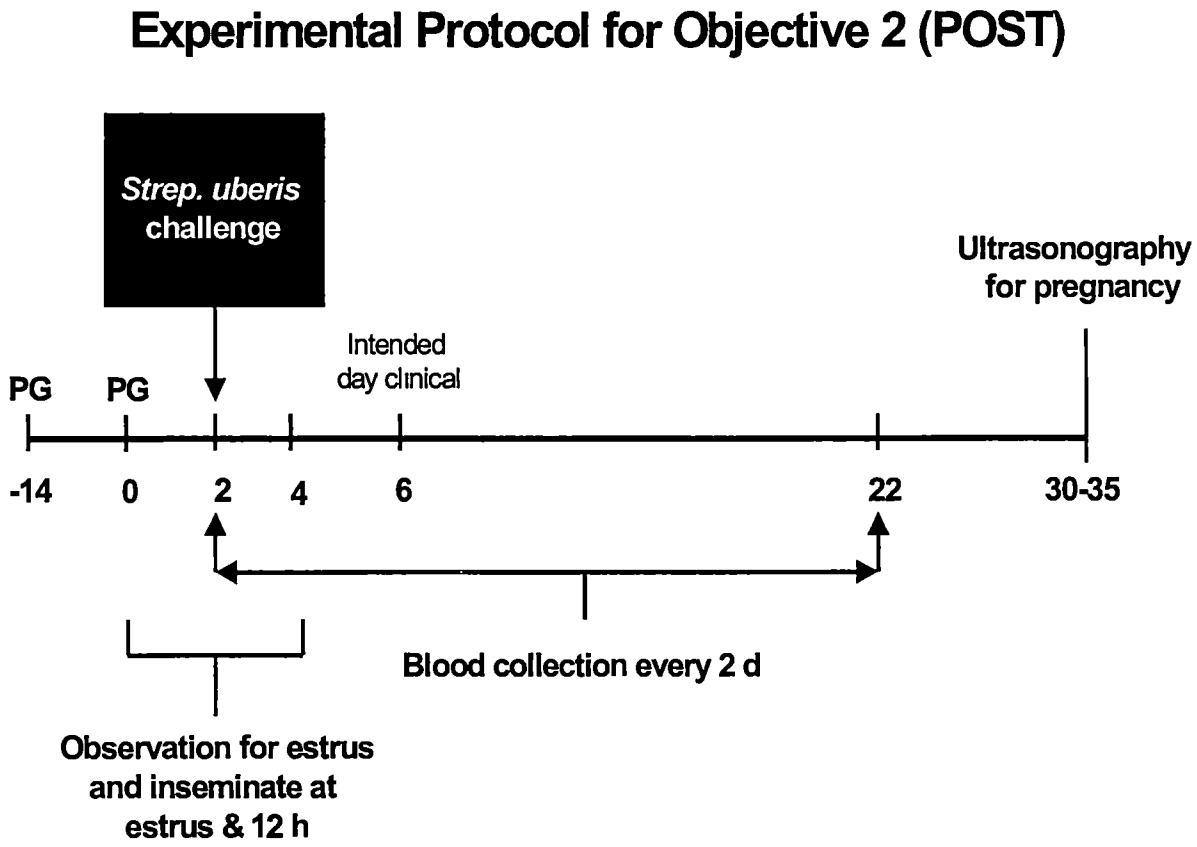


Figure 3-2. Schematic representation of experimental design for all POST challenge cows.

and after inoculation, and inoculum was maintained at room temperature up to the time of intramammary inoculation. Selection of inoculum volume and total number of CFU was based on results of several previous studies when developing the *S. uberis* challenge model in early- and mid-lactating cows (Hockett et al., 2000). The bacterial suspension was infused using sterile disposable syringes fitted with sterile disposable teat cannulas Full insertion of the teat cannula through the streak canal was employed Before inoculation, teat ends were cleaned thoroughly with swabs containing 70% ethanol The infused inoculum was massaged upward into the gland cistern The teat was immersed in a postmilking teat disinfectant when the above procedure was completed Following challenge, cows were monitored closely so that if an acute infection occurred, supportive therapy could be initiated promptly

Foremilk samples from each mammary gland were obtained aseptically for microbiological evaluation (as described below) from all challenged cows immediately before inoculation, daily for 7 d and at day 14 and 21. Cows in the untreated control (CON) group (Objective 1, n = 15, Objective 2, n = 23) were sampled at the same intervals as challenged cows

Microbiological Evaluation on Milk Samples

Samples of foremilk from quarters of cows were collected aseptically following procedures described by the National Mastitis Council (1999). Before sample collection, teats of cows were dipped in a premilking teat disinfectant,

cleaned thoroughly with individual disposable paper towels, and teat ends sanitized with swabs containing isopropyl alcohol. Milk samples were examined following procedures recommended by the National Mastitis Council (1999) as described by Oliver et al. (1994). Briefly, foremilk samples (10 μL) from each quarter were plated onto one quadrant of a trypticase soy agar plate supplemented with 5% defibrinated sheep blood (Laboratory Supply, Nashville, TN). Plates were incubated at 37°C and bacterial growth was observed and recorded at 24-h intervals for three days Bacteria on primary culture medium were identified tentatively according to colony morphologic features, hemolytic characteristics, and catalase test. Isolates identified presumptively as staphylococci were tested for coagulase by the tube coagulase method. Isolates identified presumptively as streptococci were evaluated initially for growth in 6.5% NaCl, hydrolysis of esculin and CAMP-reaction. Streptococcal organisms were identified to the species level using the API 20 Strep System (bioMerieux Vitek, Inc , Hazelwood, MO) upon first and last isolation of the organism from experimentally infected quarters Streptococcal organisms isolated between the first and last isolation were identified by sodium hippurate hydrolysis for *S uberis.* All other *Streptococcus* and *Enterococcus* species were identified using the API 20 Strep System (bioMerieux Vitek, Inc., Hazelwood, MO) for subsequent isolates. Gram-negative isolates were plated on MacConkey's agar

(Becton Dickinson Microbiology Systems, Sparks, MD) and evaluated by the following biochemical tests. triple sugar iron, urea, oxidase, motility, indole, and ornithine decarboxylase

Clinical Observations

Clinical assessment of all animals was performed before experimental bacterial challenge and when milk samples were collected. Clinical status of all mammary glands and appearance of milk were evaluated by qualified farm personnel at each milking when milk samples were collected using the following scheme 1 = normal mammary gland and normal milk, 2 = normal mammary gland and slight alterations in milk (a few flakes), 3 = abnormal mammary gland and abnormal milk (clots, clumps, changes in milk color), and 4 = swollen mammary gland, abnormal milk and systemic signs (hyperthermia, depression, dullness) of infection. Cows that developed clinical mastitis were monitored closely and antibiotic treatment was initiated after day 7 post-challenge if required. Data evaluated by Schrick et al. (2001) in comparing clinical and subclinical mastitis cows indicated similar detrimental effects of mastitis on reproductive performance It should be noted that cows with clinical mastitis in the study by Schrick et al. (2001) received antibiotic therapy following onset of mastitis whereas subclinical cows were not treated, however, similar associations of mastitis with reproduction were observed regardless of antibiotic therapy.

Clinical mastitis was diagnosed for experimentally-challenged quarters when *S. uberis* was isolated from the challenged quarter at any milking post-

challenge and the challenged quarter had a mastitis score of 2 or 3 for two consecutive milkings during the 7 d post-challenge period. Acute clinical mastitis was scored for experimentally challenged quarters when *S. uberis* was isolated from the challenged quarter at any milking post-challenge, milk from the challenged quarter had a mastitis score of 4 for two consecutive milkings during the 7 d post-challenge period, and the cow was hyperthermic Cows were diagnosed with subclinical mastitis if *S. uberis* was isolated from milk but clinical signs of mastitis were not observed Rectal temperatures were obtained for all cows daily throughout the study at milking time.

Determination of Estrus, Insemination and Pregnancy Detection

All cows were evaluated three times daily to determine onset of estrus. All cows were artificially inseminated at onset of estrus and 12 h later utilizing semen from the same bull at each insemination to maximize fertility Semen was allotted randomly across treatments from 3 to 4 bulls. Pregnancy was determined by ultrasonography (Aloka 500, 7.5 MHz linear transducer, Schrick et al , 1993; Seals et al., 1998) at days 30 to 35 postmating.

Blood Collection

Blood samples (10 mL) were collected via jugular venipuncture every other day beginning on day of challenge to evaluate P_4, cortisol, blood urea nitrogen (BUN), nonesterified fatty acids (NEFA) and insulin concentrations from

all experimentally-infected and control cows Blood samples were centrifuged (2500 x g) and serum harvested and stored at -20°C until assayed.

Hormone and Metabolite Assays

Concentrations of cortisol (Hockett et al , 2000), P_4 (Seals et al , 1998) and insulin (Fazio et al., 2000) were determined on serum utilizing solid phase radioimmunoassays (Diagnostic Products Corp., Los Angeles, CA) Sensitivity of the cortisol assay was 2 ng/mL and inter- and intra-assay CV of 0 2% and 10%, respectively Sensitivity of the insulin assay was 0 05 ng/mL with intraassay CV of 4% and interassay CV of 17% Sensitivity of the progesterone assay was 0 02 ng/mL with intraassay CV of 8% and interassay CV of 4%. Serum concentrations of BUN and NEFA were determined utilizing spectrophotometry as validated in our laboratory (Fazio et al., 2000). Blood urea nitrogen concentrations were determined utilizing a commercial kit with sensitivity of 15 mg/dL, intraassay CV of 6% and interassay CV of 12% (Sigma Diagnostics, St Louis, MO) Concentrations of NEFA were determined using a NEFA-C kit with a sensitivity of 0.125 mEq/L and intra- and inter-assay CV of 5% and 27%, respectively (Wako Pure Chemical Industries, Osaka, Japan) All of these assays have been validated and are currently utilized in our laboratory.

Statistical Analyses

Patterns of P_4, BUN, NEFA, insulin, cortisol, and days to insemination were analyzed by least squares analysis of variance for a randomized block with repeated measures. Differences in pregnancy rates, conception rates and estrous expression were determined by Chi-Square analysis The Mixed Procedure (SAS, 1996; 1997) was used for all analyses For Objective 1, 14 cows were inoculated with bacteria. Five cows that were inoculated did not develop clinical mastitis. Two of those five had abnormal progesterone profiles indicative of anestrus One of the five cows displayed estrus prior to the second injection of prostaglandins and was at a different stage of the estrous cycle than remaining cows. Thus, 2 cows were left that were challenged and did not display estrus Data from these cows were not analyzed due to the small number of animals and lack of confidence that would be associated with any statistical data generated. For Objective 2, 4 cows that were challenged and displayed estrus normally did not develop clinical mastitis. Data from these cows were analyzed to determine if differences existed between those animals that were susceptible to the bacterial challenge and those that did not develop clinical mastitis following the challenge.

Results

Objective 1

Bacterial challenge of 14 cows with *S. uberis* resulted in clinical mastitis in

10 PRE cows. Initially, 15 CON cows were synchronized for purpose of the study. Three CON cows were eliminated due to anestrus and one CON cows developed *S. uberis* mastitis concurrent with challenged cow and was subsequently moved to the PRE group for further data analysis For data analysis, this yielded 10 PRE cows and 11 CON cows Intramammary challenge resulted in elevated milk somatic cells (5 7 vs. 4 5 ± 0 2 $\log_{10}$ cells/mL, $P < 0.0001$) in PRE vs CON, respectively Furthermore, somatic cells increased over time following bacterial challenge in PRE cows, but not in CON cows Table 3-1, ($P < 0.0001$) Mean rectal temperatures were also elevated in challenged cows compared to CON (39.2 ± 0.4 vs. 38.0 ± 0.4°C, respectively; Table 3-1; P = 0 04).

Estrous expression and pregnancy

Expression of estrus within five days of $PGF_{2\alpha}$ administration was decreased in clinically-infected cows (12.5%, 1/8) compared to CON (66 7%, 8/12; $P < 0.02$). Days from $PGF_{2\alpha}$ administration to first observed estrus and artificial insemination tended to be increased (27 1 ± 5 2 d) compared to CON (7.0 ± 4 4 d, $P < 0.06$). Conception rates at insemination at the first observed estrus following challenge were similar between PRE (57 1%, 4/7) and CON (27.3%, 3/11, P = 0 21). One cow from each group was not inseminated leaving a total of 7 PRE and 11 CON

Table 3-1. Clinical assessments of mastitis in experimentally challenged and control animals for Objective 1

	DAY							
Parameter	**CH**	**CH1**	**CH2**	**CH3**	**CH4**	**CH5**	**CH6**	**CH7**
Mammary scores[1]								
CON	1.0[a]	1.0[a]	1.0[a]	1.0[a]	1.0[a]	1.0[a]	1.0[a]	1.0[a]
PRE	1.0[a]	1 6[b,c]	1 2[a,b]	1.9[c]	2 7[d]	3.5[e]	3.9[e,f]	4 3[f]
SCC[2]								
CON	4.6[a]	4 6[a]	4 8[a,b]	4 7[a,b]	4 7[a,b]	4.7[a,b]	4.7[a,b]	4 7[a,b]
PRE	4.5[a]	5.4[b]	6.0[c]	6.1[c]	6.3[c]	6 4[c]	6 3[c]	6 2[c]
Rectal Temp[3]								
CON	39.0	39 1	39.0	39.1	39.0	39 0	38.9	NA
PRE	39.0	39.3	39 0	39.6	39 7	39.1	38 8	NA

[1] Mastitis scores with different superscripts differed [a,b,c,d,e,f](Pooled SEM = 0.25; P< 0.0001)
[2] Mean somatic cell count of challenged quarters or control quarters (log_{10} cells/ml; Pooled SEM = 0 27); [a,b,c] Somatic cell counts differed (P < 0.0001)
[3] Rectal temperature (°C, Pooled SEM = 0.94); Rectal temperatures did not differ on different days between treatments (P = 0 21)
NA = data not available

Hormonal and metabolic parameters

Concentrations of progesterone during the estrous cycle across treatments were similar for PRE and CON cows (2.8 ± 1.6 vs 2 1 ± 1.5 ng/mL; respectively; P = 0.37) Progesterone concentrations did not differ for treatment across day of experiment. Mean concentrations of cortisol were not different between PRE (13 1 ± 4.1 ng/mL) and CON (11 2 ± 4.1 ng/mL; P = 0 12) However, cortisol concentrations did differ between treatments across day of the experimental period (Figure 3-3; P = 0.01)

Mean serum concentrations of BUN were higher for CON (28.9 ± 3 3 mg/dL) than for TRT (26.1 ± 3.3 mg/dL; P = 0.007) There were no significant interactions of treatment on days of experiment Nonesterified fatty acid levels were similar between PRE and CON (0.31 ± 0.01 vs. 0.28 ± 0.01 mEq/L, respectively; P = 0.20). Mean concentrations of insulin were elevated in PRE (0.37 ± 0 04 vs. 0.25 ± 0.04 ng/mL, P = 0 0036).

Objective 2

Initially, cows (n = 52) were administered prostaglandin resulting 33 cows displaying estrus and being inseminated (CON = 14/23, TRT = 19/29). As in Objective 1, only cows that remained free from clinical mastitis infection throughout the experimental period were utilized as CON animals (n = 14) Following bacterial challenge, 15 cows developed clinical mastitis (POST) while 4 cows did not develop clinical or subclinical mastitis (NOMAS) following challenge

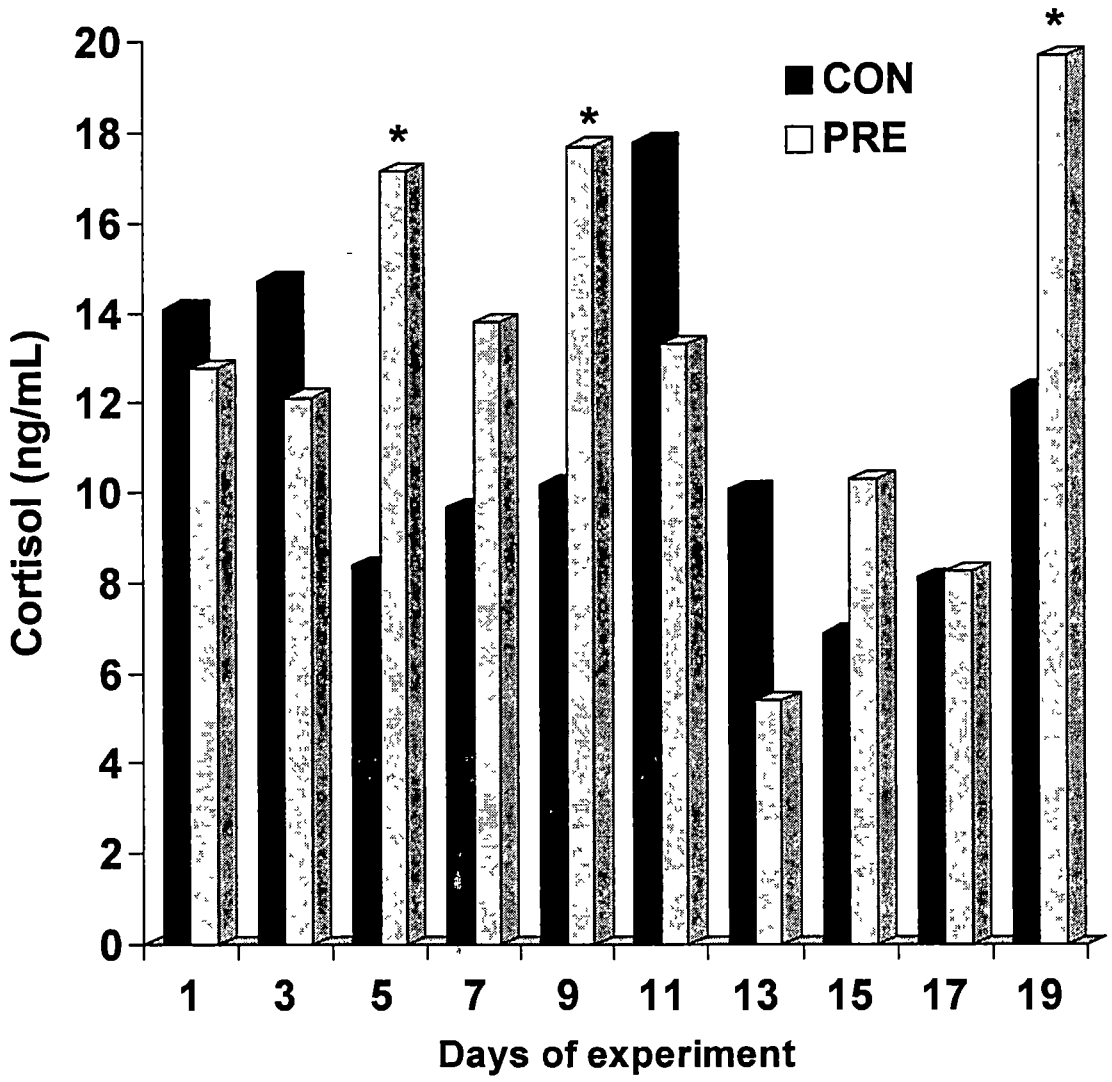


Figure 3-3 Concentrations of cortisol differed between CON and PRE on days 5, 9 and 19 of the sampling period (P = 0 01, Pooled SEM = 4 9 ng/mL). Day 1 is the day before bacterial challenge. Estrus was synchronized by administration of $PGF_{2\alpha}$ on day 6 Elevated cortisol on d 5 and 9 corresponds with timing of clinical mastitis

with *S. uberis* Data were analyzed for each of these 3 groups (CON, POST, and NOMAS)

Cows in the POST group had elevated mean concentrations of somatic cells in milk on days following the challenge compared to CON and NOMAS cows (Table 3-2, P = 0 0001). Mammary scores were increased in POST cows compared to NOMAS and CON cows (Table 3-2; P < 0 0001). Mean rectal temperatures did not differ (Table 3-2) for CON (38 8 ± 0 2), NOMAS (39 0 ± 0 2), or POST cows (38 9 ± 0.2; P = 0 14)

Estrous cycles and pregnancy

Normal estrous cycles occurred in 93% (13/14) of CON, 100% of NOMAS (4/4), and 87% of POST cows (13/15; P = 0.62). Pregnancy rates did not differ between CON (3/14), NOMAS (0/4), and POST (2/15) cows (21, 0, and 13%, respectively; Fisher's Exact Test, P = 0.16)

Hormones and metabolic parameters

Progesterone during the postovulatory time period is important for the maintenance of pregnancy. Concentrations of progesterone did not differ between CON (2 5 ± 0 4 ng/mL), NOMAS (3 7 ± 0 6 ng/mL) and POST (3 2 ± 0 4 ng/mL, P = 0.19). There was no significant day*treatment interaction, however, there was a day effect for progesterone as expected (Figure 3-4; P < 0 0001)

Table 3-2 Clinical assessments of mastitis in experimentally challenged and control animals for Objective 2

	DAY							
Parameter	**CH**	**CH1**	**CH2**	**CH3**	**CH4**	**CH5**	**CH6**	**CH7**
Mammary scores[1]								
CON	1.0[a]	1 0[a]	1 0[a]	1.0[a]	1 0[a]	1 0[a]	1 0[a]	1 0[a]
NOMAS	1 0[a]	1 0[a]	1 0[a]	1 0[a]	1 0[a]	1 0[a]	1 0[a]	1 0[a]
POST	1 0[a]	1 1[a]	1 2[a]	1 6[b]	2 4[c]	2 7[c]	2 6[c]	2 9[c]
SCC[2]								
CON	4 5[a]	4 4[a]	4.4[a]	4.4[a]	4 4[a]	4 5[a]	4 5[a]	4.4[a]
NOMAS	4 5[a,b]	4 8[a,b]	4.9[a,b]	4 8[a,b]	4 8[a,b]	4 8[a,b]	4 8[a,b]	4 7[a,b]
POST	4 5[a]	5 2[b]	5 8[c]	5 8[c]	5 8[c]	5 8[c]	5 9[c]	6 0[c]
Rectal Temp[3]								
CON	38 7	38 9	38 8	38 8	38 8	38 7	38 7	NA
NOMAS	38 8	38 9	39 0	39 0	39 2	39 0	38 9	NA
POST	38 8	38 9	38 8	39 2	39 1	39 0	38 7	NA

[1] Mastitis scores with different superscripts differed [a,b,c](Pooled SEM = 0 29, P < 0 0001)
[2] Mean somatic cell count of challenged quarters or control quarters (log_{10} cells/ml, Pooled SEM = 0 24), [a,b,c] Somatic cell counts differed (P < 0 0001)
[3] Rectal temperature (°C), Rectal temperatures did not differ on different days between treatments (P = 0 55)
NA = data not available

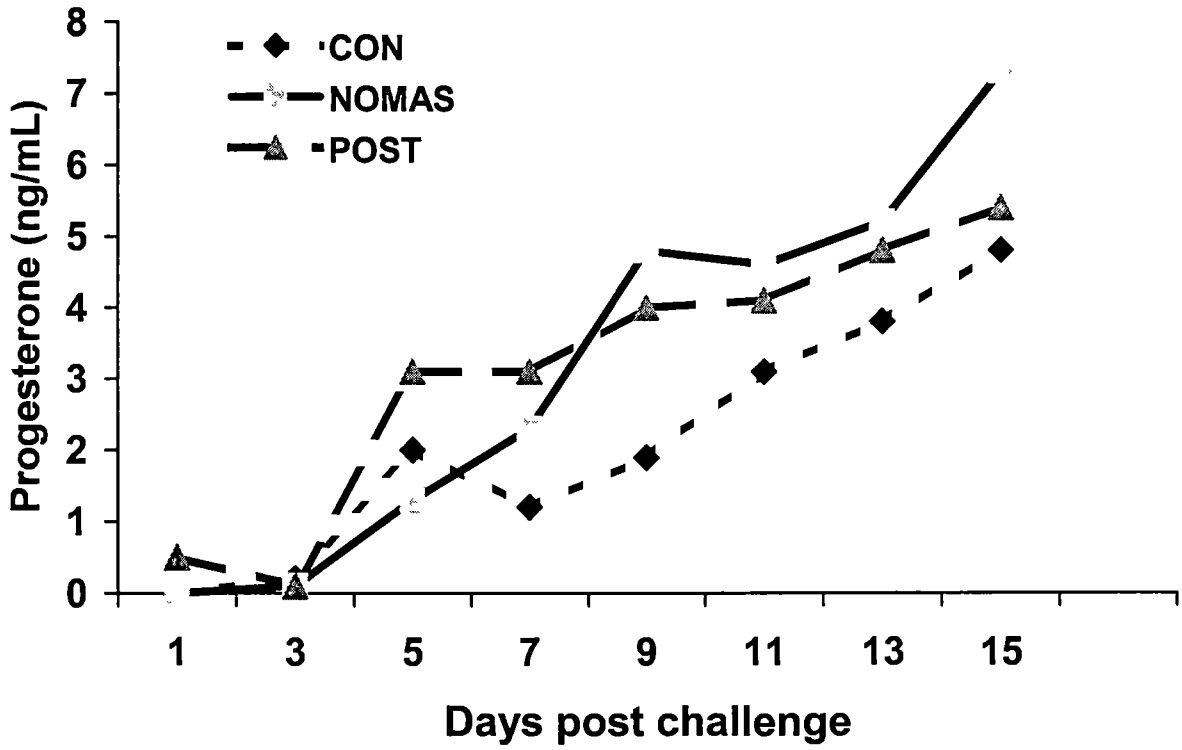


Figure 3-4. Concentrations of progesterone increased (P < 0 0001) over day of the experimental project for cows in both CON and POST groups Day = 1 time of bacterial challenge with day = 0 approximate time of synchronized estrus Concentrations of progesterone did not differ between groups throughout the experimental period (P = 0 21; Pooled SEM = 0 8 ng/mL)

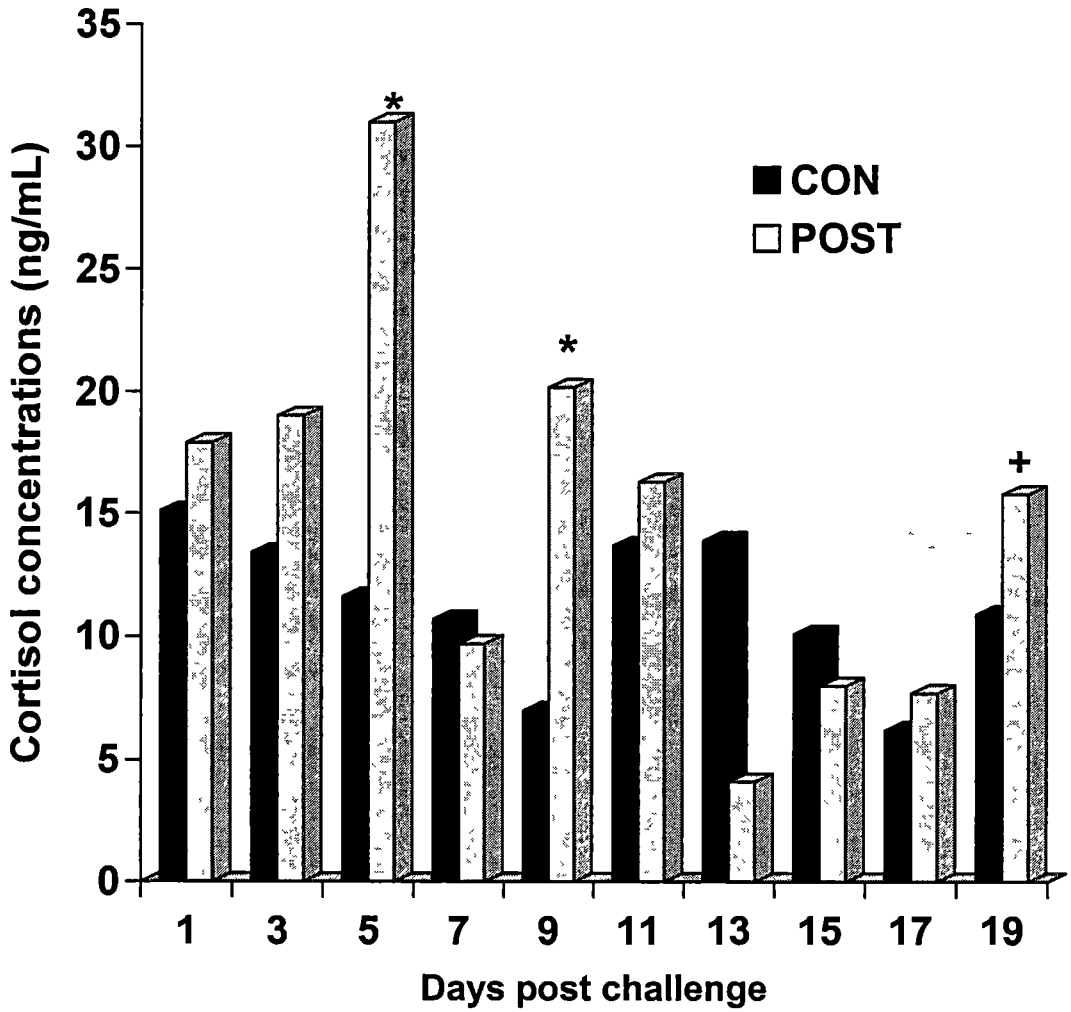


Figure 3-5. Concentrations of cortisol were higher on day 5 and day 9 for POST than for CON cows (*P = 0.0012). Concentrations of cortisol are higher for CON than for POST on day 13 ($^{+}$P = 0 0012). Mean cortisol concentrations did not differ (P = 0.15; Pooled SEM = 7.3 ng/mL)

Overall concentrations of cortisol were similar for POST (15.0 ± 5 5 ng/mL) compared with CON (11.3 ± 5.4 ng/mL; P = 0.15). However, concentrations of cortisol differed for the interaction of treatment across day of experimental period (Figure 3-5; P = 0.001).

Metabolic parameters were measured to evaluate the nutritional status of the animals throughout the experimental time period Concentrations of BUN did not differ between CON (24 0 ± 3 4 mg/dL), NOMAS (23.3 ± 3.6 mg/dL), and POST (21.5 ± 3 4 mg/dL; P = 0.15) There were no significant interactions between treatment and day for BUN concentrations Concentrations of NEFA did not differ between CON (0.23 ± 0.04 mEq/L), NOMAS (0 27 ± 0.04 mEq/L) and POST (0.25 ± 0.04 mEq/L; P = 0.25). No significant interactions existed between treatment and day for NEFA concentrations. Mean concentrations of insulin were higher for POST (0 42 ± 0 05 ng/mL) compared to CON (0 23 ± 0 05 ng/mL) and NOMAS (0.23 ± 0.09 ng/mL, P = 0 04). No significant interactions occurred between treatment and day of experiment for insulin

Discussion

The *S. uberis* challenge protocol was successful in causing clinical mastitis in dairy cows during early lactation A total of 43 cows were initially challenged and 25 cows developed clinical mastitis (58 % infection rate) and met criteria for their respective studies. Experimentally- infected cows displayed signs of clinical mastitis including elevated body temperature and increased somatic

cell counts. Cows that developed clinical mastitis had elevated concentrations of cortisol following bacterial challenge as reported previously (Hockett et al , 2000)

Cows with clinical mastitis during the preovulatory time period (Objective 1) had decreased expression of estrus. Fewer cows with clinical mastitis during the preovulatory time period displayed estrus during the five-day response period following $PGF_{2\alpha}$ administration. Cows with clinical mastitis prior to estrus had increased days from $PGF_{2\alpha}$ to insemination than did CON, in support of findings by Barker et al (1998) and Schrick et al (2001). Each of these studies reported associations of clinical mastitis prior to insemination with an increased number of days to first service. However, there were no differences in pregnancy rates of delayed services in cows with mastitis These data suggest that clinical mastitis during the preovulatory time period may negatively impact reproduction by increasing days to service; and through possible development of persistent or cystic follicles

Such developments may be due to alterations in gonadatropin function and result in altered fertility of oocytes within the ovulatory follicle. Cortisol release has been implicated in suppression of estrous expression in cows, heifers, and sheep following endotoxin challenge (Peter et al , 1990; Battaglia et al , 1998) Delays in ovulation and estrous expression may be attributed to suppressive effects of cortisol on gonadotropin secretion The follicle requires LH pulses for growth to ovulation. Any alterations in LH pulsatility or pulse size may inhibit follicle growth, estradiol-17β production, and subsequent expression of

estrous activity.

Clinical mastitis during the period of early embryonic development (Objective 2) had no obvious effects upon pregnancy rates or CL function and lifespan Cortisol was elevated on several days following development of clinical mastitis as in Objective 1. Cullor (1990) and Moore and O'Connor (1993) hypothesized that $PGF_{2\alpha}$ was released as a result of Gram-negative mastitis and might result in premature luteal regression and short cycles No differences in percent of cows with normal cycle lengths were detected between treatments in the current study, indicating that short cycles caused by $PGF_{2\alpha}$ release may not be of concern during early stages of the estrous cycle when the corpus luteum is relatively unresponsive to luteal regression by $PGF_{2\alpha}$ Baseline data of Hockett et al. (2000) showed no difference in PGFM during the luteal phase, and an elevation in PGFM was only observed following an oxytocin challenge. Additionally, $PGF_{2\alpha}$ administration during early embryonic development in progestogen-supplemented cows resulted in lowered pregnancy rates as well as decreased embryo quality and development (Hockett et al., 1998; Seals et al., 2000). Pregnancy rates in the current experiment were low and many more animals would be required to adequately test if clinical mastitis impacts pregnancy during this time period.

Conclusion

Clinical mastitis has previously been associated with increased days to

first service, days open, and services required per conception. Direct physiological effects of clinical mastitis have included increased release of cortisol and increased sensitivity for $PGF_{2\alpha}$ release following administration of oxytocin. The current experiment demonstrates that clinical mastitis has direct effects on the animal's ability to display estrus, resulting in increased number of days until insemination. This increased time period would translate into increased days open and monetary losses to the producer Further research should be aimed at determining the mechanisms by which clinical mastitis may decrease the expression of estrus Additionally, these data indicate that clinical mastitis during early embryonic development had no effect on the embryo or normal luteal function. Research should be aimed at other periods of embryonic and luteal development to determine if mastitis impacts reproductive function later in embryonic development

Chapter 4

Effects of Experimentally-Induced Clinical Mastitis during the Preovulatory Period on Endocrine Function, Follicular Growth and Ovulation

Abstract

The objective of the study was to determine if experimentally-induced clinical mastitis prior to ovulation resulted in alterations of endocrine function, follicular growth, and/or ovulation of the preovulatory follicle. Estrus was synchronized in primiparous and multiparous Jersey cows during early lactation (days 60 to 90 in milk). On approximately day 8 of the synchronized estrous cycle, cows were challenged with *Streptococcus uberis* in two quarters. Four days following bacterial challenge, prostaglandin $F_{2\alpha}$ was administered to initiate luteal regression. Forty-eight hours after prostaglandin administration, blood samples were collected every 15 min for eight hours to determine luteinizing hormone (LH) pulse frequency Following an intensive sampling period, blood sample collection continued every two hours until ovulation to determine occurrence of the LH surge. Ovaries of cows were scanned by ultrasonography every 6 h to monitor follicular growth and ovulation Sera were analyzed for cortisol, estadiol-17β, blood urea nitrogen, nonesterified fatty acids (NEFA), and insulin. Cows with experimentally-induced clinical mastitis (n = 12) had elevated rectal temperatures, somatic cell counts, and mammary scores compared to control animals (n = 12; $P < 0.05$). Estrus and ovulation occurred in 4 of 12 clinically-infected cows and all control cows. All cows that were challenged and

did not develop mastitis (n = 5) displayed estrus and ovulated Due to differences in expression of estrus, cows were further subdivided for all subsequent analyses into four groups: control, TRT-EST (infected cows that displayed estrus, n = 4), TRT-NOEST (infected cows that did not display estrus; n = 8), and NOMAS (cows that were inoculated with bacteria but did not develop mastitis; n = 4) Ovulation rate was higher for CON (100%), NOMAS (100%) and TRT-EST (100%) than for TRT-NOEST (0%) cows ($P < 0.0001$). Control and TRT-EST cows displayed similar time intervals from prostaglandin administration to estrus and ovulation. Size of the ovulatory follicle ("presumed" ovulatory follicle in TRT-NOEST cows) was similar at ovulation for all groups. Maximum concentrations of LH were higher for CON, TRT-NOEST, and NOMAS compared to TRT-EST ($P = 0.0017$). Frequency of LH pulses was decreased in TRT-NOEST compared to CON, TRT-EST and NOMAS ($P = 0.0011$) Additionally, mean concentrations of estradiol-17β decreased in TRT-NOEST (8 1 ± 2 5 pg/mL) compared to CON (12.7 ± 2.4 pg/mL) with TRT-EST cows (11.9 ± 2.7 pg/mL) and NOMAS (11.7 ± 2 6 pg/mL) intermediate ($P = 0.07$) Concentrations of estradiol-17β increased over time in CON, NOMAS, and TRT-NOEST cows, but did not increase in TRT-NOEST cows ($P < 0.0001$) Cows with clinical mastitis may exhibit estrus and ovulate normally or have disruptions in normal physiology including decreased LH pulsatility, absence of an LH surge and estrous behavior, suppressed estradiol-17β, and failure to ovulate. Reduced reproductive performance associated with the occurrence of clinical mastitis before estrus appears to result

by inhibition of estrous expression and ovulation through alterations in the hypothalamo-pituitary-ovarian axis

Key Words: cortisol, estradiol-17β, estrus, luteinizing hormone, mastitis, ovulation

Introduction

Mastitis has been described as the most economically-imposing disease facing dairy producers in the United States, costing an estimated $2 billion annually (DeGraves and Fetrow, 1993). Aside from the decrease in milk production and alterations in milk composition, studies from our laboratory have indicated an association between reproductive failure and clinical mastitis in lactating dairy cows. Days to first service, days open, and services per conception were significantly increased for cows with clinical mastitis than for cows without mammary infection or with infection after establishment of pregnancy (Barker et al., 1998, Schrick et al., 2001).

Elevated concentrations of cortisol and PGFM have been reported in cows with clinical mastitis during the luteal phase of the estrous cycle as well as altered interestrous intervals (Moore et al., 1991; Hockett et al , 2000) Cullor (1990) suggested that endotoxin might induce luteolysis and influence conception and early embryonic survival by release of inflammatory mediators. Moore and O'Connor (1993) hypothesized that Gram-negative mastitis pathogens may stimulate production of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and cause luteal regression.

Barker et al. (1998) and Schrick et al. (2001) reported that Gram-negative and Gram-positive mastitis pathogens were associated with similar decreases in reproductive efficiency of lactating dairy cows Furthermore, occurrence of subclinical mastitis had similar detrimental effects on reproductive performance as clinical mastitis (Schrick et al , 2001).

More recently, our laboratory has determined that cows with clinical mastitis during the preovulatory period had decreased expression of estrus, periods of anestrus, and decreased pregnancy rates (Hockett et al., 2002). Mechanisms by which mastitis interferes with expression of estrus are not well understood Identification of such mechanisms may lead to the development of treatment protocols to minimize additional costs of mastitis associated with decreased reproductive performance Therefore, the objective of the present study was to determine if experimental induction of clinical mastitis prior to ovulation interrupted the hypothalamo-pituitary-ovarian axis altering endocrine function, follicular growth, and ovulation of the preovulatory follicle.

Materials and Methods

General

Cows were allocated randomly by stage of lactation, lactation number and milk production prior to initiation of each replicate of experiment (n = 2 replicates) to serve as control (CON; n = 14) or mastitic-challenged (TRT, n = 19) cows Increased numbers of cows were needed for the experimentally-challenged

group to have comparable number of clinically-infected TRT cows as controls. All cows tested negative for presence of major mastitis pathogens on two occasions one week apart before allotment to treatment groups Estrus was synchronized with two intramuscular injections of 25 mg $PGF_{2\alpha}$ (Lutalyse, Pharmacia Animal Health, Kalamazoo, MI) administered at 14-d intervals Prior to initiation of treatment, cows were trained to Calan gates and were allowed a two-week adjustment period. Cows were fed a corn silage based ration and were fitted with neck transponders for individual grain feeding. Daily feed intake was monitored and recorded for individual cows.

Description of Bacterial Inoculum Preparation

Streptococcus uberis strain 888 was revived from storage at -80°C by thawing in a water bath at 37°C. The thawed bacterial suspension (20 μL) was streaked onto blood agar plates and incubated overnight at 37°C After incubation, 3 colonies were selected using a sterile loop and inoculated into 20 mL of Todd-Hewitt broth and incubated for 6 h at 37 ± 1.0°C Following incubation, broth culture was diluted in sterile phosphate-buffered saline to provide an inoculum of 600 - 1000 colony forming units (CFU)/mL

Description of Challenge Inoculum Administration

Within 0 5 h after the PM milking (four days prior to the second injection of $PGF_{2\alpha}$, Figure 4-1), 5 mL of inoculum containing 3000 to 5000 CFU of *S. uberis*

in sterile phosphate-buffered saline were infused into two uninfected mammary glands of each cow in the TRT group (n = 7 and 12 in replicate 1 and 2, respectively). Initiation of bacterial challenge four days prior to the second injection of $PGF_{2\alpha}$ was performed with the planned result of cows displaying clinical mastitis on the day of, or day following, $PGF_{2\alpha}$ administration (approximately two days prior to estrus). This time point was chosen to allow cortisol to reach peak values prior to the preovulatory rise in E_2 and LH as well as exposing the developing oocyte to inflammatory mediators prior to ovulation.

Number of bacteria was determined by viable plate count on blood agar before and after inoculation, and inoculum was maintained at room temperature up to the time of inoculation. Selection of inoculum volume and total number of CFU was based on results of several previous studies when developing the *S. uberis* challenge model in early- and mid-lactating cows (Hockett et al , 2000). The bacterial suspension was infused using sterile disposable syringes fitted with sterile disposable teat cannulas Full insertion of the teat cannula through the streak canal was employed. Before inoculation, teat ends were dipped in a teat disinfectant, teats were dried with disposable paper towels, and teat ends were cleaned thoroughly with swabs containing 70% ethanol The infused inoculum was massaged upward into the gland cistern The teat was immersed in postmilking teat disinfectant following infusion. After challenge, cows were monitored closely and supportive therapy initiated promptly if an acute infection occurred

Quarter foremilk samples for microbiological evaluation were obtained aseptically, as described below, from all mammary glands of challenged cows immediately before inoculation, daily for 7 d and at day 14 and 21. Cows in the untreated control (CON) group were sampled at the same intervals as challenged cows

Microbiological Evaluation on Milk Samples

Samples of foremilk from quarters of cows were collected aseptically as described by the National Mastitis Council (1999). Before sample collection, teats of cows were cleaned immersed in a premilking disinfectant, cleaned with individual disposable paper towels, and sanitized with swabs containing isopropyl alcohol. Milk samples were examined following procedures recommended by the National Mastitis Council and as described by Oliver et al (1994) Briefly, foremilk samples (10 μL) from each quarter were plated onto one quadrant of a trypticase soy agar plate supplemented with 5% defibrinated sheep blood (Laboratory Supply, Nashville, TN). Plates were incubated at 37°C and bacterial growth was observed and recorded at 24-h intervals for three days. Bacteria on primary culture medium were identified tentatively according to colony morphologic features, hemolytic characteristics, and catalase test. Isolates identified presumptively as staphylococci were tested for coagulase by the tube coagulase method. Isolates identified presumptively as streptococci were evaluated initially for growth in 6.5% NaCl, hydrolysis of esculin and CAMP-

reaction. Streptococcal organisms were identified to the species level using the API 20 Strep System (bioMerieux Vitek, Inc., Hazelwood, MO) upon first and last isolation of the organism from experimentally infected mammary glands Streptococcal organisms isolated between the first and last isolation were identified by sodium hippurate hydrolysis for *S. uberis* All other *Streptococcus* and *Enterococcus* species were identified using the API 20 Strep System (bioMerieux Vitek, Inc., Hazelwood, MO) for subsequent isolates. Gram-negative isolates were plated on MacConkey's agar (Becton Dickinson Microbiology Systems, Sparks, MD) and evaluated by the following biochemical tests: triple sugar iron, urea, oxidase, motility, indole, and ornithine decarboxylase

Clinical Observations

Clinical assessment of animals was performed before experimental bacterial challenge and when milk samples were collected Clinical status of mammary glands and appearance of milk were evaluated by qualified farm personnel at each milking using the following scheme: 1 = normal mammary gland and normal milk, 2 = normal mammary gland and slight alterations in milk (a few flakes), 3 = abnormal mammary gland and abnormal milk (clots, clumps, changes in milk color), and 4 = swollen mammary gland, abnormal milk and systemic signs (hyperthermia, depression, dullness) of infection Cows that developed clinical mastitis were monitored closely and antibiotic treatment initiated 7 days post-challenge. Results of a study by Schrick et al. (2001)

comparing cows with clinical and subclinical mastitis indicated similar detrimental effects of mastitis on reproductive performance It should be noted that cows with clinical animals analyzed in this study (Schrick et al., 2001) received antibiotic therapy following onset of mastitis whereas subclinical cows were not treated Therefore, authors believe that antibiotic treatment 7 days following experimental challenge should not alter endocrine responses.

Clinical mastitis was diagnosed when *S uberis* was isolated from challenged mammary glands at any milking post-challenge and the challenged mammary gland has a mastitis score of 2 or 3 for two consecutive milkings during the 7 d post-challenge period. Acute clinical mastitis was scored for experimentally-challenged mammary glands when *S. uberis* was isolated from the challenged mammary gland at any milking post-challenge, milk from the challenged mammary gland had a mastitis score of 4 for two consecutive milkings during the 7 d post-challenge period, and the cow was hyperthermic. Rectal temperatures were obtained daily throughout the study at milking time for all cows.

Determination of Estrous Expression

All cows were evaluated three times daily to determine onset of estrus Cows were monitored every two hours during intensive blood sampling to determine estrous behavior.

Blood Collection

Blood samples (10 mL) were collected daily via jugular venipuncture to evaluate P_4, cortisol, blood urea nitrogen (BUN), nonesterified fatty acids (NEFA) and insulin concentrations from all experimentally-infected and control cows. At time of second $PGF_{2\alpha}$ injection, TRT and CON cows (across 2 replicates; Figure 4-1) had jugular veins catheterized as described by Hockett et al. (2000) to allow for frequent sample collection and reduced handling stress on animals. Catheters were flushed immediately before and after each collection with heparinized saline solution (200 IU/mL) to maintain catheter function throughout the experimental period Samples were collected every 15 min from 48-56 h following $PGF_{2\alpha}$ injection (day 0) to evaluate LH pulse frequency. Following intensive blood sample collection period, samples were collected every 2 h until ovulation to determine the LH surge and preovulatory rise in E_2. Blood samples were centrifuged (2500 x g) and sera harvested and stored at -20°C until assayed.

Ultrasonography of Ovarian Structures

All cows had ovaries scanned (Corometrics Medical Systems, Inc., Aloka 500 unit, 7.5 MHz linear transducer, Wallingford, CT to ascertain development of the ovulatory follicle as follows: 1) ovaries were scanned daily beginning at intramammary inoculation to ascertain follicular and luteal characteristics (Seals et al., 1996), and 2) every 6 h beginning at visual observation of estrus until

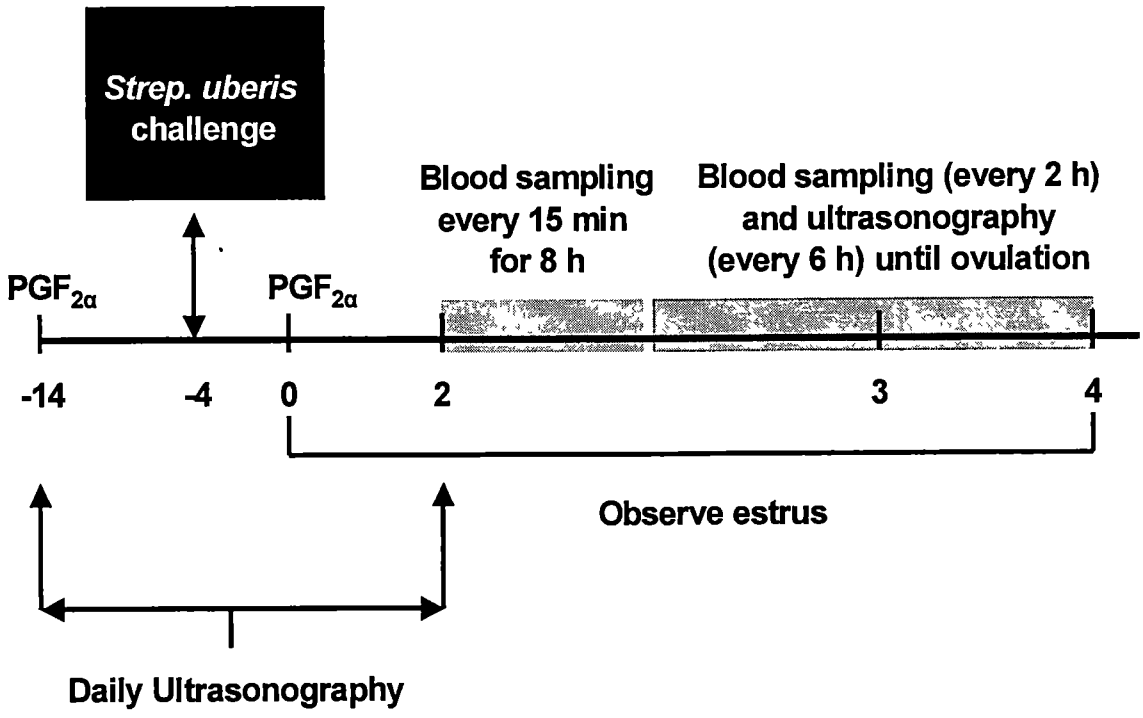


Figure 4-1. Schematic representation of experimental design for TRT and CONTROL cows

ovulation occurred (disappearance of the ovulatory follicle) Diagrams of locations and measurements of follicles ≥5 mm in diameter and corpora lutea were made for each ovary at the time of ultrasonography (Knopf et al., 1989).

Assays for Hormones and Metabolic Parameters

Samples were analyzed for LH by procedures described by Moura and Erickson (1997) with a sensitivity of 0.03 ng/mL, intra-assay CV of 6.5% and inter-assay CV of 15% Concentrations of E_2 (Moura and Erickson, 1997) were determined on serum samples utilizing antibody for E_2 supplied by Manson (Lilly Research Laboratories, Indianapolis, IN) Sensitivity of the E_2 assay was 0 15 pg/ml with intra-assay CV of 20% and inter-assay CV of 4%. Concentrations of cortisol (Hockett et al., 2000), P_4 (Seals et al., 1998) and insulin (Fazio et al., 2000) in serum were determined utilizing solid phase radioimmunoassays (Diagnostic Products Corp , Los Angeles, CA). Sensitivity of the cortisol assay was 2 ng/mL and inter- and intra-assay CV of 0.2% and 10%, respectively. Sensitivity of the progesterone assay was 0.02 ng/mL with intra-assay CV of 8% and inter-assay CV of 4% Concentrations of BUN and NEFA were determined in serum utilizing spectrophotometry as validated in our laboratory (Fazio et al , 2000) Blood urea nitrogen concentrations were determined utilizing a commercial kit with sensitivity of 15 mg/dL, intraassay CV of 6%, and interassay CV of 12% (Sigma Diagnostics, St Louis, MO) Concentrations of NEFA were determined using a NEFA-C kit with a sensitivity of 0.125 mEq/L and intra- and

inter-assay CV of 5% and 27%, respectively (Wako Pure Chemical Industries, Osaka, Japan). All assays are validated and currently utilized in our laboratory

Statistical Analyses

Patterns of P_4, E_2, LH, BUN, NEFA, insulin, cortisol, SCC, feed intake, LH maximum, LH pulse frequency, rectal temperature, mastitis (mammary) score, time periods from $PGF_{2\alpha}$ to estrus, $PGF_{2\alpha}$ to ovulation, $PGF_{2\alpha}$ to LH maximum, estrus to ovulation and differences in size of ovulatory follicles were analyzed by least squares analysis of variance for a randomized block with repeated measures Size of "presumed" ovulatory follicles in TRT-NOEST cows was determined by calculating average time of ovulation in CON cows and utilizing that time for selection of the largest follicle in TRT-NOEST cows. Concentrations of estradiol-17β were normalized for cows that displayed estrus (CON and TRT-EST) for the 24 h period prior to maximum observed LH release Due to absence of estrus and LH surge in TRT-NOEST, concentrations of estradiol-17β were calculated for TRT-NOEST based upon the average time of LH maximum in CON cows (82 h following $PGF_{2\alpha}$) Pulse frequency of LH was determined as described by Schrick et al (1990) with modifications Briefly, mean LH concentrations were established for each 8 h period with a pulse defined as a point > .5 standard deviations above the mean Differences in estrous expression and ovulation rates were determined by Chi-Square analysis. The Mixed Procedure (SAS, 1996) was used for all analyses

Results

Control cows (CON) that remained free of mammary infection (n = 12), challenge cows that developed clinical mastitis prior to estrus expression (n = 12) and cows that were inoculated with bacteria but failed to develop mastitis (n = 5) were utilized for data analysis During the experiment, two control animals displayed signs of mastitis and were culled from the experiment. Furthermore, two cows that were challenged developed subclinical mastitis (1/replicate) and these animals were not utilized for statistical analysis of data due to low numbers After removal of animals that did not comply with the experimental model, 29 cows remained Following bacterial challenge, 100% of CON (12/12) cows and 80% of cows that did not display mastitis following challenge (NOMAS; 4/5 displayed estrus while only 33% (4/12; $P < 0.0001$) of challenged cows displayed estrus Within challenged cows that fit the model of developing mastitis prior to estrus, two populations existed Therefore, challenged cows were further divided into those that displayed estrus (TRT-EST; n = 4) and those that did not display estrus (TRT-NOEST; n = 8). Animals within these different groups may be different and thus four classifications allowed for four "treatments" for analysis of data: 1) CON, 2) NOMAS, 3) TRT-EST, and 4) TRT-NOEST.

Clinical assessment of mastitis

Mammary scores, an indicator of severity or absence of mastitis, were elevated in TRT-EST and TRT-NOEST compared to control and NOMAS animals

(Table 4-1; P < 0.0001). Following bacterial inoculation, mean somatic cell counts were higher in TRT-NOEST and TRT-EST than CON and NOMAS (Table 4-1, P = 0.0009) Severity scores of 4 within TRT-EST and TRT-NOEST resulted in higher somatic cell counts over the experimental period (P < 0 0001). Somatic cell counts differed (increased) for treatment over days of the experiment (Table 4-1, P < 0 0001) Rectal temperatures were increased in TRT-NOEST and TRT-EST on multiple days of the experiment compared to CON and NOMAS cows (Table 4-1, P = 0 04) Mean rectal temperatures were higher for TRT-EST cows (38.9 ± 0 2°C) and TRT-NOEST cows (38.6 ± 0 2°C) with mastitis score of 4 than for score of 2 (38.4 ± 0 2 and 38.4 ± 0 2, respectively; P = 0 04)

Events associated with estrus

Because animals in the TRT-NOEST group did not display estrus, only data from CON, NOMAS, and TRT-EST are reported for parameters associated with timing of events of estrus. Time period from $PGF_{2\alpha}$ administration to estrus, estrus to ovulation, $PGF_{2\alpha}$ to ovulation, and $PGF_{2\alpha}$ to LH surge were similar for CON, NOMAS, and TRT-EST (Table 4-2) Size of the ovulatory follicle did not differ between CON, NOMAS, TRT-EST and TRT-NOEST (Table 4-2, P = 0 46). However, ovulation rates were higher for CON (100%), NOMAS (100%) and TRT-EST (100%) than for TRT-NOEST (0%, Fisher's Exact Test P < 0.0001).

Table 4-1. Clinical assessment of mastitis in experimentally challenged and control animals

	DAY							
Parameter	**CH**	**CH1**	**CH2**	**CH3**	**CH4**	**CH5**	**CH6**	**CH7**
Mammary scores[1]								
CON	1.0[a]	1.0[a]	1 0[a]	1.0[a]	1 0[a]	1.0[a]	1.0[a]	NA
NOMAS	1 0[a]	1.0[a]	1 0[a]	1.0[a]	1.0[a]	1.0[a]	1.0[a]	NA
TRT-EST	1.0[a]	1 0[a]	1.0[a]	2.0[b,c]	2 0[b,c]	1.8[b,c]	2 8[d]	NA
TRT-NOEST	1.0[a]	1 1[a]	1.4[a,b]	1.9[b,c]	2 8[d]	3 1[d]	3.7[e]	NA
SCC[2]								
CON	4.4[a]	4.6[a]	4.7[a]	4 4[a]	4 5[a]	4.5[a]	4 5[a]	4.5[a]
NOMAS	4.5[a]	4 8[a]	4 8[a]	4.6[a]	4 9[a]	4 8[a]	4 9[a]	4 7[a]
TRT-EST	3.8[a]	4.4[a]	4 9[a]	5.5[b]	6.0[c]	6.2[c]	7.0[d]	7.2[d]
TRT-NOEST	4 4[a]	4 7[a]	5.3[b]	5 5[b]	5 7[b,c]	5.9[c]	6 1[c]	6.8[d]
Rectal Temp[3]								
CON	38.0[a,b]	38.7[c,d]	38.3[b,c]	38.5[c]	38 6[c,d]	38.2[a]	38 5[c]	NA
NOMAS	38.1[a,b]	38.7[c,d]	38 4[b,c]	38 4[b,c]	38.4[a,b]	38.2[a,b]	38.1[a,b]	NA
TRT-EST	37.8[a]	38.7[c,d]	38.6[c,d]	38.7[c,d]	38.5[c]	38.7[c,d]	39.5[d,e]	NA
TRT-NOEST	37.8[a]	39.1[d,e]	38 4[b,c]	38.8[d]	38.3[b,c]	38.2[a,b]	38.1[a,b]	NA

[1] Mastitis scores with different superscripts differed [a,b,c,d,e](Pooled SEM = 0.27 P < 0.0001)

[2] Mean somatic cell count of challenged quarters or control quarters (log_{10} cells/ml; Pooled SEM = 0 28); [a,b,c,d] Somatic cell counts differed (P < 0.0001)

[3] Rectal temperature (°C, Pooled SEM = 0.26), [a,b,c,d] Rectal temperatures with different superscripts differed (P = 0.04)

NA = data not available

Table 4-2. Events associated with estrus for treatment groups

Parameter	CON	NOMAS	TRT-EST	TRT-NOEST
$PGF_{2\alpha}$ to estrus[1]	91.5 ± 6 1	81 8 ± 9.7	84 3 ± 11.2	NA
Estrus to ovulation[2]	28 1 ± 2.2	27.6 ± 2 9	27 6 ± 3.8	NA
$PGF_{2\alpha}$ to LH surge[3]	88.0 ± 5.9	84.5 ± 10.1	93.2 ± 9.1	NA
$PGF_{2\alpha}$ to ovulation[4]	114 1 ± 5 8	113 2 ± 9.7	115 1 ± 10 4	NA
LH max value[5]	$10.9 \pm 1\ 2^{a}$	8.2 ± 1.9^{a}	9.0 ± 1.9^{a}	$1.8 \pm 1\ 6^{b}$
LH pulse frequency[6]	$8.3 \pm 0\ 6^{a}$	$8\ 8 \pm 1.5^{a}$	7.5 ± 0.8^{a}	$4.4 \pm 0\ 7^{b}$
Follicle size[7]	$17\ 3 \pm 1.3^{a}$	$15\ 3 \pm 0\ 7^{a}$	17.3 ± 1.3^{a}	$16\ 2 \pm 1\ 8^{a}$

[1] Mean time from prostaglandin $F_{2\alpha}$ injection until estrus (hours) did not differ between treatment groups (P = 0.66)

[2] Mean time from onset of estrus to ovulation (hours ± standard error) did not differ between treatment groups (P = 0 97).

[3] Mean time from prostaglandin $F_{2\alpha}$ to LH surge (hours ± standard error) did not differ between treatment groups (P = 0.81).

[4] Mean time from prostaglandin $F_{2\alpha}$ to ovulation (hours ± standard error) did not differ between treatment groups (P = 0 94).

[5] Maximum concentration of LH observed during 2 h blood collection (ng/mL ± standard error), [a,b] concentrations with different superscripts differed (P = 0.0017)

[6] Mean number of pulses of LH during 8 h intensive blood collection (number/8h ± standard error); [a,b,c] concentrations with different superscripts differed (P = 0.0011).

[7] Mean follicle size (mm ± standard error) at ovulation (CON or TRT-EST) or at 114 h post prostaglandin (TRT-NOEST) did not differ (P = 0 46).

Hormones

Concentrations of progesterone were similar for all treatments throughout days of the experiment (P = 0.23) Progesterone declined for all treatments owing across day of experiment following $PGF_{2\alpha}$ administration (P < 0 0001) Concentrations of cortisol did not differ (P = 0.37) for any treatment group across days of the experiment. Mean concentrations of estradiol-17β during the 24 h period prior to LH maximum were significantly increased towards ovulation for CON, NOMAS, and TRT-EST while concentrations remained lower for TRT-NOEST (Figure 4-2; P < 0 0001).

Maximum observed value of LH (surge) was higher for CON, NOMAS and TRT-EST than for TRT-NOEST (Table 4-2, P = 0.0017). Luteinizing hormone pulse frequency over the 8 h intensive sampling period (Table 4-2) was higher in CON, NOMAS, and TRT-EST animals than TRT-NOEST (P = 0 001).

Metabolic parameters

Serum NEFA, BUN, and insulin concentrations were similar for all treatments throughout the experimental period (Table 4-3; P = 0.11, P = 0.85, P = 0.14, respectively). There was no effect of severity of mastitis score on any metabolic parameters (P = 0.28, P = 0.92, P = 0.96, respectively). Feed intake was highly variable among animals and would require additional animals for

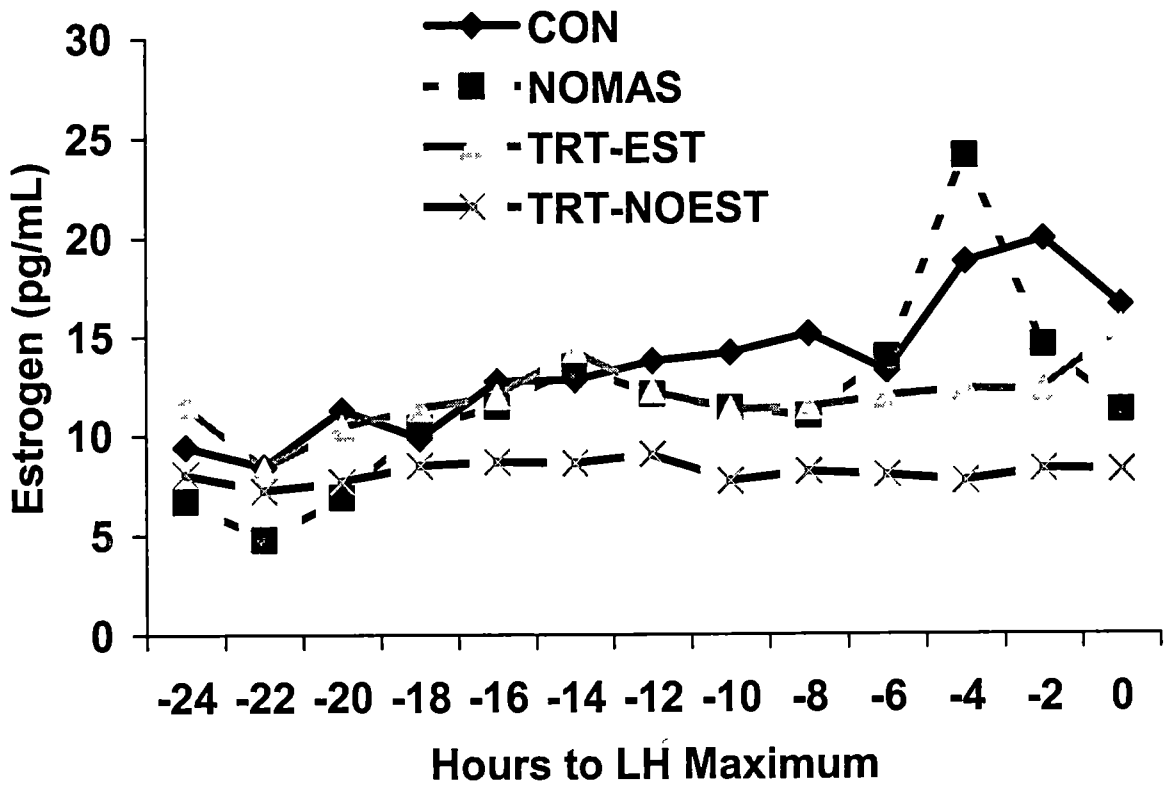


Figure 4-2 Concentrations of estradiol-17β during the 24-h period prior to LH surge. Concentrations increased similarly for CON, TRT-EST, and NOMAS. Concentrations did not increase over time for TRT-NOEST and were lower (P = 0 0001) than all other treatments (Pooled SEM = 2 91pg/mL).

adequate analysis. A representation of feed intake is depicted for 3 CON and 3 cows with clinical mastitis (Figure 4-3).

Discussion

Cows utilized in the current study became infected (infection rate· 12/19, 63%) with clinical mastitis following bacterial inoculation of *S. uberis* into two mammary quarters, consistent with previous studies (Hockett et al., 2000, 2002). Mean somatic cell counts, rectal temperatures, and mastitis scores were all elevated in cows with clinical mastitis and remained low for control animals.

Estrus was observed to occur in all control cows and in 5/12 challenged cows that developed clinical mastitis This rate is consistent with earlier studies that indicated that clinical mastitis resulted in decreased estrous expression within 8 d following prostaglandin administration (Hockett et al., 2002). Subdividing challenged cows with clinical mastitis into those that did and did not show estrus and challenged cows that did not exhibit signs of clinical mastitis resulted in 4 treatment groups for analyses of hormone data and parameters associated with estrus. These groups were subdivided to account for differences that may occur within different physiological states resulting in altered ability to display estrus or alterations that may occur following challenge in cows that did not develop clinical mastitis

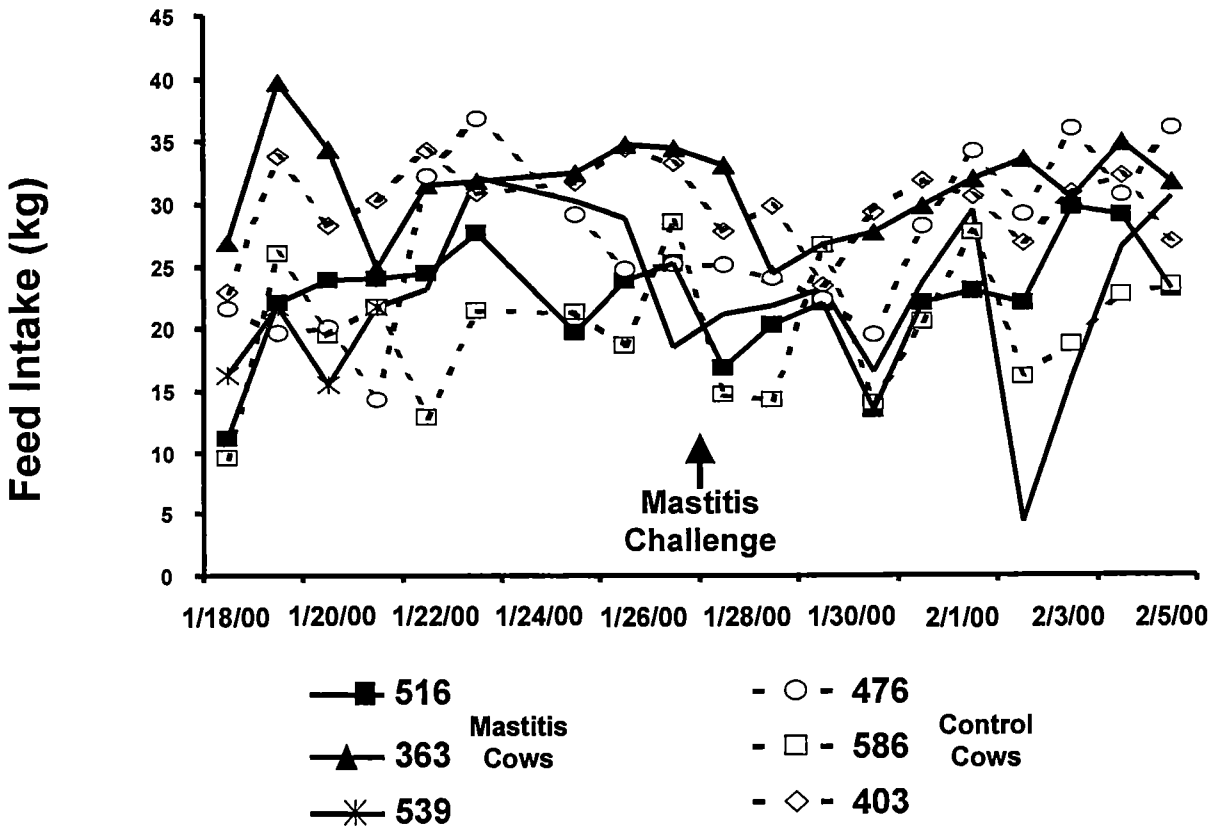


Figure 4-3 Daily feed intake determined in a subset of cows utilizing Calan gates and feed weighback Considerable variation was noted within cow regardless of treatment.

Time period from $PGF_{2\alpha}$ to estrus, estrus to ovulation, and $PGF_{2\alpha}$ to ovulation were similar between CON, NOMAS, and TRT-EST These data are not surprising as Hockett et al. (2002) reported that pregnancy occurred in mastitic cows that displayed estrus and were artificially inseminated. Cows with mastitis not displaying estrus following luteal regression would result in increased number of days to first service. This may explain similar associations between clinical mastitis, elevated days to first service, and increased days open reported previously (Barker et al., 1998; Schrick et al , 2001)

Decreased concentrations of estrogen observed in the current experiment are helpful to delineate the mechanism of decreased estrous expression observed by Hockett et al. (2002) in cows with clinical mastitis. Additionally, decreased expression of estrus and failed ovulation further explain increased days to first service associated to cows with clinical mastitis prior to first service (Barker et al , 1998, Schrick et al., 2001) Interestingly, follicle development was not different between CON, NOMAS, TRT-EST, and TRT-NOEST However, LH pulse frequency was decreased in TRT-NOEST compared to CON cows, which had LH pulsatility similar to that reported by Rahe et al (1980) Therefore, clinical mastitis immediately prior to ovulation has a negative impact on normal endocrine and follicular function.

Inflammatory or infectious diseases often result in stimulation of an immune response that interferes with LH function by releasing cytokines such as tumor necrosis factor alpha (TNF-α) or interleukins Darbon et al (1989) reported TNF-

α inhibited the stimulating action of FSH on LH receptor formation in cultured rat granulosa cells and had an inhibitory effect on FSH-induced cAMP production Darbon et al (1989) hypothesized that TNF-α released during infections may reduce ability of granulosa cells to differentiate upon FSH stimulation and to respond to LH because of alterations in LH receptors during follicle growth and maturation. Granulosa cells are important for production of estrogens that subsequently impact LH production via estrogen concentrations

Cytokines such as interleukins are key mediators associated with effects of Gram-negative and Gram-positive bacteria (Nathan, 1987; Zerbe et al., 2001). Zerbe et al (2001) reported that Gram-positive and Gram-negative have similar effects on polymorphonuclear granulocyte function *in vitro* McCann et al. (1997) reported interleukin-1 (IL-1), normally released during infection, blocks the pulsatile secretion of LH, but not FSH, through alterations in nitric oxide production to inhibit GnRH Furthermore, IL-1 disrupted normal estrous cyclicity, GnRH gene expression, and LH and FSH secretion (Rivest et al , 1993) Interleukin-6 is a cytokine produced by macrophages during an immune response and is a potent inhibitor of proliferation and FSH-induced E_2 production from small and large follicles *in vitro* (Alpizar and Spicer, 1994). Cows in the current experiment that did not display estrus had decreased concentrations of estradiol-17β following luteal regression, however, follicular size was similar in all groups. Release of inflammatory mediators and cytokines during the immune response may alter the ability of GnRH to signal gonadotropin release and

subsequently for gonadatropins that are released to transduce signals required for normal production of estrogens. Pulsatility of LH may be decreased as a secondary response of decreased estrogen production.

Intravenous endotoxin infusion interrupted the preovulatory rise of estradiol and also delayed or completely blocked the preovulatory LH surge, decreased LH pulsatility, and suppressed expression of estrus in ewes (Battaglia et al., 2000). A subsequent study (Battaglia et al., 2001) reported that low doses of endotoxin were sufficient to slightly decrease GnRH release, yet LH pulses were completely inhibited in some animals. Therefore, activation of the immune system may alter GnRH release from the hypothalamus as well as hinder the ability of the pituitary to respond to GnRH signals. In fact, administering exogenous GnRH while blocking endogenous GnRH during endotoxin infusion in sheep resulted in decreased amplitude of LH pulses. Endotoxin and clinical mastitis caused by S. uberis may have similar modes of action through release of cytokines and less of a direct action upon the endocrine system themselves.

Data support cortisol as having the major negative impact on normal follicular growth and endocrine function. However, cortisol concentrations did not appear to be elevated in the current study. More frequent sampling of cortisol and sampling over time may be necessary to properly determine alterations which occur in free cortisol Stressful conditions associated with mastitis and activation of the immune response have resulted in increased concentrations of cortisol For example, ewes treated with endotoxin had significantly lower GnRH pulse

amplitude, lower concentrations of GnRH and LH, elevated concentrations of cortisol and progesterone, and elevated body temperature (Battaglia et al , 1997) Peter et al (1989) reported that cycling heifers that received intrauterine infusion of endotoxin had elevated cortisol concentrations and smaller follicles on day 12 of the estrous cycle Peter et al (1989) also observed that follicles that failed to ovulate from $PGF_{2\alpha}$-induced luteal regression resulted in cysts, which persisted for 7 to 21 d. Formation of follicular cysts on ovaries could result from cortisol suppression of the LH surge (Lopez-Diaz and Bosu, 1992). In a previous study, expression of estrus was delayed up to 21 days in cows with experimentally induced clinical mastitis prior to estrus (Hockett et al , 2002)

Negative correlations of cortisol and suppressed LH have been observed during handling stress (Echternkamp, 1984). More directly, infusion of cortisol in ewes decreased follicular development and the ovulatory surge of LH was absent (Macfarlane et al , 2000). When cortisol infusion ceased, follicular development returned to normal and LH surge occurred. The impact of cortisol infusion was much more profound during the early follicular phase than during the luteal phase (Macfarlane et al , 2000) Daley et al (1999) reported that infusion of cortisol in sheep blocked the LH surge in 54% of sheep and estrus either did not occur or was delayed. Furthermore, administering pulses of GnRH during cortisol infusion increased concentrations of estrogen and presence of the LH surge as in controls. A similar percent of cows with clinical mastitis displayed estrus in the current study. Animals that did not display estrus had no apparent LH surge

during the intensive sampling period and LH pulsatility decreased to one-half that of control cows.

There were no observed alterations in metabolic parameters in the current study for any group of cows These data would suggest that previous finding of changes in metabolic parameters might be due to longer periods of evaluation following onset of clinical mastitis. In the present study, cows were monitored and samples were collected for approximately 10 d following challenge Decreased feed intake of animals during clinical mastitis may lead to negative energy balance and subsequent decreased reproductive performance through changes in uterine environment and $PGF_{2\alpha}$ release Feed intake was monitored in the current study with large daily variations occurring within and between cows. Conclusions about feed intake would require more animals than available in this trial and conclusions cannot be made concerning energy balance of mastitic cows.

Conclusion

Previous studies have indicated that cows with clinical mastitis had decreased expression of estrus and increased number of days until opportunity for conception to be achieved (Hockett et al., 2002) The current study was performed to determine how clinical mastitis affected endocrine function, particularly communication from the pituitary to the ovary Results of the current study suggest that clinical mastitis has multiple actions that hinder normal endocrine function Estradiol-17β production was greatly decreased and explains

the lack of estrous expression and decreased LH pulsatility. Additionally, this may explain why the LH surge was absent in cows with acute clinical mastitis and ovulation was blocked. Future studies should be aimed to determine the causative agents behind suppression of estradiol-17β, LH, and the potential role of the hypothalamus in control of the hypothalamo-pituitary-ovarian axis during clinical infection.

Chapter 5

Summary of Studies and Proposed Mechanisms

The objectives of the current studies were to determine if experimentally-induced clinical mastitis has an impact on reproductive function of lactating dairy cows. Preliminary data have indicated that clinical and subclinical mastitis are associated with increased number of days to first service, increased services per conception, and increased days open Others have speculated that clinical mastitis may result in early luteal regression. To date, no direct effects of clinical mastitis have been documented

The initial study was to determine if alterations occurred in endocrine status of cows or alterations in stress hormone profiles. Hormones of particular interest were luteinizing hormone, prostaglandin $F_{2\alpha}$, and cortisol. These data indicated that clinical mastitis did alter physiology of the lactating cow in the luteal phase of the estrous cycle by increasing concentrations of cortisol and increasing the metabolite of prostaglandin $F_{2\alpha}$ following oxytocin challenge. Therefore, a direct effect was observed on the endocrine status of cows following clinical mastitis.

The second study was aimed to determine if clinical mastitis during the immediate preovulatory period or postovulatory period altered reproductive function. Cows that developed clinical mastitis during the preovulatory time period had decreased estrous expression following synchronization of estrus, increased number of days to first observed estrus, and elevated cortisol concentrations. Meanwhile, clinical mastitis during the early postovulatory period

did not alter luteal function or establishment of pregnancy. These data indicate that clinical mastitis during the early postovulatory period is of little consequence to normal reproductive function and pregnancy However, clinical mastitis during the preovulatory time period was detrimental to the ability of cows to display estrus and decreased opportunity for artificial insemination to occur

Due to the findings of the second study, the final project was performed to determine if decreased expression of estrus resulting from clinical mastitis during the preovulatory period was a result of altered endocrine and follicular function. Following experimentally-induced clinical mastitis, mastitic cows that did not display estrus had decreased LH pulsatility, decreased LH maximum, and suppressed estradiol-17β. Data from the experiment indicate that alterations do occur in the hypothalamo-pituitary-ovarian axis such that normal endocrine function is altered

Collectively, data from these studies suggest that clinical mastitis results in alterations in normal endocrine function, especially during the preovulatory period These alterations in endocrine and follicular function resulted in decreased expression of estrus, decreased ovulation, and increased days to service opportunity Increased in cortisol were observed in 3 separate groups of animals during clinical mastitis and may exert an inhibitory effect on normal steroidogenesis. Clinical mastitis exerts effects upon reproduction via effects on the hypothalamo-pituitary-ovarian axis (Figure 5-1) Decreased estrogen may

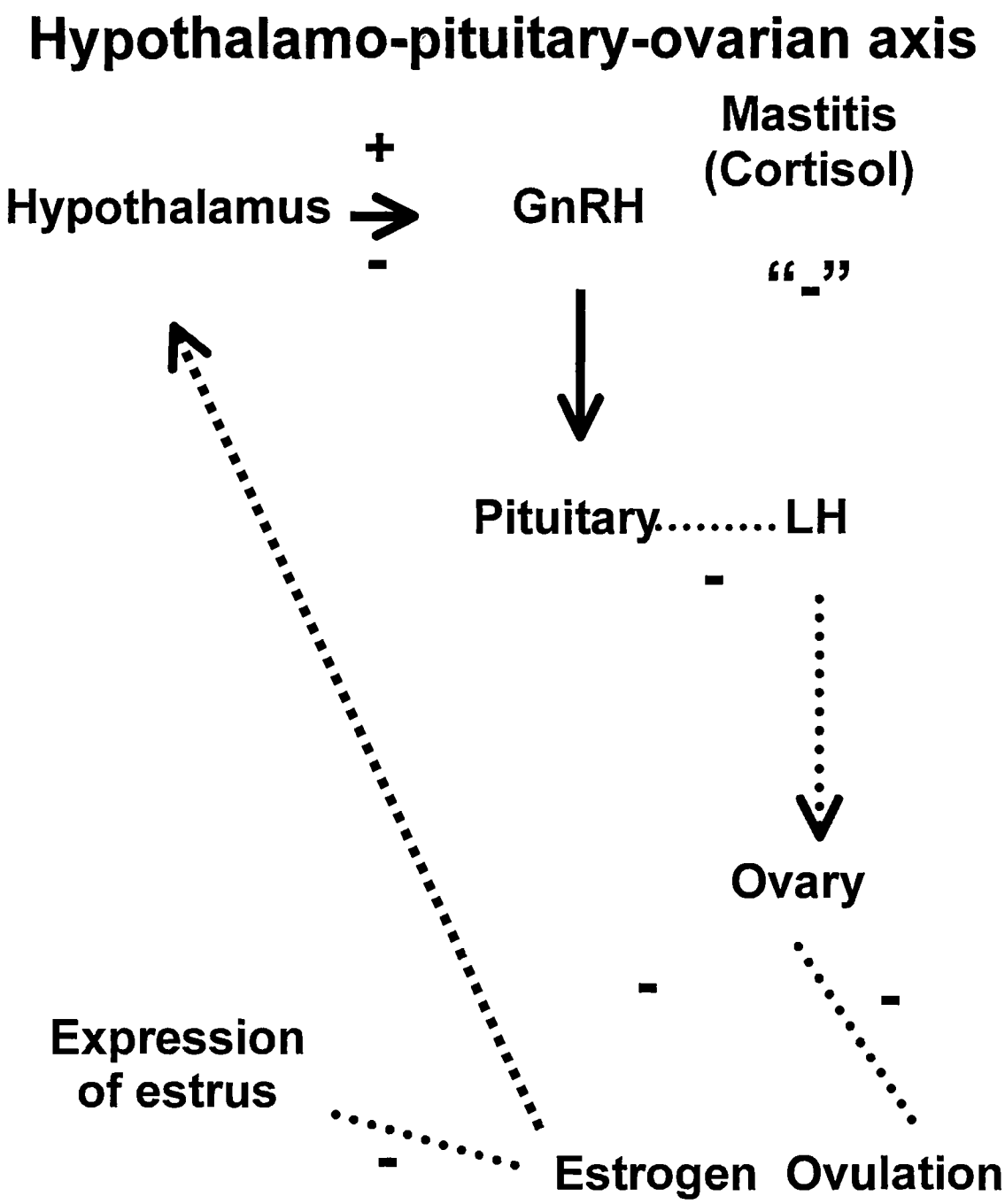


Figure 5-1. Proposed mechanism of action for clinical mastitis effects on reproduction Mastitis in inhibitory at each point where dotted line is observed. normal endocrine function at the level of the hypothalamus, pituitary, and ovary

result in a suppression of the LH surge and altered LH pulsatitilty. Cortisol has been observed to be inhibitory to normal endocrine function at the level of the hypothalamus, pituitary, and ovary Other factors, such as cytokines, involved in the ımmune response may have similar negatıve effects on steroid production Elevated body temperature during acute clinical mastitıs may result ın alterations in responsiveness of the hypothalamus to stimuli or in alterations in normal feed intake whıch may result in altered metabolism Our data do not support nutrition as beıng compromised during clinical mastitis, however, larger number of animals may be required to test such a hypothesis

In conclusion, reproduction of cows with experimentally-induced clinical mastitis during the preovulatory period is negatively impacted by direct or indirect effects of the infection. Inflammatory mediators, immune mediators, and physiologıcal changes all may play a role to decrease reproductive efficiency of animals during and following a clinical ınfection Future research should be directed to further delineate mechanisms of decreased steroidogenesis during clinical infection as well as to develop treatments that may be effective to decrease the impact clinical mastitis exerts on reproduction of cows during early lactatıon

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Vitae

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