THE EFFECTS OF FEEDING A NEGATIVE DIETARY CATION-ANION DIFFERENCE DIET AT TWO DIETARY CALCIUM INCLUSION RATES TO CLOSE UP DRY COWS ON THE SUBSEQUENT LACTATION UTERINE HEALTH AND FERTILITY

BY

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THESIS

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ABSTRACT

Feeding a negative dietary cation-anion difference (DCAD) diet in the prepartum phase been associated with increased biological active blood Ca levels (ionized Calcium; iCa) in the first days postpartum, linked with decrease incidences of hypocalcemia (HC) and subclinical hypocalcemia (SCH), as well as a host of other periparturient disorders. This is achieved by altering the mineral composition of the prepartum diet to induce a slight metabolic acidosis environment to attenuating PTH sensitivity to circulating Ca levels. While this concept has been established in literature, the rate of inclusion of dietary Ca in a negative DCAD diet has not. The objective of this study was to determine at what concentration of dietary Ca to feed with a close-up dry cow negative DCAD diet and the effects on the subsequent uterine health, circulating inflammatory blood metabolites, ovulation dynamics, and fertility.

Multiparous Holstein cows (n = 76) were enrolled at 50 days before expected calving date and followed until 75 days in milk (DIM). Treatments began at 28 days before expected calving and were: CON (n = 24), a positive DCAD diet with low dietary Ca (DCAD = 9.46 mEq/ 100 g DM; 0.4% DM); LOW (n = 26), a fully acidified DCAD diet (DCAD = -24.13 mEq/ 100 g DM; urine pH = 5.7) with low dietary Ca (0.4% DM); HIGH (n = 24), a fully acidified DCAD diet (DCAD = -23.97 mEq/ 100 g DM; urine pH = 5.7) with high dietary Ca (2.0% DM). Vaginal discharge was evaluated was at 4, 7, 10, 13, 15, 17, and 30 DIM via Metricheck (MC; 0-3 scale: 0 = clear mucus; 1 = mucus containing non-purulent material; 2 = mucus containing ≤ 50% purulent material; 3 = mucus containing > 50 % purulent material). Polymorponuclear (PMN) cell concentration in the uterus was evaluated at 15 and 30 DIM, and endometrial tissue samples were harvested at 30 DIM for glandular morphology and assessment of superoxide dismutase (SOD) and glutathione peroxidase (GPX) activity as markers of oxidative stress.

Blood plasma and serum samples were harvested at -28, -21, -14, -7, 15 and 30 DIM and were assessed for concentrations of lipopolysaccharide binding protein (LBP), serum amyloid A (SAA) and haptoglobin (HP). Ovarian dynamics was assessed at 7, 9, 11-17, 20, 30, 55, 62, and 69 DIM. Data collected were analyzed using PROC MIXED in SAS. Contrasts were CONT1 (CON vs the average of cows fed LOW and HIGH diets) and CONT2 (LOW vs HIGH). Cows fed CON tended to have a lower MC score ($P = 0.06$) than the average of cows fed LOW and cows fed HIGH. Cows fed LOW tended to have a higher MC score than cows fed HIGH. There were differences in uterine gland epithelial height where cows fed HIGH had greater epithelial height ($P = 0.02$) than cows fed LOW and cows fed CON tended to have shorter epithelial height ($P = 0.06$) than the average of cows fed LOW and cows fed HIGH. Cows fed HIGH also had a greater number of epithelial cells per gland ($P = 0.05$) than cows fed LOW. Anti-oxidative enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPX) relive oxidative stress in cells. Cows fed HIGH had increased activity of SOD ($P = 0.05$) decreased activity of GPX ($P < 0.001$) than cows fed LOW calcium diet. Cows fed LOW had higher HP concentrations than cows fed HIGH in the prepartum ($P = 0.01$) and post-partum ($P = 0.03$) periods. Cows fed CON diet had higher ($P = 0.01$) HP concentration than the average of cows fed LOW and cows fed HIGH (contrast CON *vs.* LOW and HIGH) postpartum. Cows fed HIGH tended to have an increased likelihood of being pregnant at the first timed artificial insemination. Cows fed HIGH seemed to have an improved uterine environment due to alleviation of oxidative stress, an enhanced immune response to parturition and uterine discharge comparable to cows fed CON.

In conclusion, the periparturient period is a challenging time to the dairy cow with numerous metabolic changes that affects lactation and reproductive performance. Calcium metabolism and homeostasis is an important factor and can be influenced by prepartum feeding

management. Strategies such as feeding a negative DCAD diet to close-up dry cows has been credited with decreasing incidences of HC and SCH and improving reproductive performance. Additionally, feeding a negative DCAD diet with 2% dietary Ca inclusion seems to attenuate the reproductive health and fertility of the dairy cow.

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Nearly seven years ago, I decided to pursue my career in animal sciences by coming here to the University of Illinois. From that point to now, my path has changed quite a bit, some for the better, and some not. However, with those changes, there are new challenges, and with those challenges comes the opportunity to learn and to grow.

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"The fact is, there is no foundation, no secure ground, upon which people may stand today if it isn't the family." – Morrie Schwartz (*Tuesdays with Morrie* by Mitch Albom)

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LIST OF ABBREVIATIONS

°C	=	Celsius
AI	=	Artificial insemination
APP	=	Acute phase protein
ATP	=	Adenosine triphosphate
BW	=	Body weight
Ca	=	Calcium
Cl	=	Chloride
CL	=	Corpus Luteum
cSCH	=	Chronic sub-clinical hypocalcemia
d	=	Day
DCAD	=	Dietary cation-anion difference
DIM	=	Days in milk
DM	=	Dry matter
DMI	=	Dry matter intake
DRC	=	Days relative to calving
DRO	=	Days relative to ovulation
FSH	=	Follicle stimulating hormone
g	=	Gram
GnRH	=	Gonadotropin-releasing hormone
GPX	=	Glutathione peroxidase
H&E	=	Haematoxylin and eosin
HC	=	Hypocalcemia
HP	=	Haptoglobin
IACUC	=	Institutional Animal Care and Use Committee
IFN-τ	=	Interferon-τ
IL-10	=	Interlukin-10
IL-6	=	Interlukin-6

K	=	Potassium
kg	=	Kilogram
LBP	=	Lipopolysaccharide binding protein
LH	=	Luteinizing hormone
LPS	=	Lipopolysaccharide
MC	=	Metricheck
mEq	=	milliequilvalents
Mg	=	Magnesium
mL	=	Milliliters
n	=	Number
Na	=	Sodium
NADPH	=	Nicotinamide adenine dinucleotide phosphate
NEB	=	Negative energy balance
NEFA	=	Non-esterified fatty acid
P	=	Phosphorus
PAMP	=	pathogen associated molecular pattern
PGF2α	=	Prostaglandin F2-α
PMN	=	Polymorphonuclear cells
PTH	=	Parathyroid hormone
ROS	=	Reactive oxygen species
S	=	Sulfur
SAA	=	Serum amyloid A
SCH	=	Sub-clinical hypocalcemia
SOD	=	Superoxide dismutase
TAI	=	Time artificial insemination
TLR-4	=	Toll-like receptor 4
TMR	=	Total mixed ration
TNF-α	=	Tumor necrosis factor-α

US = Ultrasound

VWP = Voluntary waiting period

INTRODUCTION

The periparturent period is defined in dairy cows as a state of near maintenance requirements in late gestation to that of rapidly increasing metabolic and nutrient demands needed for the onset of lactation. At parturition, the dairy cow is subjected to various challenges that can affect her future lactations and fertility. Failure or tardiness to adapt to these changes can leave the cow predisposed to metabolic disorders such as ketosis, acidosis, and displaced abomasum [1,2], or infectious disorders such as metritis and mastitis [3,4], calving related disorders such as dystocia, retained placenta, and decreased fertility [5,6]. What is thought to be an underlying issue in these disorders is an imbalance in calcium homeostasis at the onset of lactation. Calcium, an important macromineral involved in smooth muscle contraction, milk synthesis, and immune cell activation, reaches a nadir in the first days after parturition at the onset of lactation. An estimated 5-10% [7] of dairy cows experience hypocalcemia (HC; milk fever, parturient paresis) at the time of parturition, with an estimated cost of $246 per case [8]. The prevalence of subclinical hypocalcemia (SCH; estimated at 33% of all 1st lactation or greater [9]) is considerably higher, therefore, its economic impact is more significant than HC. Subclinical hypocalcemia is often undiagnosed and primes the cow for other various disorders as well as decreased milk production [10,11]. The homeostasis of Ca is often challenged at parturition due to a decrease in Ca signaling sensitivity from an oversaturation of Ca in the prepartum diet [12]. Parathyroid hormone (PTH) is a calciotropic hormone that is important in absorption of dietary Ca and reabsorption of Ca stores (bones, skeletal muscle). Prepartum nutritional management is paramount in a successful transition from non-lactating to lactating cow [1,13]. Treatments to lessen the effects of HC and SCH have been in use for decades, however, feeding management as a prevention of Ca imbalance at parturition has become more

commonly used in the recent years [9,10,11]. Overfeeding Ca in the last four weeks before parturition has been associated with high incidences of SCH and HC [12,13]. However, formulating a prepartum diet that only meets and not exceeds dietary Ca is challenging.

In recent years, effective dry cow management has been a focal point in dairy cow research. Due to challenges faced by the periparturient cow, she is susceptible to a host of metabolic diseases [1, 2] as well as infection from opportunistic bacteria [3, 4]. This susceptibility to disease is not easily defined to a single factor, rather, it is a complex system of changing metabolic demands, resistance to bacterial contamination of the uterine lumen, and coordination of various tissues to meet the demands of lactation and a return to a heathy state. Failure to adapt can delay cow's uterine involution process, return to ovarian cyclic activity, and diminish her immune response [4,17,18]. At the time of parturition, the cow must successfully transition from fairy low energy requirements to rapid mobilization of her endogenous energy stores [19], as well as increasing her exogenous energy intake [20,21]. Failure to transition through this period leaves the cow in a negative energy balance (NEB) for a prolonged period of time [22], thus increasing her likelihood of disease [23].

Perhaps one of the more affected macrominerals during this period, Calcium (Ca), is an extremely important mineral in a multitude of biological functions during the peripartuient period. These functions include smooth muscle contraction, lactogenesis, and act as secondary signaling molecules in various immunological pathways. At the onset of lactation, Ca requirements increase 4-fold to meet the demands of lactogenesis [10]. To satisfy this demand the cow will first increase absorption of exogenous Ca from her diet, and second, pull Ca from her own stores (bone and skeletal muscle). However, this action can be disrupted if the cow is exposed to a high dietary calcium in her prepartum diet. This is due to the actions of the

calciotropic hormone, parathyroid hormone (PTH). Parathyroid hormone regulates calcium homeostasis by increasing intestinal Ca absorption and renal Ca reabsorption [14, 15]. If exogenous Ca does not meet the Ca demand, PTH will act upon endogenous Ca stores. If exposed to high dietary calcium in the prepartum phase, sensitivity to PTH diminishes as well as PTH receptor numbers [16, 17], leaving the cow unable to adapt effectively to the increased Ca demands from colostrum and milk synthesis and instead must rely on her endogenous sources. Prolonged dependency on endogenous Ca sources puts the cow in a negative Ca balance, and in severe cases, induce clinical hypocalcemia (HC), or more often, sub-clinical hypocalcemia (SCH).

An increasingly popular dry cow management practice is to feed an acidogenic diet to cows entering the close-up period up until parturition. This is achieved by manipulating the dietary cation-anion difference (DCAD) in the diet. Acidification of the diet is realized when the concentration of anions is greater than that of the concentration of cations. This increases anion absorption in circulating blood and leaves the cow in a slight state of metabolic acidosis. Various studies [2, 18, 19] have examined this effect and observed that this practice decreases the chances of the cow developing HC and SCH. The mechanisms of this action is achieved by maintaining PTH sensitivity to calcium homeostasis and increasing calciotropic receptor numbers [20, 21]. Feeding a DCAD diet in the prepartum phase of the peripartiuent period has been observed to increase the subsequent reproductive performance of the cow as well as improve the calcium status of the cow [22, 23].

In the current study, multiparous Holstein cows were selected to evaluate the effects of feeding an acidogenic diet in the prepartum phase at two different rates of dietary Ca inclusion on the subsequent reproductive performance of the cow. Evaluation of reproductive performance

included evaluation of blood metabolites related to inflammation, evaluation of vaginal discharge, cytological evaluation of endometrial cells, antioxidant enzyme activity in endometrial tissue, uterine gland development, follicular dynamics, and likelihood of pregnancy at first timed artificial insemination (TAI). Overall, cows fed a negative DCAD diet with a high rate of dietary Ca inclusion tended to have increased likelihood of pregnancy at first TAI, improved redox environment within uterine tissue, and an attenuated immune response after parturition than cows fed a negative DCAD with low dietary Ca inclusion or cows fed a positive DCAD with low dietary Ca inclusion.

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CHAPTER 1. LITERATURE REVIEW

THE TRANSITION PERIOD

During the periparturient period, the cow undergoes a transition from non-lactating to lactating status at calving. During this transition, the cow drastically changes in endocrine status and energy metabolism [1] to prime her for lactogenesis, however, in the same instance; she becomes highly susceptible to diseases [2]. The energy demand required for lactation shifts the periparturient cows' metabolic processes to encourage hepatic gluconeogenesis, lipolysis, and fatty acid mobilization, mainly in the form of non-esterfied fatty acid (NEFA) [3], all of which are essential to milk synthesis. Grummer et al., among others [4–7], have established that periparturient cows experience decreased dry matter intake (DMI) in the days leading up to parturition, at a time when the fulfillment of the energy and nutrient requirements are crucial to the health and productivity of the postpartum cow. Marquardt et al. [8] noted a 52% drop in DMI in multiparous cows in the final two weeks before parturition. More recently, Hayirli et al. [5] used mathematical models to exemplify the predictable depression in DMI in the final days leading up to parturition. The decrease in DMI and the energy demands required for milk synthesis places the postpartum dairy cow in a state of negative energy balance (NEB) immediately following parturition [7].

To make up for the NEB, the cow will increase her energy intake, mainly in the form of increased DMI of a high energy diet in the days following parturition. However, if this response is delayed, the cow will spend a considerable amount of time in a NEB, which in turn, leaves the cow susceptible to an onslaught of metabolic disorders [9–11] and incidences of infection from pathogens [12–14]. This susceptibility is mainly due overutilization of unsustainable energy

sources to compensate for the NEB, i.e. β-oxidation of NEFA and/or ketone bodies [15,16], that leaves the cow in a state of metabolic stress. Failure on the cows part to increase her energy and nutrient intake can prolong the metabolic stress into development of a multitude of disorders such as ketosis, acidosis, displace abomasum, mastitis, metritis, retained placenta, and laminitis, to name a few.

It is thought that the initiation of aforementioned disorders are due to suppression of the immune response or depression in immune cell activation during this periparturient period. Kvidera et al. [17] indicated the activated immune system of mid-lactation cows (169 ± 7 days in milk (DIM)) infused with lipopolysaccharide (LPS; 1.5 μg/kg of body weight (BW)) - a pathogen associated molecular pattern (PAMP) molecule - required 1.09 kg of glucose in a 12-hour period. In the same experiment, Kvidera et al. [17] observed a ~80% reduction in milk yield in cows infused with LPS, partially attributing the drop in yield to the milk glucose deficit but also to direct binding of LPS to toll-like receptor 4 (TLR-4) present on mammary epithelial cells. This huge energy requirement of activated immune cells is only half the stress that the periparturient cow experiences. It has been observed that there are large deficits in nutrient availability to immune cells as well during the periparturient period. Perhaps the most effected nutrient in this period is Calcium, a macro-mineral critical to various functions in the body. Deviation from calcium homeostasis reduces the cows' ability of smooth muscle contraction [18] (Figure 1.1), blunts immune response [19], and increases risk for disease [20].

HYPOCALCEMIA

Hypocalcemia (HC) is one of the most prevalent and perhaps the most costly metabolic disorders to affect dairy cows [21]. Dairy cows generally have 8.5-10 mg/dL of circulating Ca [22]. Hypocalcemia, as defined by the Merck Veterinary Manual [22], as < 7.5 mg/dL of

circulating Ca [22]. Oetzel [21] review of the disease observed 5-10% of all dairy cows in the periparturient period were affected by clinical hypocalcemia (milk fever), resulting in economic losses from deaths, increased culling rates, and decreased milk production, in addition to treatment costs. Symptoms of milk fever, as it is defined by Merck Veterinary Manual, can be separated into in 3 stages: Stage 1: fine muscle tremors, restlessness, with excessive ear twitching and head bobbing; Stage 2: unable to stand, decreased body temperature, cold ears, and constipation; Stage 3: loss of consciousness, complete muscle flaccidity, and sever bloat. If left untreated, HC can result in cow death. Subclinical hypocalcemia (SCH) effects about 33-47% of 2nd lactation or greater ([23] dairy cows around the time of calving. Subclinical hypocalcemia is defined as depressed Ca concentration in the blood without showing clinical signs of milk fever. While the outward effects of SCH are not as pronounced as HC, the economic effect is profound. Affecting half of the milking herd in the United States, it often goes undiagnosed and the disease can manifest into increased susceptibility to secondary diseases such as ketosis, metritis, mastitis, retained placenta, lameness, and milk fever, as well as long-term losses in production efficiency (decreased DMI and milk productivity; [18]).

Caixeta et al. [24] examined the effect of Ca status through the first 3 days postpartum and observed that cows that had SCH (cows with blood Ca $\leq$ 8.6 mg/dL for at least one of the first 3 days postpartum) had an elevated number of cows in NEB when compared to eucalcemic animals. that the study also revealed that chronic SCH (cSCH) cows (cows with blood Ca $\leq$ 8.6 mg/dL for all first 3 days postpartum) had lower odds of being pregnant at first service than eucalcemic cows. Moreover, Caixeta et al. [24] observed cows that had SCH also had higher incidences of disease when compared to eucalcemic cows andthis effect was more pronounced in cSCH.

Feeding behavior of the cow as well as feeding strategies employed by the producer in the close-up dry period can influence the cows' ability to affectively use Ca in the days following parturition. Bell [25] estimation of nutrient and energy demands to increase dramatically from day 250 of gestation to 4 days postpartum (tripling the demand for glucose, doubling the demand for amino acids, and approximately quintupling the demand of fatty acids). In addition to this, Ca requirements increase about fourfold on the day of parturition [26]. It has been reported by multiple studies [24, 28, 29] for Ca concentrations in the blood to reach a nadir on the first day of lactation as the cow adapts to the metabolic changes. However, as was examined by Caixeta et al. [24], the ability of the cow to adapt to these changes, i.e. failure to do so, will negatively affect the reproductive performance and incidence of disease of the cow.

Calcium metabolism in the periparturent period is extremely important for the cow to have a successful transition to lactation. Horst [29] examined calcium homeostasis during the transition period and observed that to minimize incidence of clinical hypocalcemia, he recommended to keep dietary Ca intake at $\leq$ 50 g/d in the prepartum phase. At the onset of lactation, the cow will secrete up to 50 g/d of Ca [22] in the production of colostrum and milk. This sudden outflow of Ca into milk will cause plasma Ca levels to drop and to increase the release calciotropic hormones such as parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D_3. The parathyroid hormone first promotes Ca absorption in the intestines and Ca reabsorption through the renal system, however if PTH continues to be released, it will stimulate the resorption of Ca from skeletal muscle and bones [21,30,31]. High circulating plasma Ca concentrations (due to high dietary Ca intake) in the prepartum phase can decrease the sensitivity of PTH on target cells in the intestines and renal system (decreasing dietary Ca absorption/reabsorption), thus increasing Ca release from reservoirs in the bones and skeletal

muscles [31]. The loss of sensitivity of the PTH can be unsustainable for the cow at onset of lactation as she pulls more Ca from her own reserves to meet the demands of milk production and will result in parturient paresis [18].

At the onset of HC, there are various options for treatment. Due to the paralysis experienced as a result of HC, the weight of the cow will cut off blood supply to the down side of the cow which will begin to cause necrosis in as little as 4 hours, therefore, treatment should begin at first signs of HC. Goff [18] discussed the efficacy of these different treatments of HC extensively. The most effective method is to administer an IV injection on Ca salts (Ca borogluconate being the most common) at a dosage of 2 g Ca/100 kg of body weight at a rate of 1 g/min. Other methods include subcutaneous injection of Ca salts, however these have falling out of favor as they require 6-10 separate, 50-75 mL shots to be administered for an effective dosage. Oral administration of Ca can fall between treatment and preventative measure. This concept is if the cow's ability to absorb dietary calcium is compromised due to intercellular transport (due to loss of PTH sensitivity), administration of an oral bolus of upwards of 125 g Ca/dose will cause an osmotic difference in the intestines and allow the possibility of passive diffusion of Ca through epithelial tight junctions and into the vascular system.

Recently, there have been numerous studies aimed in identification and prevention of SCH and HC in the transition period [20,21,24,32]. The most prolific of these strategies in the last 3 decades has been to feed an acidogenic diet in the prepartum phase of the transition period [4,18,20,26,33,34]. This is achieved by feeding the cow an anionic salt supplement to obtain a negative dietary cation-anion difference (DCAD) in the formulation of the diet. The increased concentration of circulating cations (K^+, Na^+, Ca^{2+}, and Mg^{2+}) prepartum can effectively alkalinize the blood and induce conformational changes in the PTH receptor in target tissues and

a loss of sensitivity to the hormone [35]. In order to achieve the acidogenic diet, the diet will need to be formulated so that the dietary anions (Cl^-, S^{-2}, and P^{-3}) are in greater concentration than the dietary cations. Dietary cation-anion difference is commonly measured as milliequivalents per kilogram (mEq/kg) of DM, however, there are various ways to calculate the DCAD in the diet, with one of the earliest equations being from Ender et al. [36], a four variable equation:

$$DCAD = (Na^+ + K^+) - (Cl^- + S^{-2}).$$

It took into consideration the four largest DCAD influencers to be represented equally. Mongin [38] iteration of this equation assumed dietary sulfur was met (0.2% S DM, 13 mEq of S/100 g DM; NRC [4]):

$$DCAD = (Na^+ + K^+ - Cl^-).$$

Finally, if we truly want to incorporate all the major cations and anions fed in the diet, the equation could be expressed as:

$$DCAD = (Na^+ + K^+ + Ca^{2+} + Mg^{2+}) - (Cl^- + S^{-2} + P^{-3})$$

However, with this equation, we assume that Ca^{2+} and Mg^{2+} are as strong of alkalizing agents as Na^+ and K^+ and that S^{-2} and P^{-3} are as strong of acidifying agents as Cl^-. Goff and Horst [38] and Goff et al. [39] examined the acidifying/alkalizing capacity of the different anions and cations in various forms and observed differences, and assigned coefficients to these variables based on their acidification/alkalizing capacity:

$$DCAD = (Na^+ + K^+ + 0.15Ca^{2+} + 0.15Mg^{2+}) - (Cl^- + 0.6S^{-2} + 0.5P^{-3}).$$

When formulating prepartum diets with a negative DCAD most nutritionists and ration formulation software follow either Goff and Horst [38] or Ender et al. [36] iteration of the equation. Mulligan et al. [40] determined three criteria to be met when formulating a negative DCAD diet: 1) keep DCAD between -100 to -200 mEq/kg DM; 2) dietary Ca concentration should be approximately 1.2% of the diet; and 3) urine pH should stay between 6-6.8. There has been debate surrounding the second criteria in the last couple of two decades, as Oetzel et al. [41] cautioned against having Ca at 1.16% DM as it increased risk of milk fever. Since then, determination of dietary Ca inclusion with a negative DCAD has not been clearly defined [42–46].

The NRC for dairy cattle recommends the required absorbed Ca for a pregnant cow in her third trimester to be represented by the exponential equation:

$$Ca\ (g/day) = 0.02456e^{(0.05581-0.00007t)t} - 0.02456e^{(0.05581-0.00007(t-1)(t-1))}$$

Where t = day of gestation

Whereas the recommended requirement for a lactating cow Ca absorbed to be 1.22 g Ca/kg of milk produced. Meeting these necessary absorbed values depends greatly on the bioavailability of Ca in feedstuffs as well as inorganic Ca sources in the diet. While it is difficult to control for Ca available in feedstuff, particularly in fresh cow diets, many producers rely on inorganic sources to meet Ca requirements. Calcium absorption will generally equate to the body requirement if the diet has enough Ca available for absorption. However, depending on the Ca source, the rate of inclusion of the inorganic Ca source may vary (Table 1.1). There has not been much studied on the effect of a negative DCAD diet on different Ca sources, however, Oetzel et al. [47] observed that feeding a negative DCAD (-75 mEq/kg DM) when compared to feeding a

positive DCAD (189 mEq/kg DM) increased the rate of absorption and increased circulating total and ionized Ca concentrations, improving overall Ca absorption and usage. While not a direct inorganic Ca source, vitamin D is heavily involved in Ca metabolism. When sourced from calcidiol, vitamin D supplementation, when paired with a negative DCAD (-124 ± 11 mEq/kg DM) prepartum diet, increased circulating vitamin D metabolites and improve reproductive performance of transition dairy cows [48,49]. It has been observed that feeding a negative DCAD (-100 mEq/kg DM) diet in prepartum diets increases the rate of Ca excretion in urine [50], however it also has been observed to increase plasma Ca concentrations in the first day postpartum, when the cow is at most risk of hypocalcemia or SCH [50].

UTERINE HEALTH

During the process of parturition, the uterus of the cow becomes open to the outside as the cervix, vagina, and vulva open for the passage of the calf. This opening allows for the introduction of pathogens into the uterine environment and could result in infection [13]. While there is evidence of a bacterial symbiotic relationship in the uterus [13], development of infection occurs in states of uterine dysbiosis in the days following parturition [13, 48]Clinically, incidences of metritis is defined as vaginal discharge with purulent or brown characteristics, and a fetid odor accompanied by a fever (rectal temperature ≥ 39.2°C. [13,45]. Pathologically, metritis is defined as inflammation in the entirety of the uterine wall, whereas endometritis is defined as inflammation limited only to the endometrium lining of the uterus. This inflammation is often accompanied with bacterial contamination; however it is not a prerequisite for diagnosis [51]. Inflammation after parturition is expected, however, if sustained over long periods of time, it can result in decreased fertility and reproductive performance [13,51–53] and that in the absence of bacterial infection, inflammation can still have detrimental effects on fertility [54].

Figure 1.2 is a compilation of data from four different studies complied by Sheldon and Dobson [48] which details the proportion of dairy cows with bacteria contamination of the uterus in the first 60 days postpartum. While it is evident that the first 2 weeks postpartum is when we can expect bacterial contamination, this does not always result in uterine infection [51]. Sheldon and Dobson [48] discuss that the process of inflammation of the uterine lining is common and a necessary part of the innate immune system to for the clearance and sloughing of tissue and bacterial contaminants.

Various methods exist for evaluation of uterine health in order to classify the severity of bacterial contamination or inflammation. Williams et al. [55] linked characteristics of vaginal mucus with uterine bacterial contamination, by associating purulent or fetid odor of the mucus with pathogenic bacteria. This evaluation and comparison was achieved by evaluating and scoring the color and content of the vaginal mucus (Figure 1.3) as well an additional binary score of fetid odor. Williams et al. [52] also associated increased peripheral plasma concentrations of α_1-acid glycoprotein (an acute phase protein (APP)) with a fetid mucus score and with increased growth of uterine pathogens cultured from uterine swabs.

Brodzeki et al. [56] examined the response of pro-inflammatory cytokines and APPs both in peripheral blood and in uterine washings in cows diagnosed with subclinical endometritis and found significant increases in blood pro-inflammatory cytokines TNF-α, IL-6, and IL-10, as well as APP's serum amyloid A (SAA), and haptoglobin (HP) when compared to healthy cows. From the uterine washings they observed increased concentrations of IL-6 and IL-10, HP when compared to healthy cows. As mentioned before, the immune response of the cow is suppressed at parturition and even further perturbed by HC [19]. This "blunted" response delays the clearance of bacterial contamination in the uterus, and thus prolongs the involution process.

Hepplemann et al. [57] observed cows with SCH had delayed reduction in uterine length between days 8-21 postpartum when compared to normocalcemic cows.

In addition to evaluation of vaginal mucus, pro-inflammatory cytokines, and APP, a cytological approach can be used to evaluate for inflammatory stress from metritis and endometritis. Recent studies have utilized cytological methods to better understand the immune response and function in the uterus of the cow in the periparturient period [11,58–60]. Gilbert and Santos [59] observed the proportion of polymorphonuclear (PMN) cells present in the uterus from 0-7 days postpartum was negatively correlated with proportion of PMN cells at 5 or 7 weeks postpartum ($r = -39$) and positively correlated with fertility ($r = 0.41$). Dourey et al. [60] observed that cows with a low proportion of PMN cells ($\leq 8\%$) at 25 days postpartum had significantly shorter interval from calving to first ovulation (32.4 vs 45.3 days) than cows with a high proportion of PMN cells ($> 8\%$).

Endometrium tissue function is extremely important in maintaining a physical barrier in the innate immune system following parturition. Sustained bacterial contamination of the uterine lumen can stress the endometrium tissue, resulting in upregulation of inflammation related genes, failure of barrier functions, and prolonged exposure to endotoxins. The increased metabolic needs during the periparturent period requires a considerable increase in oxygen. Molecular oxygen is required as an electron acceptor for any cell/organism that undergoes aerobic metabolism. Once used, a subset of free radicals called reactive oxygen species (ROS) are formed as end products of normal metabolic processes such as formation of ATP in the mitochondria or the electron transfer facilitated by NADPH oxidase on the plasma membranes of of the cell. Reactive oxygen species are vital in some aspects of cell signaling (redox signaling) and cell homeostasis [61]. The most prominent of the ROS molecules is the superoxide

anion $^{\bullet}O_2^-$. However, an accumulation of superoxide can result in oxidative stress by increasing the production of additional ROS such as OH^- , and cause positive feedback of lipid peroxidation in the plasma membrane of cells [62]. There are biological mechanisms that catalyze superoxide and diminish its oxidative potential. Superoxide dismutase (SOD) is an antioxidant enzyme found in three different isoforms in the mitochondria (SOD2), cytosol (SOD1), and extracellular plasma membrane (SOD3). All three isoforms of SOD derive from distinct genes and specific localization within the cell, however all three forms catalyze the same reaction of dismutation ofsuperoxide to H_2O_2 and further reducing it to H_2O via catalase or glutathione peroxidase (GPX). Superoxide dismutase and GPX both work in concert to maintain a proper redox environment within the cell [61]. Ramos et al. [63] study in beef cows observed that endometrium tissue of cows with reduced GPX and catalase activity are more prone to lipid peroxidation and associated with smaller follicles and smaller corpus luteum (CL). Bacterial contamination has been associated to induce oxidative stress in tissues, via inflammatory cytokine release in response to PAMPs from bacteria [64].

UTERINE GLAND MORPHOLOGY

A critical role of the endometrium tissue is to facilitate glandular secretion into the uterine lumen. These glands, located throughout the uterus, densely populate the areas between caruncles (superficial sites of implantation and placentation) and continually undergo hyperplasia throughout gestation [65]. Gray et al. [66] observed that sheep that had uterine adenogensis inhibited, had failure of conceptus implantation, receptivity to the uterus, and decidualization. One of the most critical glandular secretions is the secretion of prostaglandin $F_{2\alpha}$ (PGF) to cause luteolysis or regression of the CL when the animal is not pregnant. If the unbound conceptus released from the ovulatory follicle reaches the uterus, it will release IFN-τ to inhibit oxytocin

receptors so that oxytocin cannot stimulate PGF synthesis and thus halting the estrus cycle. At the time of parturition, the uterine tissue is subjected physical stress (detachment of the placenta and movement of the calf), oxidative stress and infection from bacterial contamination, and a dampened immune response [17,19,51]. If the uterus of the periparturient cow is unable to heal or involute properly, the tissue damage, specifically of the glandular epithelial cells can decrease the functionality of the uterine endometrium and glands, which might reduce fertility postpartum [58,67,68].

OVARIAN FOLLICULAR DYNAMICS

Aside from maintaining a healthy status throughout the transition period, synchronization protocols have been implemented to improve conception and pregnancy rates. Approximately 80% dairy producers in the United States utilize artificial insemination (AI) to breed their herds and approximately 55% of these producers breed cows after detections of estrus in their natural estrus cycle [69]. After parturition, the cow will be placed in a voluntarily waiting period (VWP) to allow for the involution of the reproductive tract and return to ovarian cyclicity. The VWP on average is 50 to 60 days [69] in the United States. At the end of the VWP the producer will utilize numerous reproductive management practices to detect estrus to maximize reproductive efficiency. To achieve a successful insemination, the breeder will have to time the moment of AI with the time the dominant follicle ovulates. Aforementioned, a little over half of dairy producers use natural estrus detection methods (standing to be mounted, pedometers, tail chalk) to time artificial insemination. Generally, most producers will breed within 24 hours of the onset of estrus behavior with about 70% success rate (measured as pregnant vs non-pregnant at first service; [69]).

To try to optimize on breeding conception rates, various hormonal synchronization protocols have been developed that would allow for a timed artificial insemination (TAI). This allows the producer control over the ovulation cycle of the cow so that the ovulation of the dominant follicle is almost guaranteed with the TAI. The goal of synchronization programs are to administer reproductive hormones at critical points in the ovulation cycle to encourage ovulation of the dominant follicle at a set time range. Gonadotropin-releasing hormone (GnRH) and prostaglandin $F_{2\alpha}$ (PGF) are the two most common reproductive hormones used in synchronization protocols. Gonadotropin-releasing hormone stimulates the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary gland to act on the ovary of the cow to stimulate follicular growth (FSH) and stimulate ovulation of the dominant follicle and maintenance of the corpus luteum (CL). Prostaglandin $F_{2\alpha}$ acts upon the CL to initiate luteolysis.

In cattle, ovary follicles develop in wave-like patterns with an ovulation or atresia ending each wave and initiating a new wave. At the start of each wave, follicles are recruited from a pool of growing follicles on the ovary. From the recruited follicles, one will begin to dominate in growth. This dominant follicle will continue to grow and in the absence of progesterone, will become the ovulatory follicle and will ovulate following standing estrus. In the presence of progesterone, the dominant follicle will undergo atresia and initiate a new follicular wave. Often two to three waves will occur before ovulation of the dominant follicle (Figure 1.4). The cells that make up the ovulated follicle become luteal cells to form the CL. The primary purpose of the CL is to produce progesterone, which maintains an environment in the uterus for conception and maintenance of pregnancy if fertilization occurs. Perhaps one of the most successful synchronization protocols is the Double-Ovsynch™ protocol. This protocol

allows tight control of the follicular development and luteal regression. This protocol is initiated with an injection of PGF, this will cause regression of the existing CL (if present) and begin a new ovulating cycle. After 14 days, another injection of PGF is given to cause regression of the new CL, from the previous PGF injection. Fourteen days is necessary to allow the cow time to grow a dominant follicle, ovulate, and create a new CL. After 12 more days, the first injection of GnRH is given to either cause ovulation of the existing dominant follicle or continued growth of a non-dominant follicle. After 7 more days, the last injection of PGF is given to cause CL regression, and prime the growing dominant follicle for ovulation. Finally, after 48 hours, the last GnRH injection is administered to promote the growth and ovulation of the dominant follicle. After, 12-16 hours the cow is bred to ensure the sperm are present in the reproductive tract for when the egg from the ovulated follicle arrives.

Conversely to the genetic selection of high producing milking cows, reproductive efficiency in dairy cows has been in decline for the last two decades [70–72]. While the underlying reasons to the negative association between milk production and fertility is multifaceted, the physiological changes of various tissues of the dairy cow undergo to support the mammary gland are taxing to the reproductive system of the cow, particularly during the periparturient period [73–75]. Butler et al. [76] associated a NEB with lower fertility and reduced circulating progesterone at critical points of the ovulation cycle and failure to return to normal cyclicity of the ovaries. After calving, there are two primary objectives that the cow must accomplish to maximize successful breeding: restoring uterine health and a return to normal ovarian cyclicity. As aforementioned, the restoration and involution of the uterus post calving has numerous challenges to overcome in the first 4 weeks postpartum. However, numerous studies have linked ovarian cyclicity with uterine health [68,71,75,77]. It can be expected that

there is a direct relationship between uterine health and ovarian function. Hypocalcemia and SCH at parturition has been strongly associated with decreased odds of pregnancy at first service and that chronically SCH cows had a negative effect of return to ovarian function [24]. Sheldon et al. [77] observed that uterine bacterial contamination was associated with decreased dominant follicle diameter and circulating estradiol and FSH concentrations. Herath et al. [78] observed that the granulosa cells of a recruited or dominant ovarian follicle express TLR-4, CD14, MD-2 receptor complex (PAMP receptors) throughout follicular development. Herath et al. [78] also observed that granulosa cells exposed to LPS *in-vivo* produced less estradiol *in vitro* when cultured in absence of immune cell contamination. The localized immune capability of the follicular granulosa cells perturbs the follicle steroidogenesis. Bromfield and Sheldon [68] mimicked the LPS concentrations observed in follicular fluid during an active uterine infection by culturing freshly harvested ovarian cortexes in medium with 0.1, 1, and 10 μg/mL of LPS. Bromfield and Sheldon observed that the primordial follicle pool was reduced in the ovarian cortex, and that there was an accumulation (inflammatory response) of inflammatory cytokines with increasing concentrations of LPS in the ovarian cortex.

TABLES AND FIGURES

Table 1.1. Composition of inorganic mineral sources and absorption coefficient of Calcium

Calcium Sources	Dry Matter (%)	Crude Protein	Calcium Content (%)	Absorption Coefficient
Bone meal, steamed, fg[2]	97	13.2	30.71	0.95
Calcium carbonate, fg	100	-[4]	39.39	0.75
Calcium chloride anhydrous, cp[3]	100	-	36.11	0.95
Calcium chloride dehydrate, cp	100	-	27.53	0.95
Calcium propionate, fg	94	-	21.50	0.90
Calcium hydroxide, cp	100	-	54.09	0.55
Calcium oxide, fg	100	-	71.47	0.50
Calcium phosphate, fg	97	-	16.40	0.95
Calcium sulfate dihydrate, cp	97	-	23.28	0.70
Curacao, phosphate, fg	99	-	34.34	0.71
Dicalcium phosphate, fg	97	-	22.00	0.94
Dolomitic limestone, fg	99	-	22.30	0.60
Limestone, ground, fg	100	-	34.00	0.70
Magnesium oxide, fg	98	-	3.07	0.70

[1]Table derived from NRC ([4]; Table 15-4)
[2]Fg = feed grade
[3]Cp = chemically pure
[4]Not present

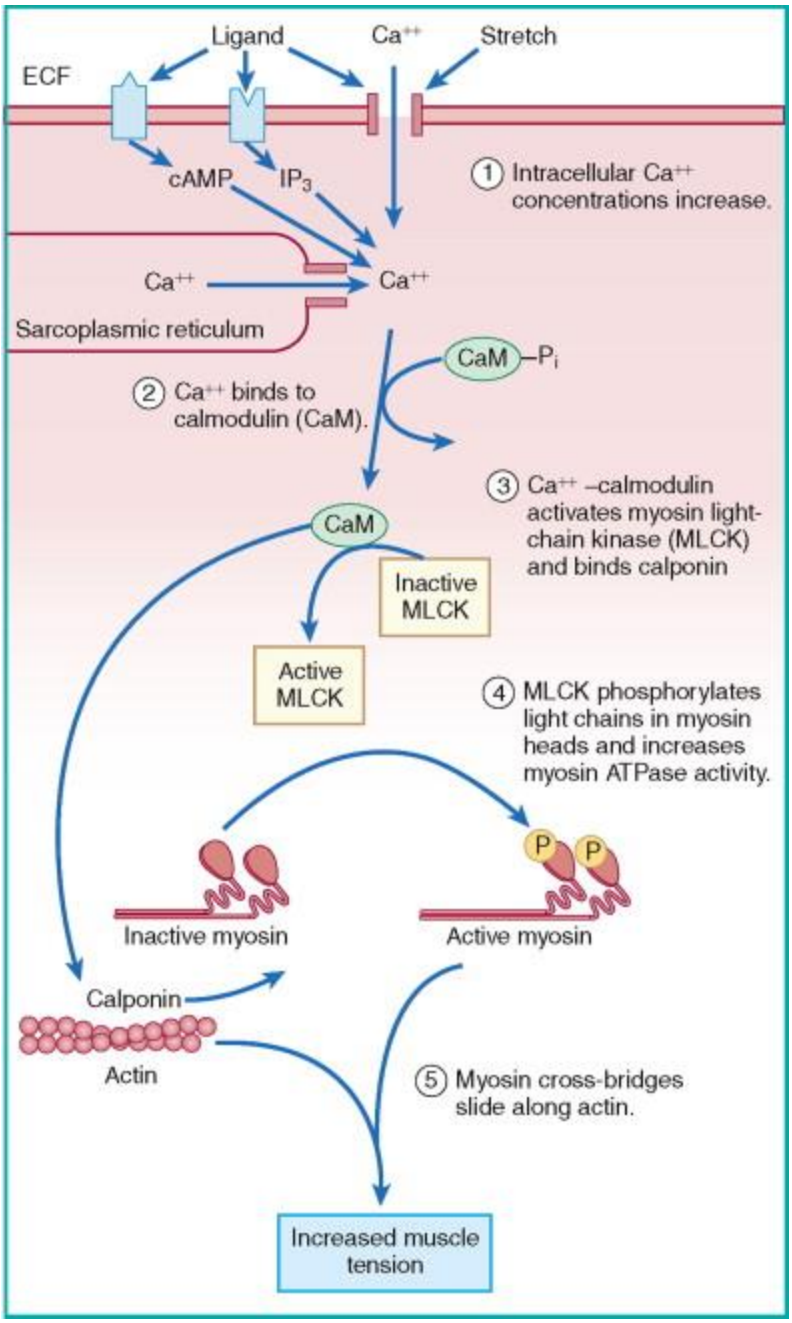

Figure 1.1. Calcium mechanism of smooth muscle contraction. Ca^{2+} enters the cell of the smooth muscle (1) and binds to calmodulin (2; CaM) from where CaM acts on muosin light-chain kinase (3; MLCK) to phosphorylate light-chains in the myosin head to the active form (4) to increase ATPase activity, and finally, the myosin cross-bridges slide along the actin chains (5) to increase muscle tension (contraction). Credit: Goff (2008)

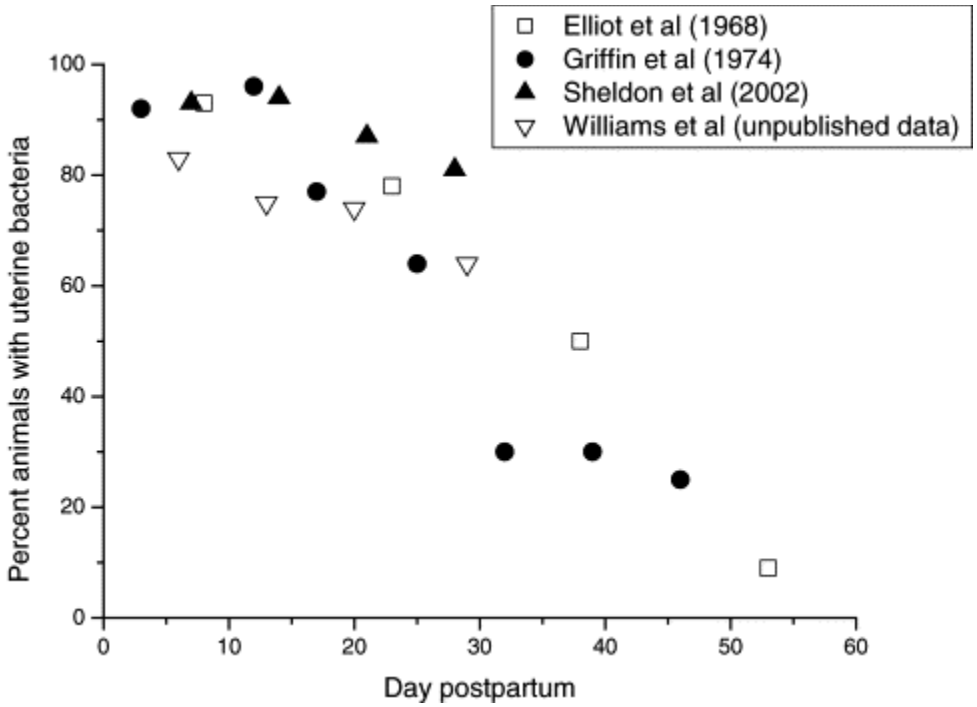

Figure 1.2. Proportion of uteri contaminated with bacteria in the first 60 days postpartum from data of Elliot et al. [79], Griffin et al. [80], Sheldon et al. [81], and Williams et al. [55]. Complied by: Sheldon and Dobson [48].

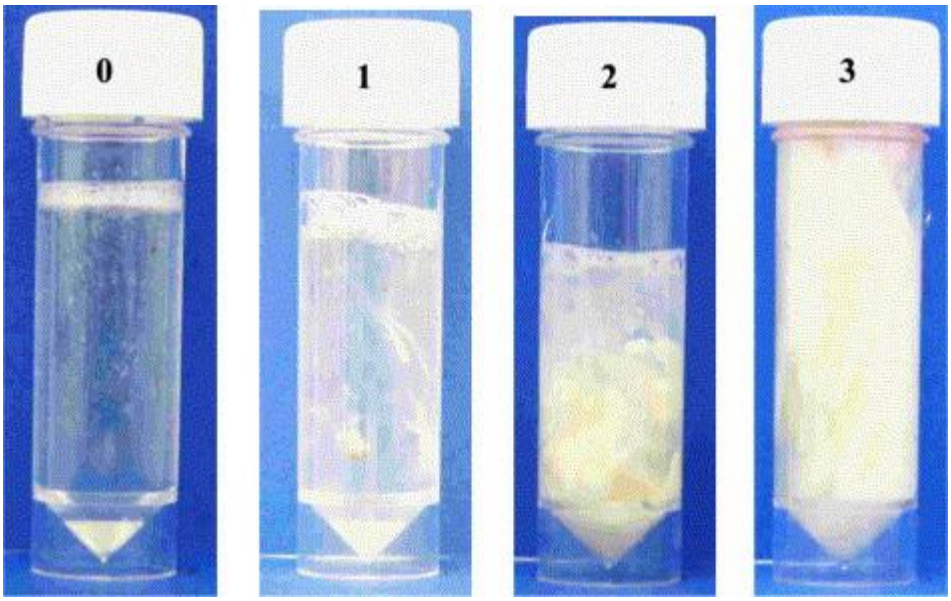

Figure 1.3. Scoring system for evaluation of vaginal mucus. Credit: Williams et al. [55].

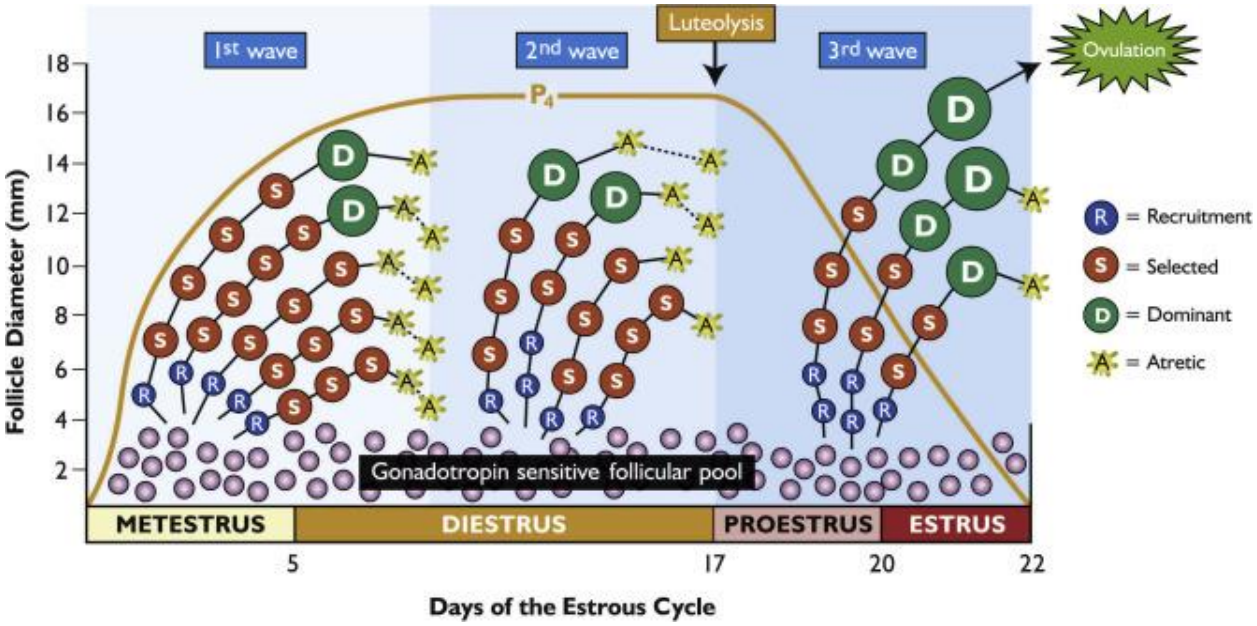

Figure 1.4. Follicular waves of the bovine dominant follicle through the stages of estrus. P4 = progesterone. Image adapted from Pathways to Pregnancy & Parturition [8]

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CHAPTER II. EFFECTS OF FEEDING AN ACIDOGENIC DIET AT TWO DIETARY CALCIUM INCLUSION RATES TO CLOSE UP DRY COWS ON THE SUBSEQUENT LACTATION, UTERINE HEALTH, AND FERTILITY

INTRODUCTION

The transition period is a crucial point in time for the cow in ongoing health and production of the current and future lactations of the cow. During this period of time, the cow is subjected to various stressors that include metabolic disorders, prolonged inflammation, and most prevalently, subclinical hypocalcemia (SCH) [1–4]. As the periparturient cow transitions from a pregnant and non-lactating status, to lactation, her energy and nutrient demands multiply [5,6]. In the first 3 days following parturition, the cow is often in a negative energy balance as well as negative calcium status [4,7,8]. If chronic or prolonged, the cow becomes predisposed to disorders observed around calving such as retained placenta, displaced abomasum, ketosis, metritis and mastitis [6,9,10]. If left untreated, SCH can develop into clinical hypocalcemia [11], which can reduce the ability of immune cells to respond to stimuli [10], thus increasing susceptibility to infections such as metritis [11]. Many strategies have been employed to treat SCH such has Ca boluses, or injections of Ca salts [9], however, these are often only treatments to the to clinical signs. An effective preventative strategy in controlling SCH is to feed an acidogenic diet with a negative dietary cation-anion difference (DCAD) in the prepartum phase of the transition period [12,13]. This diet effectively reduces Ca absorption in the prepartum phase to maintain calciotropic hormones (parathyroid hormone; PTH) sensitivity so that the cow has the capacity to mobilize and absorbed Ca in the post-partum period [1,9,14,15].

Inflammation related blood metabolites, such as lipopolysaccharide binding protein (LBP), serum amyloid A (SAA), and haptoglobin (HP), are key indicators of systematic

inflammation [16,17]. These indicators can be related to inflammation throughout the body, however, at parturition, the uterus of the dairy cow will be contaminated with a host of pathogens [18] and this contamination can develop into severe uterine inflammation (metritis). The immune response during the peripartum period is known to be suppressed due to the sudden change to a negative energy balance system [3]. The depression in the immune response can diminish the ability of the dairy cow to tolerate the pathogens within the uterus [19], and furthermore enhance the severity of inflammation within the uterus, thus, prolonging the return to normal cyclic activity of the ovaries [20].

The return of normal ovarian cyclic activity is dependent on the uterine involution process and return to a non-inflammatory state. Uterine infection and inflammation prolongs the uterine involution process [21] with detrimental effects on ovarian function [22] as well as uterine function (hormone secretion and uterine adenogenesis). At the time of parturition, the dairy cow experiences physical trauma and bacterial contamination of the endometrium. This damage and contamination of the endometrium initiates an immune response in the sites of infection and trauma,and reactive oxygen species (ROS) signaling might be activated. Reactive oxygen species, a common byproduct in cellular metabolism, production is tightly controlled by antioxidant enzymes,primarily superoxide dismutase (SOD) and glutathione peroxidase (GPX), to maintain the reduction-oxygenation (redox) environment within the cell [23]. However in high concentrations, such that it overwhelms the tissues antioxidant capacity, ROS can cause damage to cellular macromolecules (lipids and proteins) and induce cell death (apoptosis) [23]. Pathogen associated molecular patterns (PAMPs), such as lipopolysaccharides from the cell wall of gram-negative bacteria, also produce ROS once bound to the cell PAMP receptors [24]. Kimura et al. [10] observed that cows diagnosed with

HC experience a blunted immune cell response at the time of parturition, possibly due to the unavailable Ca^{2+}, an important secondary signaling molecule in immune response pathways.

The objective of this study was to determine the effects of feeding a negative DCAD diet in the 4 weeks before expected calving date at two different concentrations of dietary Ca inclusion. Ovarian development, likelihood of conception, uterine health, and inflammatory blood metabolite evaluations were used as markers to determine the effect of feeding a negative DCAD diet at 2 concentrations of Ca inclusion on overall reproductive health.

MATERIALS AND METHODS

ANIMALS AND EXPERIMENTAL DESIGN

The following procedures were approved by the University of Illinois Institutional Animal Care and Use Committee (IACUC # 16115). A diagram with the experiment design is in Figure 1. Holstein cows (n = 81) entering their second or greater lactation were included in the experiment from 50 days before calving until 75 days in milk (DIM). The experiment began in September 2016 and concluded in December 2017. Descriptive characteristics of the cows enrolled can be found in Table 2.1. Cows were housed in a free stall style barn at the University of Illinois Dairy Research Farm during the dry period before being moved to a tie stall style barn until 30 DIM and then finally moved into free stall style lots for the remainder of the trial. A complete total mixed ration (TMR) was provided during the dry and lactation period that met but did not exceed the energy requirements of the cows. Cows were blocked (n = 28) for treatment assignment based on parturition, with cows entering into their second lactation in one block and cows entering into their third or greater lactation in another block. Cows were also blocked based on expected calving date, with all three treatments represented in each group of three cows within the

previously described blocks. The treatments were further balanced based on previous milk production and pre-partum body condition score. Previous milk production was calculated through a PC Dart (Dairy Records Management Systems, Raleigh, NC) report of past 305 d milk production and determined as high if it was above 25,000 lbs. Body condition score was assigned at the beginning of the close-up period and was labeled high if above 3.5 on the 5 point dairy scoring scale. Treatments were balanced to be equally represented in high and low milk producers, as well as under and over conditioned cows. Treatment began at 28 days before expected calving and ended at parturition. Treatments were CON: Positive DCAD (+6 mEq/100g of DM; urine pH > 8.0) with low dietary Ca (0.40% of DM; 46.2 ±15.2 g Ca/d; n = 26); LOW: negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with low dietary Ca (0.40% of DM; 44.1 ± 16.1 Ca/d; n = 24); HIGH: Negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with high dietary Ca (2.0% of DM; 226.6 ± 96.0 g Ca/d; n = 26).

VAGINAL DISCHARGE AND EVALUATION

Evaluations of vaginal discharge were performed at 4, 7, 10, 13, 15, 17, and 30 DIM via the Metricheck® device (MC, Simcro, New Zealand). The device is composed of a 50 cm long stainless steel rod with a 4 cm rubber hemisphere to collect vaginal contents. The MC was disinfected before and after each use in a single cow with chlorhexidine diacetate disinfectant (Nolvasan Solution, Zoetis Animal Health, Florham Park, NJ). The evaluation began with cleaning the perineal region of the cow with a paper towel and disinfectant solution. The tail was then moved to the side and the MC was inserted into the vaginal canal until the cervix was reached. The device was then retracted and removed from the reproductive tract with the vaginal contents remaining in the rubber hemisphere. With the vaginal content in the rubber hemisphere, evaluation of smell was quantified (smell 0 = no odor or smell 3 = fetid odor). The vaginal

contents was then examined and scored on a scale of 0 – 3: score 0 = clear or translucent mucus; score 1 = mucus containing flecks of white or off-white pus; score 2 = discharge containing ≤ 50% white or off-white mucopurulent material; and score 3 = discharge containing ≥ 50% purulent material, may be white, yellow or sanguineous [20; https://youtu.be/jIhd8buSpHU]. Rectal temperature and ultrasonography of uterine content (yes = contained hyperchogenic material or no = contained no visible material) were also obtained after vaginal evaluation. The MC was not used if the cow had retained placenta or visible vulvar damage at the time of evaluation.

Cows were evaluated for metritis based on farm protocol, which consisted of visual inspection of exuded vaginal content for purulent material accompanied by fever (≥ 54°C). Cows were also considered to have metritis based on MC parameters if score = 3, smell = 3, or the combination of score plus smell was ≥ 3 on 4-17 DRC or ≥ 2 on 30 DRC.

CYTOLOGY OF THE UTERINE ENDOMETRIUM

Cytology of the endometrium was performed using a cytology brush (Andwin Scientific, CA) at 15 and 30 DIM. The sterile cytology brush was mounted to a sterile stainless steel cytology rod and inserted into a larger sterile stainless steel rod (SSR) covered with a plastic sleeve for easy passage through the cervix and into the uterine body without contamination. Prior to the procedure, the cow was restrained and the vulva was cleaned with water and 70% ethanol. After passage of the cytology rod through the first ring of the cervix, the SSR was exposed through the plastic sleeve and was advanced into the uterine body. Once inside the uterine body, the outer SSR was pulled back to expose the cytology brush. The SSR that was mounted to the cytology brush was then rotated three times while the cytology brush remained in contact with the endometrium. Finally, the cytology brush was retracted back into the outer SSR and removed

from the reproductive tract. The SSRs were washed and autoclaved between each day of use. If multiple samples were being taken within a single day, the SSRs were sanitized in a chlorhexidine diacetate disinfectant solution between each animal.

Cytology slides were prepped immediately following the procedure by rolling the cytology brush onto a clean glass microscope slide and fixed using a cytology fixative (Cytoprep, Fisher Scientific, Pittsburg, PA). Once the fixative was dry, the samples were transported to the laboratory where they were stained with a differential stain (Camco Quik Stain 2 – Self Buffered Differential Wright-Giemsa Stain, Cambridge Diagnostic Products, FL). After being allowed to dry for 24 hours, the slides were covered using a mounting medium (Permount, Fisher Scientific, Pittsburg, PA) and dried for at least 48 hours before being scanned. All slides were scanned at the Institute for Genomic Biology at the University of Illinois with 20 magnification using whole slide imaging (Nanozoomer Digital Pathology System, Hamamatsu Photonics, Japan). Five areas were captured at 20 magnification from five separate locations, one image from each corner of the sample area of the slide and one image from the center, to represent the entire population of the slide (NDP.view software, Hamamatsu Photonics). A minimum of 100 cells were counted within the individual areas (ImageJ, National Institutes of Health, MD) and the percentage of PMN to epithelial cells was determined. The cell counting was performed by the same technician for all samples. Endometritis was classified if the proportion of PMN at 15 DIM was greater than 40% and at 30 DIM if the proportion was greater than 18% [25]. Likelihood analysis of being classified as having endometritis at 15 or 30 DIM was then determined based on these values.

ENDOMETRIUM BIOPSY

Endometrial tissue was collected transcervically from the body of the uterus from all eligible cows at 30 ± 1.32 DIM. The biopsy was not performed if the cow had a MC score of 3, a smell score of 3, or if the body temperature, pulse or respiration rates were abnormally high. The biopsy instrument (Aries Surgical, Davis, CA) were covered with a sanitary disposable sleeve and inserted into the vagina. The biopsy forceps was positioned at the cervical opening, and the sleeve was retracted to force the instrument through the end of the sleeve. The exposed biopsy forceps was then threaded through the cervix and into the uterine body. Endometrial tissue was collected at a location approximately 1 cm beyond the end of the cervix. The endometrium tissue, if it was an adequate size, were split into 3 portions. The first piece was placed into a cryovial (ThermoFisher Scientific, Waltham, MA) and immediately placed into liquid nitrogen, where it was kept until analysis. The next two portions of the sample were placed into formalin for 24 hours or until fixed in the solution. One portion of the sample was set in a paraffin block at the University of Illinois Veterinary Diagnostic Lab for analysis of glutathione peroxidase (GPX), superoxidase dismutase (SOD), and haematoxylin and eosin staining (H&E).

Tissue samples that were stained with GPX and SOD antibodies were then evaluated for concentration of cells stained positive for their respective enzyme activity (Figure 1) following the same protocol as aforementioned PMN cell concentration from cytology samples. Samples were further evaluated to determine samples with high concentration of cells with GPX and SOD activity (HGPX and HSOD, respectively), and low concentration of cells with GPX and SOD activity (LGPX and LSOD, respectively). High concentration of cells with GPX activity were classified when concentrations was ≥74%, high concentration of cells with SOD activity were

classified when concentrations was ≥64%. Classification of high and low concentrations were determined from median values.

ULTRASONOGRAPHY OF OVARIAN STRUCTURES

The first postpartum follicular growth cycle was monitored at 7, 9, 11-17, 20, and 30 DIM via transrectal ultrasonography (7.5-MHz linear array probe, E.I. Medical Imaging, Loveland, Colorado). Follicular growth continued to be monitored in tandem with the synchronization protocol until 75 DIM at which the cow was bred. Ultrasonographic videos of ovarian structures were recorded to allow drawing of ovarian structures and measurement of the follicles present. Once the dominant follicle was ≥ 5 mm in diameter in the recordings, measurements were continually recorded at all relevant time points until the end of the first follicular wave. Ovulation was classified as the disappearance of the previously identified dominant follicle and the appearance of a corpus luteum (CL) in the subsequent examinations.

BLOOD SAMPLING AND ANALYSIS

Blood was sampled from the coccygeal vein or artery at 21 and 7 days before expected calving and at 15 and 30 days after calving from each cow for serum and plasma collection (BD Vacutainer; BD and Co., Franklin Lakes, NJ). Additional time points for blood sample were shared from the other portion of this study. These serum and plasma samples were obtained by centrifugation of the tubes at 699 × *g* for 15 minutes and stored at −80℃. Plasma lipopolysaccharide-binding protein (LBP; Human LBP Multispecies Reactive ELISA Kit, Cell Sciences, Newburyport, MA) and serum amyloid A (SAA; PHASE^{TM} Range Multispecies SAA ELISA kit, Tridelta Development LTD, Maynooth, Ireland) were assessed at 30, 21, and 7 days before expected calving and 15 and 30 days after calving. Haptoglobin (HP; PHASE^{TM} Range

Haptoglobin kit, Tridelta Development LTD, Maynooth, Ireland) was assessed at 14, 7, 4, 2, and 1 days before expected calving and 1, 2, 4, 7, and 14 days after calving.

STATISTICAL ANALYSIS

Variables with measurement over time, the MIXED procedure of SAS was used to model the fixed effects of treatment, day, and block using the following model:

$$Y_{ijk} = \mu + T_i + D_j + T_i \times D_j + B_k + \varepsilon_{ijk}$$

Where Y_{ijk} = the observations for dependent variables; μ = the overall mean; T_i = the fixed effect of the i^{th} treatment; D_j = the repeated measurement (days relative to calving) effect; $T_i \times D_j$ = the interaction of treatment and repeated measurement; B_k = effect of the k^{th} block; and ε_{ijk} = the random residual error. The estimation method was restrictive maximum likelihood (REML) and the degrees of freedom method was Kenward-Rogers [26]. Variables were subjected to 5 covariance structures: compound symmetry, autoregressive order 1, autoregressive heterogeneous order 1, unstructured, and toeplitz. The covariance structure that yielded the lowest corrected Akaike information criterion was compound symmetry and used in the model (Littell et al., 2002). Cow was the experimental unit and considered as a random effect. Two contrasts were used: Contrast 1 (CONT1): CON compared with the average of LOW and HIGH; contrast 2 (CONT2): LOW compared with HIGH.

A logarithmic transformation was used for pre- and postpartum circulating LBP and HP concentrations for normality and homogeneity of residuals. A square root transformation was used for prepartum SAA concentration, and the number of uterine glandular epithelial cells for normality and homogeneity of residuals. Least squares means and standard errors shown for these variables were back transformed.

Multivariable logistic mixed models (PROC GLIMMIX) considering the likelihood of endometritis (based on PMN cell concentration) at 15 and 30 DRC, metritis (based on MC score, MC smell, and MC score + smell) at 4 - 17 and 30 DRC, retained placenta, development of CL at 30, 57, 64, follicle at 71 DRC, success of first TAI at 75 DRC, and high or low GPX or SOD concentration at 30 DRC. Treatments were forced into the models, with cows considered as a random effect. Significance was declared at $P \leq 0.05$ and trends at $0.05 \leq P \leq 0.11$.

RESULTS

EXCLUSIONS FROM ANALYSIS

Cows excluded from experiment based on treatment delivery are in Table 2.2. Cows excluded from metricheck (CON: n = 1; LOW: n = 2; HIGH: n = 2). Cows excluded from PMN cell evaluation (CON: n = 5; LOW: n = 6; HIGH: n = 4). Cows excluded from endometrial biopsy (CON: n = 9; LOW: n = 6; HIGH: n = 8).

BLOOD METABOLITES

Treatments did not differ for LBP or SAA in both prepartum and postpartum periods (Table 2.3). Haptoglobin concentration was higher in LOW than HIGH cows in both prepartum (CONT2: $P = 0.01$) and postpartum (CONT2: $P = 0.03$) periods. The HP concentrations postpartum were greater (CONT1: $P = 0.01$) in CON cows than LOW and HIGH cows. There was a DRC effect for LBP, SAA, and HP (Figures 2.3a, 2.3b, and 2.3c, respectively) in both prepartum ($P < 0.001$, $P < 0.0001$, and $P = 0.024$, respectively) and postpartum ($P = 0.006$, $P = 0.02$, and $P < 0.001$, respectively). There was a tendency for treatment × DRC interaction ($P = 0.06$), indicating cows fed CON diet had increased circulating HP concentrations in the first 4

days postpartum. There was a tendency ($P = 0.06$) for HP concentration postpartum to be greater CON cows than cows fed LOW or HIGH calcium diets.

UTERINE HEALTH EVALUATION

Treatments differed for MC ($P = 0.02$; Table 2.4), indicating cows fed CON had a lower a MC score than the average of cows fed LOW and cows fed HIGH. There was a tendency ($P = 0.11$) for cows fed LOW to have a higher MC score than cows fed HIGH. There was a tendency ($P = 0.10$) for cows fed CON to have lower proportion of PMN cells in the uterus than the average of cows fed LOW and cows fed HIGH. There was a tendency for a treatment effect ($P = 0.06$; Figure 2.4a) for cows fed LOW to have a higher MC score than cows fed CON and cows fed HIGH. There was a DRC effect for MC score (Figure 2.4a; $P < 0.001$), MC score + smell (Figure 2.4b; $P < 0.0001$), and proportion of PMN cells in the uterus (Figure 2.4c; $P < 0.001$). Tables 2.5a-2.5c shows the likelihood of cows developing metritis based on MC score, smell, and score + smell. At 13 DRC, cows fed CON were more likely to develop metritis based MC score (Table 2.5a) and MC score + smell (Table 2.5c) than cows fed HIGH (OR = 6.01; $P = 0.01$) or LOW (OR = 7.35; $P < 0.01$). There was no treatment effect (Table 2.6) for the likelihood of endometritis at 15 DRC or treatment effect on the likelihood of retained placenta postpartum. However, at 30 DRC, cows fed LOW tended ($P = 0.08$) to be more likely to have endometritis than CON.

FOLLICULAR GROWTH AND DEVELOPMENT

Ovulation dynamics (Table 2.7) differed in days to first ovulation ($P = 0.05$), postpartum, indicating cows fed CON had more days to first ovulation than the average of cows fed LOW and cows fed HIGH. There was a DRO effect (Figure 2.4; $P < 0.001$) in that the diameter of the

first dominant follicle postpartum was increasing in size until ovulation. There was a tendency for a treatment × DRO interaction (Figure 2.4; $P = 0.11$) indicating cows fed CON had a slower rate of growth in days 4-2 before ovulation of the first dominant follicle postpartum than cows fed LOW or HIGH. There was a tendency (Table 2.8; $P = 0.09$) for cows fed HIGH to be more likely (OR = 0.22; CON: 3 of 15 confirmed pregnant; HIGH: 11 of 21 confirmed pregnant) to be pregnant at the first timed artificial insemination than cows fed CON.

UTERINE GLANDULAR MORPHOLOGY AND IMMUNOLABELING

Uterine glandular morphology and immunolabeling results are in Table 2.9. There was no treatment difference for endometrial glandular area and perimeter; however, cows fed CON tended ($P = 0.06$) to have shorter glandular epithelial height at 30 DIM when compared to the average of cows fed LOW and cows fed HIGH. Cows fed LOW had ($P = 0.02$) shorter glandular epithelial height than cows fed HIGH at 30 DIM. Cows fed LOW had ($P = 0.05$) fewer epithelial cells per gland than cows fed HIGH. There were no differences of endometrial tissue oxidative stress indicators glutathione peroxidase (GPX; $P = 0.32$) and superoxide dismutase (SOD; $P = 0.31$) between cows fed CON and the average of cows fed LOW and cows fed HIGH. Cows fed LOW had ($P < 0.0001$) higher concentration of cells stained positive for GPX than cows fed HIGH, and cows fed LOW had ($P = 0.05$) lower concentration of cells stained positive for SOD than cows fed HIGH. Finally, cows fed CON tended to have an increased likelihood of being classified as having a high concentration of SOD than cows fed HIGH (Table 2.10; $P = 0.08$) or cows fed LOW ($P = 0.06$)

DISCUSSION

The goal of this study was to evaluate the effectiveness of feeding a negative DCAD at two levels of dietary calcium inclusion on dairy cow reproductive function and health. Lipopolysaccharide binding protein, SAA, and HP are all acute phase proteins found in blood that are commonly used as an indicator of systemic inflammation. There were no treatment differences in circulating LBP concentration, however, as reported in previous literature, in the last week before parturition, there is an overall marked decrease in dry matter intake (DMI) [27]. Kvidera et al. [28] observed that feed restricted cows had increased concentrations of circulating LBP. In the current study, a decrease in DMI in the week before parturition can explain the marked increase of LBP observed just before parturition. Ametaj et al. [29] noted that circulating SAA and HP concentrations increases around parturition (from -4 – +3 days relative to calving) as a part of an acute phase response to parturition. Haptoglobin, which is essential in preventing iron uptake by bacteria which is critical to bacterial growth [30] and SAA, which binds to circulating toxins and is associated with clearance of lipoproteins by the liver [31], are essential in relieving inflammatory related stress that is associated with parturition. The differences observed in the current study indicates that cows receiving the negative DCAD treatment experience parturition-related inflammatory stress to a lesser extent than cows fed CON.

Bacterial contamination of the uterus after parturition is inevitable. However, not all cows develop metritis as a result. The continuation and degree of infection and the possible eventual development of metritis depends greatly on the bacterial load, pathogenicity, and the immune response in the uterus [18]. Decreased immune functions during the peripartum period has been documented in previous research [10,28,32] and has been suggested as the major contributor to the higher incidences of bacterial infection (i.e.; mastitis and metritis) in early-lactation cows. As

the uterus involutes. The severity of contamination lessens over time as the tissue sloughing and hemorrhaging of the endometrium ceases [20]. In the current study, the decrease in proportion of PMN cells in the uterus from 15 to 30 DRC agrees with previous literature. Polymorphonuclear concentration paired with the results of metricheck data over this period affirms this assumption. In the current study, cows fed a negative DCAD diet had significantly higher MC scores than cow fed CON, however, cows fed HIGH tended to score lower than cows fed LOW. As was observed by Caxieta et al. [43] the development of SCH or HC in early postpartum can attribute to increased incidences of metritis further into the fresh period. Glosson et al. [44] documented that feeding a negative DCAD (-24 mEq/ 100g of DM) pre-partum increases urinary Ca excretion (LOW: 8.4 g/d; HIGH: 13.4 g/d) in the pre-partum phase when compared to feeding a positive DCAD diet, (+6 mEq/ 100g DM) and that feeding higher amounts of Ca increases the rate of Ca excretion. In the same study, Glosson et al. observed circulating iCa concentrations were greater ($P < 0.01$) post-partum of cows fed LOW and cows fed HIGH, averaged together, when compared to cows fed CON. Glosson et al. also documented that incidences of SCH was greater in cows fed CON, when based on iCa. The increased iCa concentrations in cows fed the negative DCAD diets pre-partum could indicate a stronger immune response post-partum and explain the lower concentration of PMN in the uterus of cows fed CON observed in the current study and also the decreased likelihood of development of metritis at 13 DRC based on diagnosis from MC parameters.

Post-partum gland development and function are essential to uterine reproductive function. The tendency of effect of a negative DCAD (comparing cows fed CON vs cows fed LOW and cows fed HIGH) on the glandular epithelial height suggests that the negative DCAD alleviated tissue damage to the glandular epithelial cells. A negative DCAD coupled with supplementation

of high Ca (2.0% DM) amplified this alleviative effect. To our current knowledge, this effect of a negative DCAD and high Ca supplementation on glandular development has not been previously studied, however Martinez et al. [33,34] suggests that an improved Ca status around parturition led to lower incidences of endometritis, thus, less tissue damage. Our study also indicates differences in glandular epithelial cell numbers increased with increasing Ca supplementation while on a negative DCAD diet. These findings could indicate that a negative DCAD diet may alleviate uterine tissue damage and is amplified by increasing Ca supplementation.

Superoxide dismutase activity in cows fed HIGH was increased in comparison to cows fed LOW, while GPX activity was decreased in the same comparison. Superoxide dismutase and GPX work in concert to maintain a proper redox environment in tissue by reducing reactive oxygen species (ROS) that cause oxidative stress in high concentrations [23]. Ramos et al. [35] study on beef cows, indicated an increase in GPX activity with larger follicles and larger CL, which does not agree with the results from our current study. The loss or decrease of SOD activity has been linked with controlling tissue damage signaling by perturbing reactive oxygen species (ROS) signaling by lowering H_2O_2 concentrations [36]. Brigelius-Flohé [37] review of GPX function and activity in different tissues indicates that cytosolic GPX reduces cytosolic H_2O_2 concentrations to H_2O. In eukaryotic species, H_2O_2 is not only a mild ROS, but it is an important signaling molecule in immune cell activation [38]. Glutathione peroxidase, under oxidative stress conditions, is activated via nuclear factor-κB (NFκB), a key regulator in immune response to infection and inflammation [39]. The inverse relationship of the enzyme activities of SOD and GPX observed from cows fed HIGH in the current study is a part of the controlling mechanism of the redox environment within cells, and is an intrinsic controller to immune response activation.

Development of the dominant follicle of the first follicular wave postpartum is highly correlated with conception rates [40–42]. Butler [42] described a positive association of the early commencement of the ovulatory cycle and increased conception rates for the cow is able to have multiple ovulatory cycles before the first timed artificial insemination. In the current study, the average days to first ovulation, postpartum, of cows fed HIGH and cows fed LOW was less than cows fed CON. Caixeta et al. [43] observed cows that had abnormal Ca levels at the first three days after calving tended to take longer before returning to normal cyclicity. Martinez et al. [33] indicated that there were no differences in likelihood of pregnancy at first TAI between a positive DCAD (145 ± 11 mEq/kg DM) and a negative DCAD (-129 ± 11 mEq/kg DM) diet. However, in this study we observed a tendency for increased likelihood of cows fed HIGH to be pregnant after the first TAI in comparison to CON.

CONCLUSION

In conclusion, cows that received a negative DCAD diet prepartum had decreased days to ovulation of the dominant follicle of the first follicular wave after calving. Cows in HIGH had improved uterine immune response (i.e.; PMN at 15 DIM) than cows fed LOW. Additionally, cows that received DCAD diets had lower plasma haptoglobin concentrations and improved uterine glandular epithelial cell morphology than cows that received CON; moreover, cows fed HIGH had increased uterine glandular epithelial cells than cows fed LOW. Cows fed HIGH had greater SOD activity and lower GPX activity than cows fed LOW indicating an improved redox environment in the uterine tissue. Overall, providing a DCAD diet prepartum with increased calcium concentration (HIGH) enhanced the immune response in the days following parturition

TABLES AND FIGURES

Table 2.1. Descriptive characteristics of cows enrolled in the experiment

	Treatment[1]				
Variable	CON	LOW	HIGH	SEM	Totals
Initial body weight, kg	786.1	761.2	763.0	13.9	-
Body condition score, high (>3.5)	18	14	16	-	48
Previous 305 d milking equivalent, kg/lactation	12,275	12,077	12,304	124	-
Lactation number > 2	18	17	16	-	51

[1]CON: Positive DCAD (+6 mEq/100g of DM; urine pH > 8.0) with low dietary Ca (0.40% of DM; 46.2 ±15.2 g Ca/d; n = 26); LOW: Negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with low dietary Ca (0.40% of DM; 44.1 ± 16.1 Ca/d; n = 24); HIGH: Negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with high dietary Ca (2.0% of DM; 226.6 ± 96.0 g Ca/d; n = 26). From Glosson et al. [41].

Table 2.2. Cows excluded from current analysis.

Cow	Treatment	Reason[1]	DRC[2] treated	Date[3]
8764	CON	Received calcium for treatment of milk fever	2	4/14/2017
8807	LOW	-	1	3/21/2017
96	LOW	-	1	6/1/2017
8687	LOW	-	0	8/1/2017

[1]Cows were excluded from this analysis because the treatment with the additional calcium is incomparable to cows that did not receive any additional calcium in this study.
[2]Days relative to calving.
[3]Milk fever treatments were administered post-partum for 8764, 8807 and 96. 8687 received treatment for milk fever the day of calving, before parturition.

Table 2.3. Least squares means and associated SEM for blood metabolites.

	Treatment[1]				*P*-value	
					Contrasts[2]	
Blood[3]	CON	LOW	HIGH	SEM	1	2
Prepartum						
LBP[4], µg/mL	7.31	7.32	5.84	0.47	0.98	0.20
SAA[5], µg/mL	36.07	37.09	35.29	0.89	0.65	0.84
HP[6], µg/mL	160	220	150	0.02	0.26	0.01
Postpartum						
LBP[4], µg/mL	8.71	7.82	8.76	0.48	0.33	0.40
SAA[5], µg/mL	36.69	36.36	35.05	0.96	0.32	0.40
HP[6], µg/mL	530	420	330	0.05	0.01	0.03

[1]CON: Positive DCAD (+6 mEq/100g of DM; urine pH > 8.0) with low dietary Ca (0.40% of DM; 46.2 ±15.2 g Ca/d; n = 26); LOW: Negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with low dietary Ca (0.40% of DM; 44.1 ± 16.1 Ca/d; n = 24); HIGH: Negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with high dietary Ca (2.0% of DM; 226.6 ± 96.0 g Ca/d; n = 26).

[2]Contrasts were CONT1 = CON vs. the average of HIGH and LOW; CONT2 = LOW vs. HIGH. Concentrations of prepartum lipopolysaccharide binding protein (LBP), serum amyloid A (SAA) and haptoglobin (HP) had a days relative to calving (**DRC**) effect ($P < 0.0001$, $P < 0.0001$, and $P = 0.024$, respectively; Figure 2.3a, b, and c). There was a tendency for a treatment effect for HP postpartum ($P = 0.06$), however this effect was not observed in SAA or LBP concentrations ($P = 0.44$, $P = 0.41$, respectively). There was a tendency for treatment × DRC interaction present prepartum and postpartum for HP concentration ($P = 0.08$ and $P = 0.06$, respectively). Postpartum LBP, SAA, and HP concentrations had a DRC effect ($P = 0.006$, $P = 0.02$, and $P < 0.0001$, respectively). There was no treatment effect for LBP or SAA ($P = 0.41$; $P = 0.44$, respectively).

[3]Blood samples for LBP and SAA concentration were harvested at -30, -21, -7, 15 and 30 DRC. Samples for SAA and LBP were analyzed on blood plasma. Samples analyzed for HP were harvested at -14, -7, -4, -2, -1, 0, 1, 2, 4, 7, and 14 DRC.

[4]LBP: Lipopolysaccharide Binding Protein.

[5]SAA: Serum Amyloid A

[6]HP: Haptoglobin

Table 2.4. Least squares means and associated SEM for metricheck (MC) evaluation of uterine health and proportion of polymorphonuclear (PMN) cells present in the uterus.

		Treatment[1]				*P*-value	
						Contrasts[2]	
Variable	**n**	**CON**	**LOW**	**HIGH**	**SEM**	**1**	**2**
MC Score[3]	76	1.82	2.21	1.94	0.12	0.02	0.11
MC Score + Smell[3,4]	76	2.07	2.38	2.26	0.21	0.29	0.70
PMN, %[5]	62	14.75	21.22	16.34	2.35	0.10	0.18

[1]CON: Positive DCAD (+6 mEq/100g of DM; urine pH > 8.0) with low dietary Ca (0.40% of DM; 46.2 ±15.2 g Ca/d; n = 26); LOW: Negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with low dietary Ca (0.40% of DM; 44.1 ± 16.1 Ca/d; n = 24); HIGH: Negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with high dietary Ca (2.0% of DM; 226.6 ± 96.0 g Ca/d; n = 26).

[2]Contrasts were CONT1 = CON vs. the average of HIGH and LOW; CONT2 = LOW vs. HIGH.

[3]MC Score and MC Score + Smell: CON: n = 26; LOW: n = 24; HIGH: n = 26.

Number of cows that were positive for smell score: 4 DIM: CON: n = 2; LOW: n = 1; HIGH: n = 1; 7 DIM:CON: n = 4; LOW: n = 2; HIGH: n = 1; 10 DIM: CON: n = 3; LOW: n = 1; HIGH: n = 3; 13 DIM: CON: n = 2; LOW: n = 2; HIGH: n = 3: 15 DIM: CON: n = 3; LOW: n = 2; HIGH: n = 2; 17 DIM: CON: n = 1; LOW: n = 2; HIGH: n = 3; 30 DIM:CON: n = 0; LOW: n = 1; HIGH: n = 1

[4]Proportion of Polymorphonuclear cells: CON: n = 22; LOW: n = 18; HIGH: n = 22.

Table 2.5a. Likelihood analysis of cows having metritis based on a metricheck score of 3 at 4 - 17 DRC or ≥ 2 on 30 DRC

Day	N	Level[1]	Coefficient	SEM	Odds ratio	95% CI	*P*-value
4	70	HIGH	0.01	0.61	1.01	0.30 – 3.42	0.99
		LOW	0.10	0.61	1.47	0.43 – 4.96	0.53
7	72	HIGH	0.15	0.61	1.17	0.35 – 3.89	0.80
		LOW	0.70	0.60	2.02	0.60 – 6.80	0.24
10	76	HIGH	0.51	0.58	1.67	0.52 – 5.33	0.38
		LOW	0.98	0.62	2.67	0.77 – 9.25	0.12
13	76	HIGH	1.80	0.70	6.01	1.49 – 24.25	0.01
		LOW	2.00	0.71	7.35	1.77 – 30.56	<0.01
15	76	HIGH	-0.03	0.58	0.97	0.31 – 3.07	0.96
		LOW	0.30	0.57	1.34	0.43 – 4.20	0.61
17	76	HIGH	-0.44	0.60	0.64	0.20 – 2.11	0.46
		LOW	0.94	0.60	2.56	0.77 – 8.55	0.12
30	76	HIGH	0.79	1.23	2.02	0.17 – 28.89	0.54
		LOW	1.37	1.29	3.95	0.34 – 45.96	0.27

[1]Referent is CON. Treatments: CON: Positive DCAD (+6 mEq/100g of DM; urine pH > 8.0) with low dietary Ca (0.40% of DM; 46.2 ±15.2 g Ca/d; n = 26); LOW: Negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with low dietary Ca (0.40% of DM; 44.1 ± 16.1 Ca/d; n = 24); HIGH: Negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with high dietary Ca (2.0% of DM; 226.6 ± 96.0 g Ca/d; n = 26).

Table 2.5b. Likelihood analysis of cows having metritis based on a metricheck smell of 3 at 4 - 30 DRC

Day	N	Level[1]	Coefficient	SEM	Odds ratio	95% CI	*P*-value
4	70	HIGH	-0.51	1.08	1.41	0.10 - 12.09	0.75
		LOW	0.34	1.28	0.60	0.04 - 7.8	0.69
7	72	HIGH	-1.44	1.16	0.24	<0.001 - >999.99	0.43
		LOW	-0.69	0.92	0.50	<0.001 - >999.99	0.59
10	76	HIGH	0.09	0.89	1.09	0.18 – 6.48	0.92
		LOW	-1.01	1.21	0.37	0.03 – 4.08	0.41
13	76	HIGH	0.59	0.98	1.80	0.15 – 10.55	0.55
		LOW	0.23	1.06	1.26	0.25 – 12.72	0.83
15	76	HIGH	-0.36	0.98	0.70	0.10 – 4.87	0.37
		LOW	-1.10	1.20	0.33	0.03 – 3.69	0.71
17	76	HIGH	-0.44	0.60	0.64	0.20 – 2.11	0.46
		LOW	0.94	0.60	2.56	0.77 – 8.55	0.12
30[2]	76	HIGH	-	-	-	-	-
		LOW	-	-	-	-	-

[1]Referent is CON. Treatments: CON: Positive DCAD (+6 mEq/100g of DM; urine pH > 8.0) with low dietary Ca (0.40% of DM; 46.2 ±15.2 g Ca/d; n = 26); LOW: Negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with low dietary Ca (0.40% of DM; 44.1 ± 16.1 Ca/d; n = 24); HIGH: Negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with high dietary Ca (2.0% of DM; 226.6 ± 96.0 g Ca/d; n = 26).

[2]For the purpose of this study, 30 DRC was not included in this likelihood analysis of metritis based on smell score due to insufficient n-values in CON (n = 1), LOW (n = 0), and HIGH (n = 0).

Table 2.5c. Likelihood analysis of cows having metritis based on a metricheck score plus smell ≥ 3 at 4 - 17 DRC or ≥ 2 at 30 DRC

Day	N	Level[1]	Coefficient	SEM	Odds ratio	95% CI	*P*-value
4	70	HIGH	0.01	0.64	1.01	0.42 – 5.32	0.99
		LOW	0.40	0.64	1.49	0.28 – 3.59	0.53
7	72	HIGH	0.15	0.60	1.17	0.35 – 3.90	0.80
		LOW	0.90	0.61	2.45	0.72 – 8.34	0.15
10	76	HIGH	0.69	0.59	2.00	0.20 – 6.50	0.24
		LOW	0.98	0.62	2.67	0.77 – 9.26	0.12
13	76	HIGH	1.79	0.70	6.01	1.49 – 24.25	0.01
		LOW	2.00	0.71	7.35	1.77 – 30.56	<0.01
15	76	HIGH	-0.03	0.58	0.97	0.31 – 3.07	0.96
		LOW	0.30	0.57	1.34	0.43 – 4.20	0.61
17	76	HIGH	-0.44	0.60	0.64	0.20 – 2.11	0.46
		LOW	0.94	0.60	2.56	0.77 – 8.55	0.12
30	76	HIGH	0.79	0.96	2.20	0.33 – 14.93	0.41
		LOW	1.64	0.90	5.16	0.86 – 31.13	0.07

[1]Referent is CON. Treatments: CON: Positive DCAD (+6 mEq/100g of DM; urine pH > 8.0) with low dietary Ca (0.40% of DM; 46.2 ±15.2 g Ca/d; n = 26); LOW: Negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with low dietary Ca (0.40% of DM; 44.1 ± 16.1 Ca/d; n = 24); HIGH: Negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with high dietary Ca (2.0% of DM; 226.6 ± 96.0 g Ca/d; n = 26).

Table 2.6. Odds ratio (OR) for animals classified[1] as having endometritis from uterine cytology samples.

Variable	Treatments[2]	Level	OR	95% CI[3]	*P*-value
Endometritis at 15 DIM[4]	CON	CON-HIGH	0.53	0.08 - 3.73	0.39
	–	CON-LOW	0.40	0.06 – 2.56	0.27
	LOW	LOW-HIGH	1.33	0.25 – 7.26	0.36
Endometritis at 30 DIM[5]	CON	CON-HIGH	0.17	0.02 – 1.82	0.14
	–	CON-LOW	0.12	0.01 – 1.29	0.08
	LOW	LOW- HIGH	1.40	3.01 – 6.48	0.66
Retained Placenta[6]	CON	CON-HIGH	1.00	0.05 – 18.44	0.99
	–	CON-LOW	0.96	0.92 – 17.05	0.96
	LOW	LOW-HIGH	0.91	0.06 – 20.12	0.96

[1]15 DIM: animals were classified as having endometritis if proportion of polymorphonuclear cells was >40%; At 30 DIM animals were classified as having endometritis if proportion of polymorphonuclear cells was >18%.
[2]CON: Positive DCAD (+6 mEq/100g of DM; urine pH > 8.0) with low dietary Ca (0.40% of DM; 46.2 ±15.2 g Ca/d; n = 26); LOW: Negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with low dietary Ca (0.40% of DM; 44.1 ± 16.1 Ca/d; n = 24); HIGH: Negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with high dietary Ca (2.0% of DM; 226.6 ± 96.0 g Ca/d; n = 26).
[2]Contrasts were CONT1 = CON vs. the average of HIGH and LOW; CONT2 = LOW vs. HIGH.
[3]CI: confidence interval derived from a binomial regression.
[4]Likelihood of endometritis from at 15 ± 2 DRC; CON (n = 25: Yes = 3, and No = 22); LOW (n = 22: Yes = 4, and No = 18); HIGH (n = 24; Yes = 4, and No = 20).
[5]Likelihood of endometritis at 30 ± 2 DRC; CON (n = 23; Yes = 2, and No = 21); LOW (n = 23; Yes = 5, and No = 18); HIGH (n = 23; Yes = 5, and No = 18).
[6]Likelihood of retained placenta; CON (n = 28; Yes = 2, and No = 26); LOW (n = 25; Yes = 1, and No = 24); HIGH (n = 27; Yes = 1, and No = 26).

Table 2.7. Least squares means and associated SEM for ovulation dynamics of the dominant follicle of the first follicular wave

	Treatment[1]				*P*-value	
					Contrast[2]	
Variable	**CON**	**LOW**	**HIGH**	**SEM**	**1**	**2**
Diameter of dominant follicle at first measurement, mm[3]	8.29	8.42	8.79	0.38	0.81	0.46
Diameter of dominant follicle at last measurement before first ovulation, mm[4]	17.87	18.33	17.56	0.44	0.44	0.16
Growth rate of dominant follicle before first ovulation, mm/d[5]	1.48	1.59	1.69	0.09	0.54	0.40
Growth rate of dominant follicle over last four days before first ovulation, mm/d[6]	3.71	3.82	3.78	0.34	0.30	0.66
Total growth of dominant follicle from first measurement to last measurement, mm[7]	9.58	9.91	8.77	0.53	0.64	0.12
Days to first Ovulation, d[8]	18.93	17.93	16.30	0.78	0.05	0.12

[1]Treatments were CON: Positive DCAD (+6 mEq/100g of DM; urine pH > 8.0) with low dietary Ca (0.40% of DM; 46.2 ±15.2 g Ca/d; n = 26); LOW: Negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with low dietary Ca (0.40% of DM; 44.1 ± 16.1 Ca/d; n = 24); HIGH: Negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with high dietary Ca (2.0% of DM; 226.6 ± 96.0 g Ca/d; n = 26).

[2]Contrasts were CONT1 = CON compared with the average of LOW and HIGH; CONT2 = LOW compared with HIGH.

[3]First measurement was on average 7.21 ± 0.79 days in milk (DIM): CON (n = 23); LOW (n = 22); HIGH (n = 25).

[4]Last measurement was on average 19.33 ± 4.31 DIM: CON (n = 23); LOW (n = 22); HIGH (n = 25).

[5]Growth rate of the dominant follicle from first measurement of the dominant follicle of the first follicular wave post-partum. CON (n = 23); LOW (n = 22); HIGH (n = 25).

[6]Growth rate of dominant follicle for the last 4 days of growth before first ovulation: CON (n = 23); LOW (n = 22); HIGH (n = 25). There was no treatment effect ($P = 0.57$) or treatment × day interaction ($P = 0.11$). DRO differed for growth rate ($P < 0.0001$) (Figure 2.4).

[7]Total growth of the dominant follicle from first ultrasound until first ovulation: CON (n = 23); LOW (n = 22); HIGH (n = 25).

[8]Days of growth from first measurement until first ovulation of dominant follicle: CON (n = 23); LOW (n = 22); HIGH (n = 25).

Table 2.8. Odds ratio (OR) for the presence of a Corpus Luteum (CL) at 30, 57 and 64 days in milk (DIM), presence of a follicle at 71 DIM and pregnant at first timed artificial insemination (TAI)

Variable[1]	Treatments[2]	Level	OR	95% CI[3]	*P*-value
CL at 30 DIM[4]	CON	CON-HIGH	0.86	0.86– 3.18	0.82
	–	CON-LOW	1.00	0.28 – 3.57	0.99
	LOW	LOW-HIGH	0.86	0.86– 3.18	0.82
CL at 57 DIM[5]	CON	CON-HIGH	2.59	0.55 – 12.16	0.22
	–	CON-LOW	3.00	0.65 – 13.58	0.16
	LOW	LOW-HIGH	0.86	0.248 – 3.09	0.83
CL at 64 DIM[6]	CON	CON-HIGH	2.03	0.53 – 7.69	0.49
	–	CON-LOW	1.60	0.42 – 6.13	0.29
	LOW	LOW-HIGH	1.27	0.38 – 4.27	0.70
Follicle at 71 DIM[7]	CON	CON-HIGH	0.21	0.02 - 5.16	0.18
	–	CON-LOW	1.13	0.25 – 2.14	0.87
	LOW	LOW-HIGH	0.18	0.02 – 2.80	0.14
Pregnant at First TAI[8*]	CON	CON-HIGH	0.29	0.06 – 1.22	0.09
	–	CON-LOW	0.43	0.10 – 1.89	0.26
	LOW	LOW-HIGH	0.67	0.19 – 2.38	0.53

[1]Variables are CL at 30 (n = 76), 57 (n = 74), and 64 DIM (n = 74); Follicle at 71 DIM (n = 73); Pregnant at first TAI (n = 55).
[2]CON: Positive DCAD (+6 mEq/100g of DM; urine pH > 8.0) with low dietary Ca (0.40% of DM; 46.2 ±15.2 g Ca/d; n = 26); LOW: Negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with low dietary Ca (0.40% of DM; 44.1 ± 16.1 Ca/d; n = 24); HIGH: Negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with high dietary Ca (2.0% of DM; 226.6 ± 96.0 g Ca/d; n = 26).
[3]CI: confidence interval derived from a binomial regression.
[4]CON (n = 26; Yes = 19, and No = 7); HIGH (n = 25; Yes = 19, and No = 6); LOW (n = 25; Yes = 19, and No = 7).
[5]CON (n = 23; Yes = 20, and No = 3); HIGH (n = 25; Yes = 18, and No = 7); LOW (n = 26; Yes = 18, and No = 8).
[6] CON (n = 23; Yes = 18, and No = 5); HIGH (n = 25; Yes = 16, and No = 9); LOW (n = 26; Yes = 18, and No = 8).
[7]CON (n = 23; Yes = 19, and No = 4); HIGH (n = 24; Yes = 23, and No = 1); LOW (n = 26; Yes = 21, and No = 5).
[8]CON (n = 15; Yes = 3, and No = 12); HIGH (n = 19; Yes = 10, and No = 9); LOW (n = 21; Yes = 10, and No = 11).

Table 2.9. Least squares means and associated standard error for glandular morphology and immunolabeling for glutathione peroxidase 1 and superoxide dismutase 1 in endometrial tissue harvested from Holstein cows.

	Treatment[1,2]				*P*-value Contrasts[3]	
Item	**CON**	**LOW**	**HIGH**	**SEM**	**1**	**2**
Glandular Morphology[4]						
Glandular Area, μm	8175.05	9316.83	7981.45	890.25	0.66	0.29
Glandular Perimeter, μm	348.13	384.48	376.71	18.92	0.17	0.77
Glandular Epithelial Height, μm	18.01	18.67	22.47	1.08	0.06	0.02
Number of Cells per Gland	23.58	22.93	25.93	1.07	0.51	0.05
Number of Glands	304.47	284.76	235.39	30.38	0.24	0.25
Immunolabeling						
Glutathione Peroxidase, %	56.85	68.31	32.89	5.05	0.32	<0.0001
Superoxide Dismutase, %	78.50	69.49	73.50	2.83	0.31	0.05

[1]CON: Positive DCAD (+6 mEq/100g of DM; urine pH > 8.0) with low dietary Ca (0.40% of DM; 46.2 ±15.2 g Ca/d; n = 17); LOW: Negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with low dietary Ca (0.40% of DM; 44.1 ± 16.1 Ca/d; n = 18); HIGH: Negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with high dietary Ca (2.0% of DM; 226.6 ± 96.0 g Ca/d; n = 18) fed from 28 days before expected calving until parturition.

[2]Due to sampling criteria, samples were not taken from every cow. Cows not sampled per treatment: CON: n = 9; LOW: n = 6; HIGH: n = 8.

[3]Contrasts were 1 = CON compared with the average of LOW and HIGH; 2 = LOW compared with HIGH.

[4]Endometrial tissue was harvested at 30 ± 1.32 days after calving.

Table 2.10. Odds ratio (OR) for animals classified as having high or low superoxide dismutase 1 (SOD) activity and high or low glutathione peroxidase 1 (GPX) activity in endometrial tissue harvested from Holstein cows.

Variable	Treatments[1,2,3]	Level	OR	95% CI[4]	*P*-value
Glutathione peroxidase[5,6,]	CON	CON-HIGH	- - -[7]	- - -[7]	- - -[7]
	-	CON-LOW	0.39	0.07 – 2.14	0.27
	LOW	LOW-HIGH	- - -[7]	- - -[7]	- - -[7]
Superoxide dismutase[5,6]	CON	CON-HIGH	3.79	0.85 – 16.85	0.08
	–	CON-LOW	4.42	0.96 – 20.33	0.06
	LOW	LOW-HIGH	0.85	0.20 – 3.68	0.83

[1]CON: Positive DCAD (+6 mEq/100g of DM; urine pH > 8.0) with low dietary Ca (0.40% of DM; 46.2 ±15.2 g Ca/d; n = 17); LOW: Negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with low dietary Ca (0.40% of DM; 44.1 ± 16.1 Ca/d; n = 18); HIGH: Negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with high dietary Ca (2.0% of DM; 226.6 ± 96.0 g Ca/d; n = 18) fed from 28 days before expected calving until parturition

[2]Due to sampling criteria, samples were not taken from every cow. Cows not sampled per treatment: CON: n = 9; LOW: n = 6; HIGH: n = 8.

[3]Endometrial tissue was harvested at 30 ± 1.32 days after calving.

[4]CI: Confidence interval derived from a binomial regression.

[5]Tissue samples were stained with glutathione peroxidase 1 (GPX) and superoxide dismutase 1 (SOD) antibodies to determine samples with high concentration of cells with GPX and SOD activity (HGPX and HSOD, respectively), and low concentration of cells with GPX and SOD activity (LGPX and LSOD, respectively). High concentration of cells with GPX activity were classified when concentrations was ≥74%, high concentration of cells with SOD activity were classified when concentrations was ≥64%. Classification of high and low concentrations were determined from median values.

[6]GPX: CON: n = 17 (LGPX = 6, and HGPX = 11); LOW: n = 18 (LGPX = 4, and HGPX = 14); HIGH: n = 18 (LGPX = 17, and HGPX = 1). SOD: CON: n = 17 (LSOD = 5, and HSOD = 12); LOW: n = 18 (LSOD = 12, and HSOD = 6); HIGH: n = 18 (LSOD = 11, and HSOD = 7).

[7]For the purpose of this study, HIGH group was excluded from the GPX statistical analysis because there was not a sufficient n-value (n = 1) in the HGPX group.

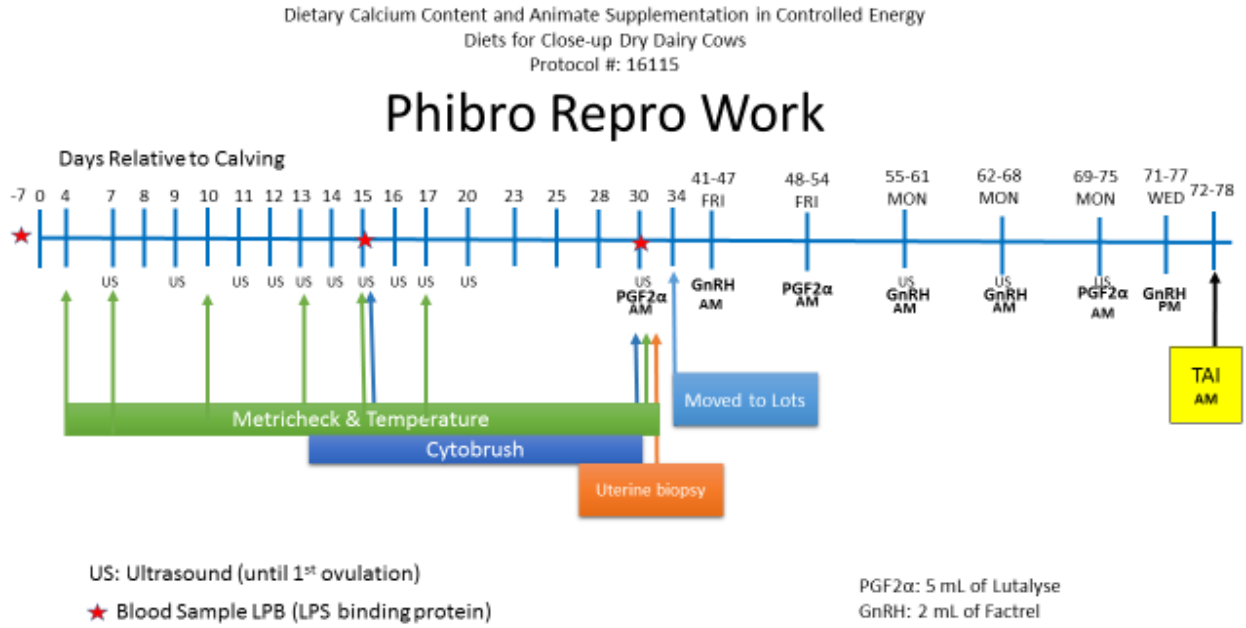

Figure 2.1. Schematic of the experimental design.

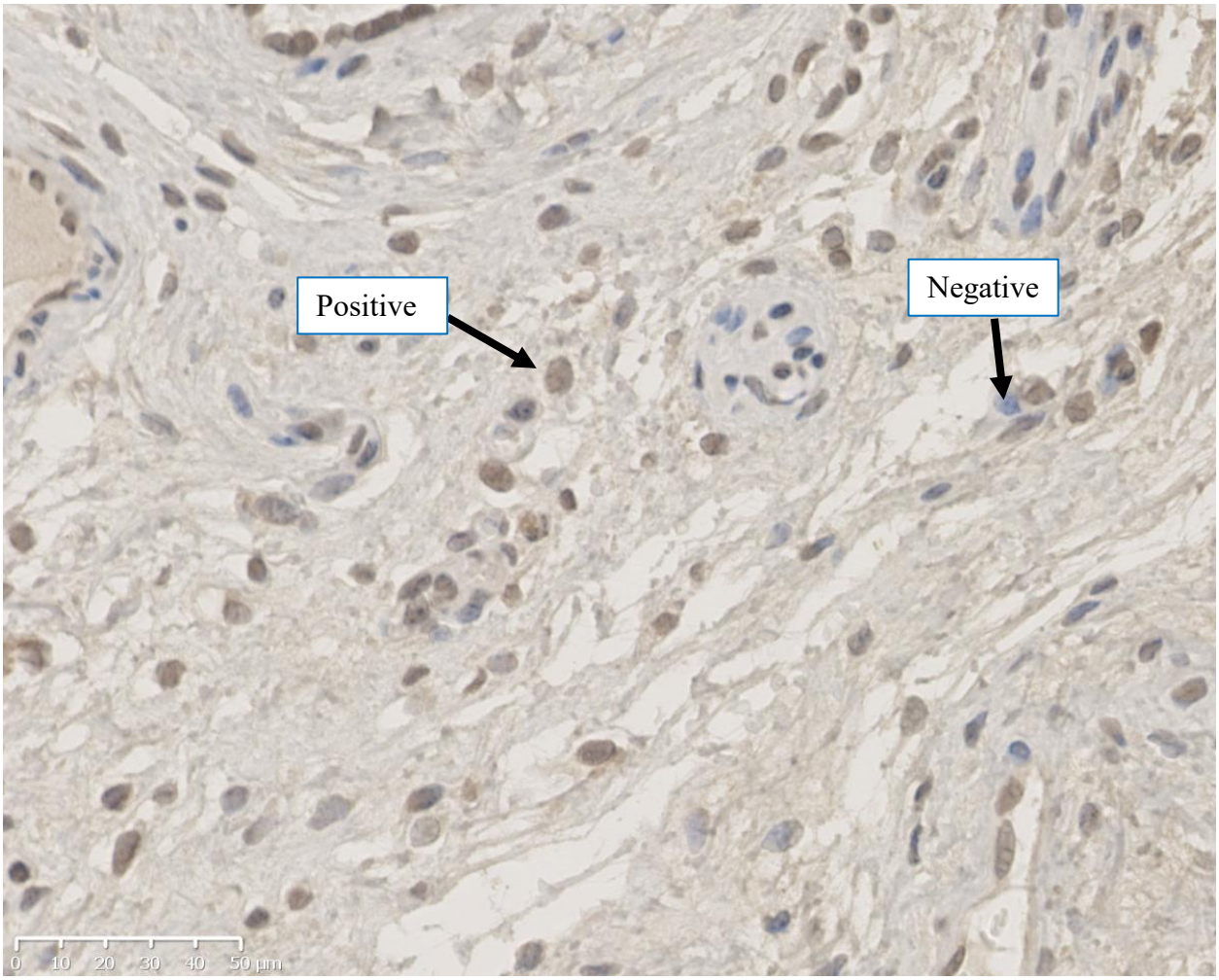

Figure 2.2. Tissue samples stained for glutathione peroxidase (GPX) activity or superoxide dismutase (SOD) activity. Cells appear blue if negative for both GPX or SOD activity and brown if positive. Stains were applied to different sections of the same tissue sample. Image taken from sample of the current study

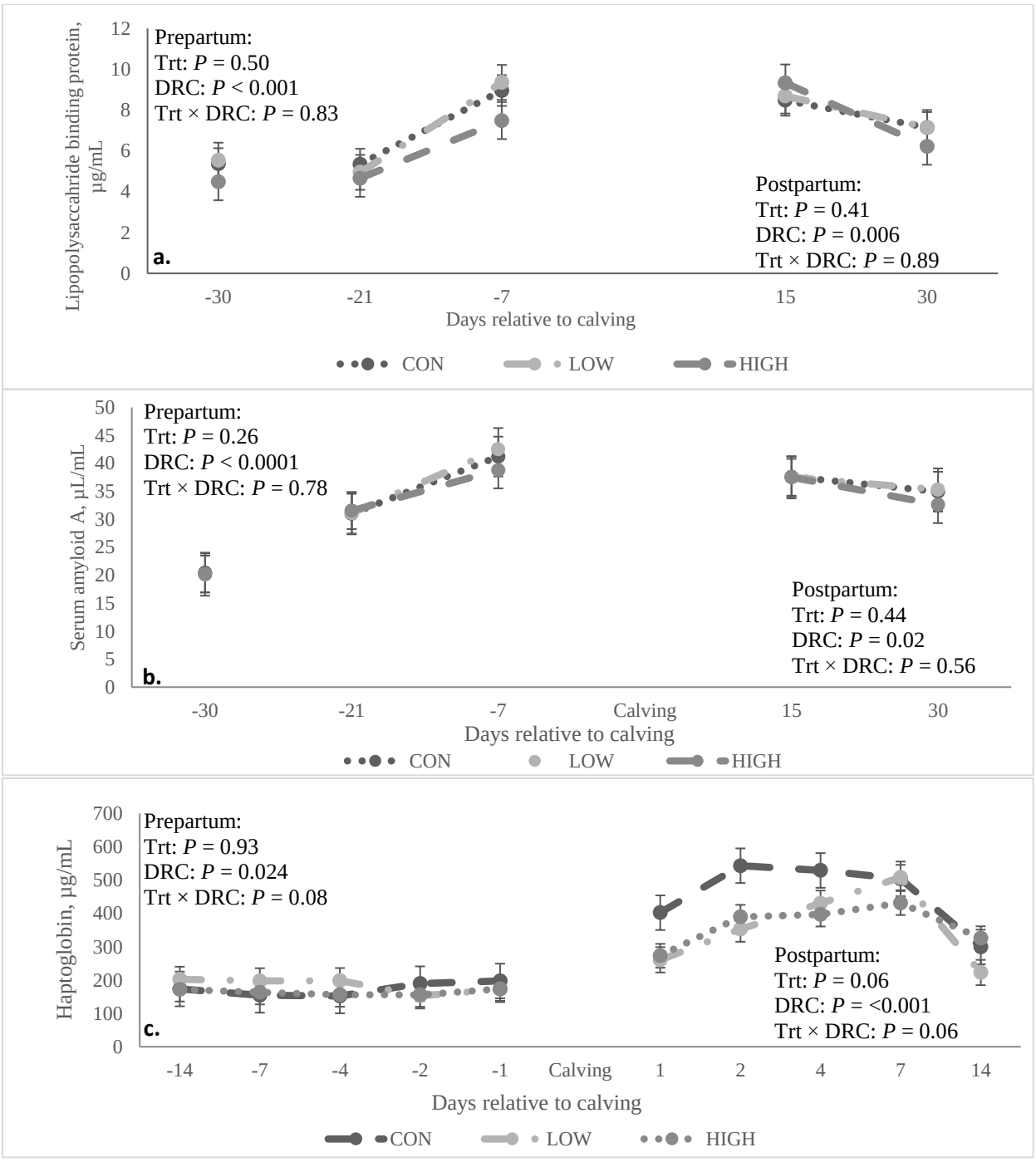

Figure 2.3. Least squares means and associated SEM for inflammatory blood metabolites found in blood plasma samples of Holstein cows fed CON: Positive DCAD (+6 mEq/100g of DM; urine pH > 8.0) with low dietary Ca (0.40% of DM; 46.2 ±15.2 g Ca/d; n = 26); LOW: Negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with low dietary Ca (0.40% of DM; 44.1 ± 16.1 Ca/d; n = 24); HIGH: Negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with high dietary Ca (2.0% of DM; 226.6 ± 96.0 g Ca/d; n = 26). Figure 2.3a: Lipopolysaccharide binding protein (LBP); Figure 2.3b. Serum amyloid A (SAA); Figure 2.3c. Haptoglobin (HP). There was a DRC effect prepartum for LBP, SAA and HP (P <0.001, P < 0.0001, P = 0.024, respectively) for increasing concentrations of each metabolite.

Figure 2.3 (cont.). A tendency for treatment × DRC interaction for HP concentration ($P = 0.08$) prepartum indicates that CON had a higher HP concentration than in the last 2 days before calving than cows in HIGH and LOW. A tendency for a treatment effect and a tendency for a treatment × DRC interaction in HP concentration postpartum indicates cows in CON had higher HP concentrations postpartum, especially in the first 4 days postpartum. The DRC effect postpartum for LBP, SAA, and HP ($P = 0.006$, $P = 0.02$, and $P < 0.001$) indicates a decreasing concentrations of all three metabolites overtime.

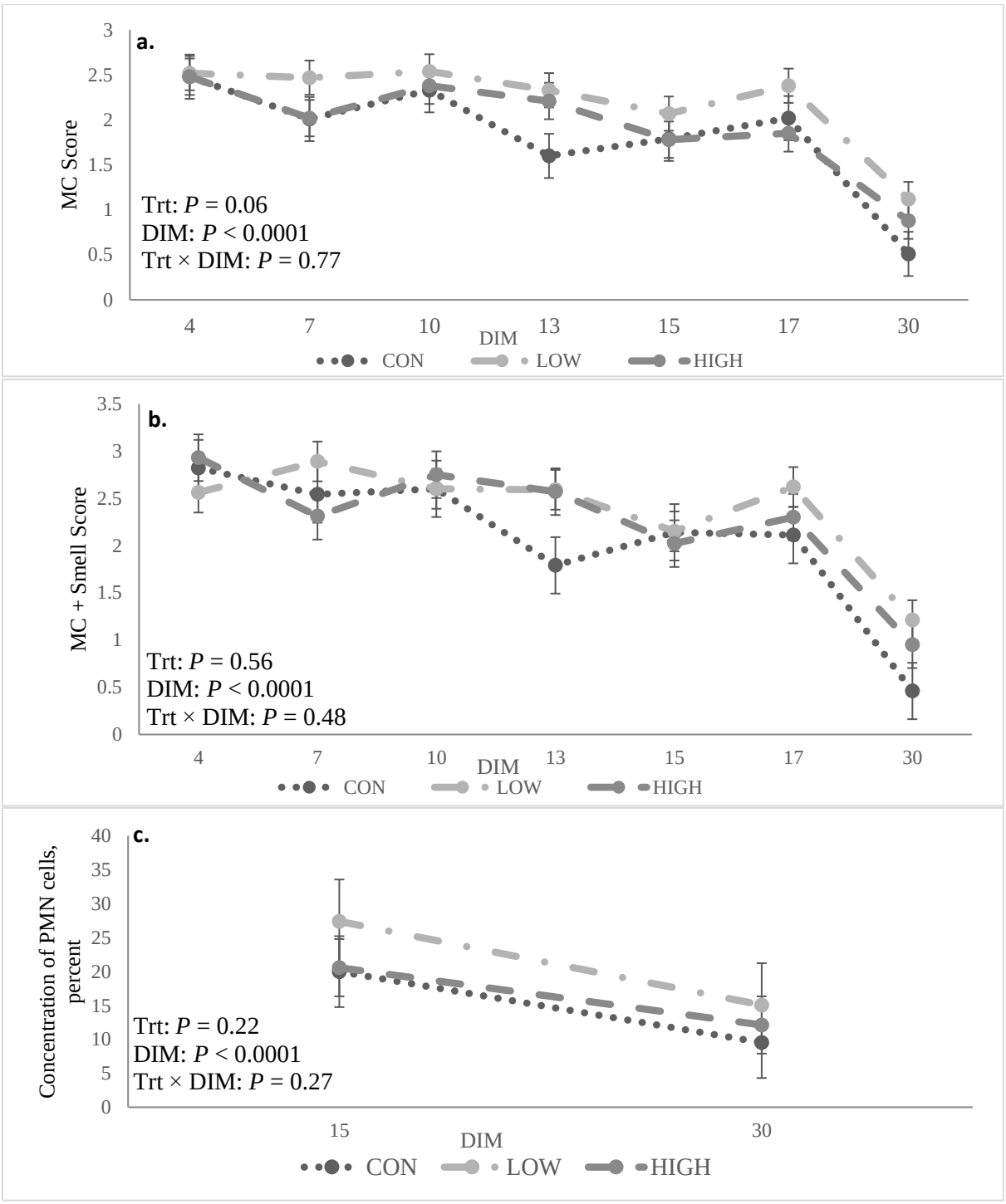

Figure 2.4. Least squares means and associated SEM of Metricheck score (MC), Metricheck score + smell (MC + smell), and proportion of polymorphonuclear cells (PMN) present in the uterus, plotted by DIM for Holstein cows fed CON: Positive DCAD (+6 mEq/100g of DM; urine pH > 8.0) with low dietary Ca (0.40% of DM; 46.2 ±15.2 g Ca/d; n = 26); LOW: Negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with low dietary Ca (0.40% of DM; 44.1 ± 16.1 Ca/d; n = 24); HIGH: Negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with high dietary Ca (2.0% of DM; 226.6 ± 96.0 g Ca/d; n = 26).

Figure 2.4 (cont.). Figure 2.4a: MC score by time; Figure 2.4b: MC + smell score by time; Figure 2.4c: proportion of PMN cells by time. There was a tendency for a treatment effect for cows in LOW to have a higher MC score than cows in CON and HIGH. A DIM effect ($P <$ 0.0001 for all three variables) indicates decreasing scores and concentration over time.

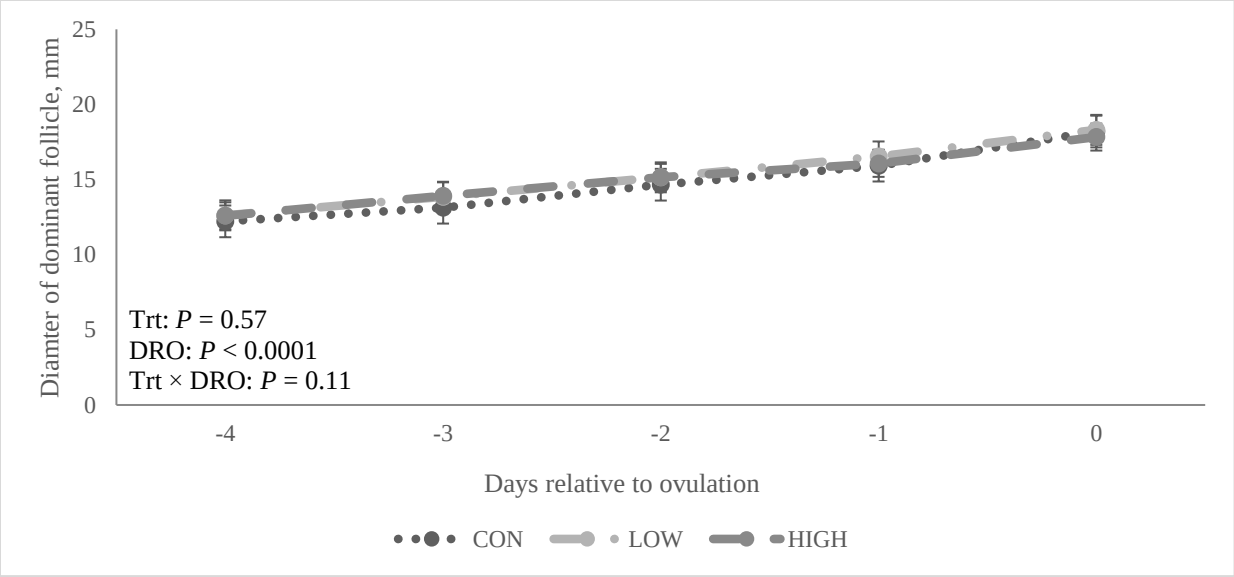

Figure 2.5. Least squares means and associated standard errors for diameter relative to the moment of the first ovulation (DRO) of the first postpartum dominant follicle for Holstein cows fed CON: Positive DCAD (+6 mEq/100g of DM; urine pH > 8.0) with low dietary Ca (0.40% of DM; 46.2 ±15.2 g Ca/d; n = 26); LOW: Negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with low dietary Ca (0.40% of DM; 44.1 ± 16.1 Ca/d; n = 24); HIGH: Negative DCAD (-24 mEq/100g of DM; urine pH = 5.8) with high dietary Ca (2.0% of DM; 226.6 ± 96.0 g Ca/d; n = 26). There was a DRO effect ($P < 0.0001$) indicates that the diameter of the first dominant follicle postpartum increased overtime. There was a tendency for a treatment × DRO interaction ($P = 0.11$) indicating cows fed CON had a slower rate of growth in days 4-2 before ovulation of the first dominant follicle postpartum than cows fed LOW or HIGH

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CHAPTER III. OVERALL SUMMARY, CONCLUSIONS, AND PERSPECTIVES

The challenges of the periparturient period in dairy cows has been exacerbated in recent years due to the increasing demands of lactation. Over the years, we have genetically selected for high producing dairy cows and have faltered on selection for other qualities, such as fertility, pathogen resistance and tolerance, and sensitivity to metabolic disorders. After decades of selection for a few production traits, we have developed a cow that is extremely efficient at converting feedstuffs to milk, however, at the same time, these cows are more prone to metabolic disorders and infertility. This has been recognized in the last two decades and various management strategies (specifically in dry cow management) have been employed to mitigate these effects. Strategies such as feeding a controlled energy diets to dry cows or feeding a negative DCAD diet to close-up dry cows, are aimed at helping the cow successfully transition through the periparturient period. While these practices are effective, the periparturient period continues to be a focal point in research of dairy cow nutrition and health.

From this study, we gained a better understanding of the effects of feeding a negative DCAD diet to close-up dairy cows and the effect the subsequent reproductive health and fertility. We also gained an understanding of the effect of feeding rates of Ca with a negative DCAD diet on the same parameters. Our observations from this study help us glean some insight the relationship of feeding a negative DCAD with differing Ca concentrations. While the mechanisms are not completely understood, this study indicates that feeding Ca at 2% inclusion in dry cow rations with a negative DCAD has alleviative effects on reproductive health, improves the likelihood of conception at first TAI, and attenuates the inflammatory response of cows at parturition. Cows fed this treatment also had improved uterine glandular development

and improved redox environment in the endometrium, which contributes to the overall reproductive health of the cow.

While there are guidelines recommended by the NRC and various studies on the amount of Ca to feed to a close-up dry cow, the actual amount of Ca fed is often times greater than the recommended amounts. This variation can come from many factors including types and stage of maturation of forages, inclusion of Ca binders, mineral additives, and environmental factors. While this variation is difficult to predict without consistent feed component analysis and updates to your rations, supplementation with anionic salts can safeguard the transitioning cow from loss of sensitivity to Ca homeostasis. The HIGH treatment in comparison to LOW and CON, more accurately reflects the amount of dietary Ca that is expected to be supplied to a dry cow ration.

Going forward, it is important to understand the importance of Ca homeostasis in the periparturient cow and how it influences, not just other metabolic systems in the cow, but her ovulatory cycles, immune response, and involution of the uterus. It is also important to understand how dietary factors influence this balance, and can either perturb or enhance Ca homeostasis. Future studies can investigate the effectiveness of feeding Ca from different sources (i.e. dicalcium phosphate, Calcium chloride dehydrate, etc.) and how that interacts with a negative DCAD diet and effects on future production performance.