MONITORING, PREVENTION AND TREATMENT OF HYPOCALCEMIA IN
PERIPARTURIENT DAIRY COWS

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The Ca demand of colostrum and milk production in the immediate postpartum period compared to the demands in late gestation result in a huge metabolic adaptation for periparturient dairy cows. This adaptation requires the coordination of several hormones and tissues and if delayed adaptation results in an excessive drop in blood Ca it can impair cow health and performance. Management of hypocalcemia requires multifaceted approaches that integrate prevention, treatment and monitoring. The objectives of this dissertation were to: 1) investigate strategies for monitoring blood Ca, 2) optimize application of established nutritional strategies for prevention, 3) evaluate new approaches to macromineral nutrition to support blood Ca recovery postpartum, and 3) identify opportunities for use of supplemental Ca postpartum. The VetTest is a tool that measures blood total Ca which has potential for field application and its use resulted in reliable identification of hypocalcemia. The variation in the relationship between ionized and total Ca in the immediate postpartum period suggest that these parameters cannot be used interchangeably for identification of hypocalcemia and ionized Ca was a better predictor of neutrophil function in the week postpartum. Cows fed prepartum rations with a negative DCAD, targeting an average urine pH between 5.5 and 6.0, had higher blood Ca concentrations, intake and milk production in the early lactation period compared to cows fed a low K or intermediate ration. Blood Ca responses to this preventative approach were more pronounced in cows entering their 3rd parity or greater compared to 2nd lactation cows. Altering dietary source of supplemental Ca and Mg, and postpartum dietary concentration of Mg, had minimal influence on

mineral status in the transition period but did improve intake and energetic status in the transition period. This suggests opportunity for strategic use of mineral sources to support the metabolic adaptations to lactation. Supplementation with oral Ca postpartum improved early lactation health for cows with increasing age, cows with high BCS and lame cows. Plasma Ca status did not differentiate response of primiparous cows to supplementation but multiparous cows with low plasma Ca had improved health in early lactation when supplemented with oral Ca.

BIOGRAPHICAL SKETCH

Brittany Leno was raised in New Haven, Vermont and attended the University of Vermont to study Animal Science where she graduated with her Bachelor's degree in 2011. In 2009, Brittany participated in the CREAM (Cooperative for Real Education in Agricultural Management) program at UVM where she first learned about the science at the foundation of dairy production systems. After spending time working on commercial dairy farms throughout college and spending a semester at the Miner Institute in Chazy, NY completing the Advanced Dairy Management Program, she decided to investigate the opportunities in agricultural research further. After graduating from UVM, Brittany spent a year at Miner Institute as a research intern. In 2012, Brittany arrived at Cornell to begin her graduate program with Tom Overton in the area of transition cow mineral metabolism. Brittany is defending her thesis in May 2017 and plans to work in the nutrition industry gaining direct experience with producers and nutritional consultants.

Dedicated to my husband, Zane Hawk Leno, who challenges me to think differently and to stay

true to myself through the most rewarding and most challenging experiences

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CHAPTER 1

INTRODUCTION

One of the challenges associated with the transition from late gestation to the early lactation period for the dairy cow is the dramatic increase in demand for Ca to support colostrum and milk production (House and Bell, 1993; Kehoe et al., 2007). The complex homeostatic system for maintaining blood Ca concentration and supplying Ca for productive functions requires coordination of parathyroid hormone (**PTH**) and 1,25-dihydroxyvitamin D synthesis and release, renal handling of Ca, intestinal Ca absorption efficiency and turnover of bone Ca (Ramberg et al., 1970). The variation in the adaptation of this system can result in varying degrees of hypocalcemia in periparturient dairy cows.

Disorders of Ca homeostasis have been recognized as important determinants of health and productivity of dairy cows for several decades (Curtis et al., 1983). Clinical hypocalcemia is a severe disorder that predisposes cows to many other clinical diseases after parturition as well as decreased productivity (Rajala-Schultz et al., 1999). With nutritional prevention strategies, the incidence of clinical hypocalcemia has been reduced to 5% or less in many parts of the U.S. (Chapinal et al., 2011; Reinhardt et al., 2011). In recent years, it has been demonstrated that a large proportion of cows can be categorized as having subclinical hypocalcemia (**SCH**), which is characterized by the lack of clinical signs while having blood Ca concentration below normal ranges (Reinhardt et al., 2011; Caixeta et al., 2015). Subclinical hypocalcemia has been associated with exacerbated negative energy balance, demonstrated by higher liver lipid accumulation and greater body fat mobilization in early lactation (Reinhardt et al., 2011; Martinez et al., 2012; Chamberlin et al., 2013), as well as further compromised immune function

in the transition period resulting in greater risk of infectious disease (Kimura et al., 2006; Martinez et al., 2012; Wilhelm et al., 2017). Compromised milk production and reproductive performance have also been demonstrated for cows with SCH (Chapinal et al., 2012; Caixeta et al., 2017). It has been estimated that due to the high prevalence and high impact, the total economic influence of SCH is much greater than that of clinical hypocalcemia (Oetzel, 2013). The identification of SCH in research has increased drastically within the past 5 to 10 years, and appropriate methods and timing for SCH identification are controversial. Some degree of hypocalcemia may represent an appropriate adaptation to lactation (Ramberg et al., 1970; van't Klooster, 1976), and peripartum disorders can result in a secondary drop in blood Ca (Waldron et al., 2003) or a delay in blood Ca recovery. These relationships need to be better characterized before SCH monitoring can be reliably implemented in the field.

Strategies for prevention of hypocalcemia have been investigated for decades and the predominant strategy in conventional systems throughout the U.S. is feeding prepartum rations with a negative dietary cation-anion difference (**DCAD**; USDA, 2014). Negative DCAD rations aid in prevention of hypocalcemia by inducing a metabolic acidosis prepartum, which alleviates tissue insensitivity to endocrine signals responsible for maintaining blood Ca (Goff et al., 2014) and by increasing whole body Ca turnover prior to parturition (Grunberg et al., 2011). This approach is highly effective in the prevention of clinical hypocalcemia (Block, 1984) and more recent work has demonstrated that improvements in overall Ca status are evident (Moore et al., 2000). Promoting optimal Ca metabolism in the transition period also requires consideration of the interaction between other dietary factors and Ca homeostasis. Magnesium plays an important role in facilitating the synthesis and release of PTH (Rude et al., 1978; Rude, 1998) and

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prepartum supply is recognized as an important factor in determining clinical hypocalcemia risk (Lean et al., 2006).

In addition to prevention and monitoring of hypocalcemia, postpartum supplementation with Ca plays a role in management of SCH. Supplementation of Ca with either injectable forms or through oral administration can temporarily alleviate the compromised blood Ca status while the homeostatic mechanisms adapt to the greater lactational demands. While the benefits for supporting immune cell function (Ducusin et al., 2003) and gastrointestinal motility (Daniel, 1983) seem intuitive, recent data suggests that excessive supplementation can impede adaptation and even predispose some cows to compromised health or performance (Blanc et al., 2014; Martinez et al., 2016).

The objective of this literature review is to establish our current knowledge of strategies for managing disorders of Ca homeostasis and to bring to light areas in need of investigation that may improve management of SCH. The primary focus will be the application and interpretation of blood Ca monitoring, the underlying physiology and application of nutritional prevention, interactions in mineral nutrition and metabolism, and strategies for Ca supplementation postpartum in the context of SCH.

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      management. J. Dairy Sci. 100:3059–3067.

CHAPTER 2

LITERATURE REVIEW

CALCIUM METABOLISM OF TRANSITION COWS AND THE MONITORING, PREVENTION AND TREATMENT OF HYPOCALCMEIA

METABOLIC CHANGES IN THE TRANSITION COW

The transition period of the dairy cow is traditionally described as the period from 3 wk prior to parturition until 3 wk after parturition (Grummer, 1995) and is characterized by the adaptation from relatively low metabolic demands during late gestation to massive demands for lactogenesis in the face of depressed nutrient intake in the immediate postpartum period (Bell, 1995). Total nutrient requirements for development of the conceptus are significantly lower than that of colostrum and milk in early lactation and in order to minimize disruption of normal physiological functions, the homeostatic mechanisms for energy and mineral status must adapt in a coordinated manner. The term "homeorhesis" has been used to describe this coordinated shift to support the predominant physiological process, in this case shifting from support of pregnancy to support of lactation (Bauman and Currie, 1980). The diversion of nutrients to these functions often comes at a cost to the animal, for example the mobilization of body tissue stores to support milk production (Bell, 1995), and is evidence of the overlying homeorhetic adaptation that ensures survival of the offspring.

One component of the homeorhetic adaptations occurring during the transition period is the shift to increased efflux of Ca for colostrum and milk production as compared to development of the fetus. Harvest studies have demonstrated that during late gestation, after approximately 190 days of pregnancy, the Ca requirement for development of the fetus increases rapidly (House and Bell, 1993). The onset of increased fetal Ca requirement typically occurs during late lactation for parous cows in conventionally managed systems when dietary Ca intake and absorption are elevated to support the demands of milk production. As a result, this increased fetal demand does not impose a significant challenge. During the dry period, fetal demands have reached the peak of approximately 10 g/d while overall Ca demand is at the nadir

for the production cycle of the cow. In contrast, Ca composition of colostrum has been shown to average 4.7 g/kg (Kehoe et al., 2007). At just 10 kg of colostrum production in the first day after parturition, the demands for Ca can greater than double in comparison to late gestation fetal demands. With approximately 2 to 4 g of Ca circulating in the plasma pool, production of colostrum requires that the flux of Ca through this pool be rapid to ensure that systemic Ca status is maintained (Horst et al., 1997). This dramatic change, accompanied by a delay in the compensatory influx of Ca (Ramberg et al., 1970), often results in some decline in blood Ca in the periparturient period (Reinhardt et al., 2011). The mechanisms responsible for increasing supply of Ca for these physiological functions will be discussed herein.

CALCIUM METABOLISM IN DAIRY COWS

In contrast to other physiologically important minerals that circulate in the blood of the dairy cow, a complex homeostatic mechanism exists to maintain plasma Ca within a narrow range. Normal blood Ca in dairy cows is approximately 2.1 to 2.5 mmol/L (Goff, 2006). Calcium sensing receptors present on the chief cells of the parathyroid gland of dairy cows are sensitive to circulating concentrations of Ca in the ionized form (Brown et al., 1993) and secrete parathyroid hormone (**PTH**) when free ionized Ca (**iCa**) concentrations in blood begin to fall (Capen, 1971). Increases in PTH have been shown to be inversely proportional to the change in blood Ca and circulating PTH increases within 15 min of the change in blood Ca concentration (Ramberg et al., 1967). Parathyroid hormone elicits effects at the kidney, gastrointestinal tract and bone and these downstream effects are coordinated to increase Ca flux into the blood through decreasing renal Ca excretion, increasing the efficiency of Ca absorption from the intestine, and increasing mobilization of bone Ca stores.

Role of the Kidney and Vitamin D in Calcium Homeostasis

The majority of information about molecular aspects of Ca handling in the kidney come from studies conducted in rodents, however, these mechanisms can likely be extrapolated to larger mammals such as the bovine because of the highly conserved nature of Ca homeostasis in mammalian physiology (Hall, 2011). Calcium that is bound to proteins or anions cannot be filtered at the kidney and therefore only approximately 50% Ca in the blood (the free iCa) is filtered. Approximately 70% of filtered Ca reabsorption occurs passively in the proximal tubule of the kidney nephron and Ca absorption occurs in parallel with Na (Hoenderop et al., 2005). Another 25 to 30% is reabsorbed in the loop of Henle (Hall, 2011). In distal sections of the nephron, including the distal convoluted tubule and the connecting tubule, active absorption of Ca against an electrochemical gradient occurs, allowing for greater control of the total amount of Ca that is retained (Costanzo et al., 1978). The active reabsorption of filtered Ca is dependent on PTH responsive expression of proteins (van Abel et al., 2005). The first and rate limiting step of the active reabsorption of Ca is the transport of Ca from the lumen into the renal epithelial cell by the Ca transporter TRPV5 (Hoenderop et al., 2002b). Second is the facilitated diffusion of Ca to the basolateral side of the epithelial cell by Ca binding proteins, primarily calbindins-D$_{28K}$ in the kidney, and the third and final step is the energy dependent extrusion of Ca on the basolateral side of the epithelial cell by the Na$^+$/Ca^{2+} exchanger, NCX1, and the Ca^{2+}-ATPase, PMCA1b (Hoenderop et al., 2005). In addition to requiring PTH stimulation, expression of these transport proteins is dependent on Ca influx into the cell and the production of the hormone 1,25-dihydroxyvitamin D (Hoenderop et al., 2002a; van Abel et al., 2005).

To facilitate downstream effects on bone resorption and intestinal absorption of Ca, PTH elicits the activation of the 1-α-hydroxylase enzyme in the kidney. This enzyme activates 25-hydroxyvitamin D, the primary circulating form of vitamin D, by forming 1,25-

dihydroxyvitamin D (Nelson and Merriman, 2014). The production of 1,25-dihydroxyvitamin D is dependent on an adequate supply of vitamin D, either from the diet or conversion of 7-dehydrocholesterol to vitamin D_3 in the skin as a result of exposure to sunlight (Stipanuk and Caudill, 2013). Vitamin D absorbed from the diet or produced endogenously is then converted to 25-hydroxyvitamin D in the liver by the 25-hydroxylase enzyme. Vitamin D circulates in blood at low levels and is quickly taken up by the liver (Horst and Reinhardt, 1983) and the hydroxylation to 25-hydroxyvitamin D does not appear to be closely regulated. Circulating levels of 25-hydroxyvitamin D are considered the indicator of vitamin D status (Nelson et al., 2012) and feeding vitamin D at or above the NRC requirement of 30 IU/kg of BW is adequate to maintain circulating 25-hydroxyvitamin D (Weiss, 1998; NRC, 2001; Nelson and Merriman, 2014). Activated 1,25-dihydroxyvitamin D circulates primarily in association with vitamin D binding protein (Bikle et al., 1984), with only 5% circulating the in the free form. Free 1,25-dihydroxyvitamin D diffuses into the cytoplasm of target cells where it acts as a steroid hormone, binding the vitamin D receptor. Upon phosphorylation, this complex binds the retinoic acid X receptor to form a heterodimer which can then bind the vitamin D response elements to initiate transcription of vitamin D dependent genes (Stipanuk and Caudill, 2013).

Intestinal Calcium Absorption in Calcium Homeostasis

Absorption of dietary Ca has been shown to occur primarily in the small intestine in dairy cows (Yano et al., 1979; Greene et al., 1983c; Rahnema and Fontenot, 1983) with net addition of Ca to digesta occurring prior to the duodenum as a result of endogenous Ca secretion (van't Klooster, 1976). Some data suggest that passive absorption of Ca can occur in the rumen at high concentrations of Ca (Greene et al., 1983b; Khorasani et al., 1997). The molecular aspects of absorption of Ca in the gastrointestinal epithelium share many similarities with reabsorption of

Ca in the renal epithelium. Activated 1,25-dihydroxyvitamin D acts on epithelial cells of the small intestine to increase the expression of transporters and Ca binding proteins in those cells which facilitate active transcellular absorption of dietary Ca (Hoenderop et al., 2005; Martin-Tereso and Martens, 2014). A decline in blood Ca of cows fed a Ca-deficient diet in the prepartum period is associated with an increase in circulating 1,25-dihydroxyvitamin D and a subsequent restoration of blood Ca (Green et al., 1981). In response to feeding a Ca-deficient diet, the restoration of blood Ca can take 2 d or more, illustrating the time required for downstream signaling and expression of the necessary proteins (van't Klooster, 1976).

Bone Calcium Resorption in Calcium Homeostasis

Bone serves as a major reservoir of Ca and an estimated 99% of whole body Ca is present in bone (Horst, 1986). In periods of Ca deficit, bone serves as a resource to stabilize blood Ca. The majority of bone Ca is present as hydroxyapatite salts $[Ca_5(PO_4)_3OH]$ although other forms of Ca are present. The more rapidly degrading Ca acts as an exchangeable pool of Ca that is available to correct minor fluxuations in blood Ca concentration through a buffering system (Hall, 2011). Formation and resorption of bone are both continual processes facilitated primarily by osteoblasts and osteoclasts, respectively, at the level of bone. These cells have significant cross talk and respond to endocrine factors including PTH and 1,25-dihydroxyvitamin D. The development, proliferation and activity of osteoclasts, and the simultaneous downregulation of osteoblast activity, is stimulated by the protein RANK ligand, produced by osteoblasts in response to PTH. This effect is further stimulated by the presence of high concentrations of 1,25-dihydroxyvitamin D. Proteolytic enzymes and acids secreted by osteoclasts solubilize the inorganic components of the bone tissue, releasing Ca into circulation (Hall, 2011). Evidence of increased bone metabolism can be found in situations of induced hypocalcemia. In dairy cows

fed Ca deficient diets, an increase in plasma hydroxyproline concentrations, a marker of collagen catabolism that is associated with bone resorption, has been observed (Green et al., 1981). This indicates that the deficiency of the ration Ca is at least partially compensated for by increased mobilization of bone Ca. Taylor et al. (2009) measured apparent bone resorption (measured indirectly through deoxypridinoline concentrations in blood) and this was increased after calving and subsequently decreased through 20 wk postpartum, and the opposite trend was observed for osteocalcin (a measure of formation of the bone protein matrix). These data demonstrate that temporal changes in both the formation and resorption of bone occur in times of systemic Ca challenges.

Regulation of Calcium Secretion in Milk

The secretion of Ca into milk requires the upregulation of Ca transporters on both the basolateral and luminal side of the mammary epithelial cells. This process is mediated by the non-neuronal serotonin-parathyroid hormone related peptide (PTHrP) axis. Serotonin is produced from L-tryptophan through two reactions, the rate limiting step is the conversion of L-tryptophan to 5-hydroxytryptophan (5-HTP) which then converted to 5-hydroxytryptamine (serotonin) (Laporta et al., 2014; Weaver and Hernandez, 2016). The production of serotonin stimulates synthesis and secretion of parathyroid hormone related protein (**PTHrP**) which has many similar functions to PTH that were described previously. The PTHrP is responsible for increasing supply of Ca for milk production by stimulating additional bone Ca resorption and stimulation of PTHrP production has been demonstrated to increase osteoclast size and number in mice (Laporta et al., 2013b). In mice with ablations of enzymes for serotonin production, abnormalities in mammary epithelial cell morphology and proliferation are evident and blood Ca concentrations in these mice are decreased (Laporta et al., 2014). Administration of 5-HTP to

these mice rescues phenotypes such as Ca transporter expression and blood Ca concentrations.

When 5-HTP is fed to transition mice, blood and milk concentrations of Ca are increased after

parturition (Laporta et al., 2013b). These data demonstrate the importance of the serotonin-

PTHrP pathway for maintaining normal Ca homeostasis during lactation. Research in this area is

active and application of this knowledge in periparturient dairy cow nutrition will be discussed

later.

Effect of Age on Ca Metabolism

Increasing parity is associated with increased susceptibility to clinical and subclinical

hypocalcemia in the early postpartum period (Erb and Grohn, 1988; Reinhardt et al., 2011;

Wilkens et al., 2013). Primiparous cows have been shown not only to maintain higher blood Ca

concentrations in the periparturient period but also to be more resilient to prepartum rations

known to predispose older cows to hypocalcemia (Shappell et al., 1987; Tucker et al., 1992;

Moore et al., 2000). Taken together, this suggests that older cows either have a more severe Ca

challenge at parturition, are less capable of adapting to the increased Ca demand, or both. While

it has been demonstrated that colostrum from primiparous cows has higher Ca concentration than

that of multiparous cows, the total yield of colostrum and milk is higher for multiparous cows

(Shin-ichi and Shinobu, 1993). However, the relationship between milk Ca output and

hypocalcemia is not direct, and some cows with clinical hypocalcemia have been shown to have

lower colostrum Ca output than their normocalcemic counterparts (Shappell et al., 1987). This

suggests that greater milk Ca output does not fully explain why some older cows succumb to

hypocalcemia and indicates that some aspect of Ca homeostasis is impaired.

Exacerbated hypocalcemia that is observed in older cows is accompanied by increases in

circulating concentrations of PTH and 1,25-dihydroxyvitamin D although whether the increases

in those hormones are appropriate for the degree of hypocalcemia is not clear (Wilkens et al., 2013). Shappell et al. (1987) observed that the increases in PTH were only slightly greater postpartum for older cows experiencing severe hypocalcemia. In the study by Rodriguez et al. (2016), PTH concentrations were higher in cows with more severe hypocalcemia, but concentrations of 1,25-dihydroxyvitamin D were lower than cows with less severe hypocalcemia. This suggests that the efficiency of PTH signaling at target tissues is an important factor in hypocalcemia susceptibility. Administration of exogenous PTH at least 60 h prior to parturition effectively eliminated hypocalcemia in multiparous cows in one study suggesting that additional PTH, or more efficient PTH signaling, could benefit multiparous cows in the prevention of hypocalcemia (Goff et al., 1986).

Studies using radiotracer techniques demonstrated that mature and aged cows in a steady physiological state had reduced absorption efficiency of dietary Ca and greater excretion of Ca from endogenous origins (Hansard et al., 1954). Calves younger than 30 d old in this study had a Ca absorption efficiency of approximately 98%. Absorption efficiency precipitously decreased to 40% for cows older than 6 months and continued to decline for mature and aged cows. While lower Ca demand for growth likely accounts for some of this difference, evidence suggests that vitamin D mediated mechanisms for increasing Ca absorption efficiency decline with age. In addition to decreased abundance of vitamin D receptors in intestinal epithelial cells of aged rats and cows, the ability to upregulate vitamin D receptor expression in aged rats is also compromised (Horst et al., 1990). Supporting evidence in rats has demonstrated that age is associated with decreased responsiveness to exogenous 1,25-dihydroxyvitamin D (Horst et al., 1978a; Armbrecht et al., 1998) and decreased responsiveness to feeding a Ca deficient diet (Horst et al., 1978a). This is on contrast to young rats which were able to rapidly adapt intestinal

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Ca transport in response to deficient diets. For the periparturient cow of advanced age, a decrease in the number of vitamin D receptors in the intestine and decreased sensitivity of those receptors to a stimuli could certainly inhibit the adaptation of that cow to lactation.

Similar indications of age related declines in metabolic activity of bone have been measured in rodent models as well as periparturient dairy cows. Decreases in the abundance of vitamin D receptors on bone cells in rats of advanced age have been measured similarly to the changes seen in intestinal epithelial cells (Horst et al., 1978a). Sensitivity of bone tissue to stimulation may be affected by the decrease in vitamin D receptor expression and delay the contribution of resorbed bone tissue to maintaining blood Ca in older cows. Taylor et al. (2009) showed that indirect markers of bone tissue resorption and bone formation were higher from 2 wk prior to calving until 11 wk postpartum in primiparous compared to multiparous cows. Although the patterns of these markers throughout the study period were similar for both parity groups, the higher concentrations in primiparous cows indicated greater total bone turnover which likely contributed to maintenance of higher blood Ca concentrations throughout the transition period in those cows.

CALCIUM HOMEOSTASIS AT THE ONSET OF LACTATION

The complexity of Ca homeostasis has been demonstrated thus far, and the challenge at parturition lies in coordination of the hormones and tissues involved in increasing the flux of Ca rapidly enough to avoid the consequences associated with compromised systemic Ca status. In a series of elegant radioisotope studies, the alterations in Ca metabolism in the periparturient period of dairy cows were characterized. Ramberg et al. (1970) described the flow of radiolabeled ^{45}Ca through a compartmental model during the late gestation period, at the initiation of lactation and during the recovery of blood Ca postpartum. A reduction in the mass

of Ca in the exchangeable pool of 33% was observed in the immediate postpartum period, reflective of the drain of Ca for colostrum and early milk production, and this was accompanied by a drop in plasma Ca. This initial drop in blood Ca concentration is an important stimulus for production of PTH (Ramberg et al., 1967) as it is the initial trigger for the mechanisms responsible for increasing influx of Ca to the exchangeable pool. Ramberg et al. (1970) measured a decline in plasma Ca for 1 to 2 d after parturition, suggesting a delay in the adaptation of Ca flux to compensate for this loss. Changes in Ca flux that occurred at the onset of lactation included a decrease in endogenous fecal Ca output, a decrease in deposition of Ca in bone, and an increase in Ca absorption from both dietary and endogenous sources. Interestingly, the contribution of bone Ca to the exchangeable pool in this study was not evident until after wk 2 of lactation. In contrast, Rowland et al. (1972) showed that clinically healthy cows had elevated bone resorption and decreased bone formation over the first 10 DIM in comparison to prepartum bone tissue activity. Black and Capen (1971) similarly showed that hydroxyproline is gradually elevated prepartum and rapidly elevated immediately after parturition. These studies suggest a much more rapid change in bone metabolism compared to that determined by Ramberg et al. (1970).

Studies by van't Klooster (1976) showed that 80% of the Ca content in milk could be accounted for by more efficient absorption of dietary Ca in the early postpartum period. Calcium absorption efficiency was shown to be approximately 20-25% prepartum with increases to around 40% by d 8 postpartum when there is no change in dietary Ca concentration. In this study, the adaptation of Ca absorption efficiency did not begin until approximately 2 to 3 d after parturition, consistent with the depression in plasma Ca that was observed in the experiments of Ramberg et al. (1970). The response of PTH to changes in blood Ca have been shown to require

only approximately 15 min and is not the cause for delayed adaptation (Ramberg et al., 1967). Evidently, mechanisms for activation of bone Ca resorption and increased efficiency of intestinal Ca absorption discussed previously require as long as 2 to 3 d to take effect. In addition to the depression in DMI that occurs around parturition, the onset of lactation is accompanied by a decreased flow of digesta through the gastrointestinal tract (van't Klooster, 1976), at least partly due to decreased gut motility as a result of hypocalcemia (Daniel, 1983). These dynamics likely contribute to the lag in contribution of dietary Ca to milk Ca outflow. Although these studies provide a frame of reference for the time needed to activate homeostatic mechanisms, the production and feeding of cows in these studies was quite different than that of cows in current systems. The relative contribution of bone Ca and dietary Ca to maintenance and recovery of blood Ca is likely quite different in the two settings. Factors influencing these pathways in periparturient cows will be discussed in more detail in the context of disorders of Ca homeostasis and prevention of hypocalcemia.

DISORDERS OF CALCIUM HOMEOSTASIS IN TRANSITION COWS

Some decline in blood Ca postpartum is an important stimulatory factor for increasing the supply of Ca to the exchangeable pool in support of early lactation Ca demands. However, as discussed previously, some delay in this adaptation occurs and some cows experience severe declines in blood Ca or are unable to recover blood Ca concentrations rapidly enough to avoid negative consequences. Degrees of severity in this condition have been identified based on clinical symptoms and in the absence of clinical symptoms, the associations between blood Ca measurements in the days following parturition and the subsequent health and performance of the cow.

Clinical Hypocalcemia

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Clinical hypocalcemia has classically been defined as blood Ca concentration below 1.375 mmol/L (5.5 mg/dL), although some cows fall below this threshold without showing clinical signs (Goff, 2008; Ramos-Nieves et al., 2009). True cases of clinical hypocalcemia are accompanied by paresis which is the result of insufficient Ca for skeletal muscle function and the condition progresses from weak muscles and lethargy to recumbency and possibly death if left untreated (Oetzel, 2013). The occurrence of clinical hypocalcemia is associated with an increased risk of several other metabolic and infectious diseases including dystocia, retained placenta, mastitis, ketosis, and displaced abomasum (Curtis et al., 1983). Consequences of the disorder also include reductions in milk production that can be sustained for 4 to 6 wk after calving (Rajala-Schultz et al., 1999) and can amount to a 14% reduction in milk yield compared to non-paretic counterparts (Block, 1984). Clearly this clinical condition has a massive influence on the well-being, health and productivity of dairy cows.

Certain risk factors that predispose cows to hypocalcemia have been identified. Ramberg et al. (1970) showed that cows that developed parturient paresis did not have greater total outflow of Ca to milk postpartum, nor did they have more rapid increase in Ca outflow, compared to non-paretic cows. Further, Shappell et al. (1987) found that the concentration of Ca in colostrum, and Ca output in colostrum as a fraction of metabolic body weight, was higher only for cows that did not experience a severe decline in blood Ca postpartum. As opposed to greater Ca output, parturient paresis is characterized by significant deficiencies in the adaptation of Ca homeostasis to the onset of lactation. The comparison of cows that experience parturient paresis to apparently unaffected cows shows that production of PTH is not the limitation that restricts the cow from adapting to the increased Ca demand since PTH production is higher than cows with mild reductions in blood Ca (Mayer et al., 1969; Horst et al., 1978b). In the study by Horst

et al. (1978b), cows that developed parturient paresis had higher concentrations of PTH and 1,25-dihydroxyvitamin D but the correlation between PTH and 1,25-dihydroxyvitamin D in that study was weak (r=0.31) suggesting that responsiveness of target tissues to PTH stimulation was not optimal in paretic cows.

Further evidence suggests that tissues of paretic cows become refractory to PTH stimulation in the period around calving. Upon comparison of bone samples analyzed via microradiography from cows that became paretic around calving and those that did not, it was found that paretic cows had very low bone turnover rates despite elevated PTH. The bone turnover of cows that did not develop parturient paresis was elevated around calving with a significant increase in resorption and decrease in formation, especially postpartum (Rowland et al., 1972). Similar comparisons were demonstrated in another study which measured plasma hydroxyproline and found increases prior to calving in non-paretic cows but decreases in paretic cows (Black and Capen, 1971) suggesting that cows that ultimately develop parturient paresis are unable to respond adequately to the increased demand for Ca. The cause of this apparent hypoparathyroidism will be discussed later.

Subclinical Hypocalcemia

A large proportion of dairy cows in current management systems are able to avoid clinical hypocalcemia but still experience a decline in blood Ca to subclinically low levels. Recent surveys indicate that incidence of clinical hypocalcemia has been reduced to approximately 5% in U.S. dairy herds (Reinhardt et al., 2011) and as low as 2.6% in other studies (Chapinal et al., 2011). Of much greater estimated economic importance to the dairy industry is the incidence of subclinical hypocalcemia (**SCH**) which appears to occur in approximately 25% of primiparous cows and at least 50% of older cows (Reinhardt et al., 2011; Oetzel, 2013;

Caixeta et al., 2015). In contrast to clinical hypocalcemia, cows affected by SCH can only be identified through the measurement of blood Ca since these cows do not display any outward signs of the disorder. Recent work has established thresholds for identification of hypocalcemia based on subsequent risk of compromised health or performance. These thresholds range from 2.0 mmol/L to 2.2 mmol/L (Chapinal et al., 2011; Chapinal et al., 2012; Martinez et al., 2012; Wilhelm et al., 2017). The merits and nuances of identifying hypocalcemia based on these thresholds will be discussed in the next section.

Factors that predispose cows to SCH likely are similar to those discussed for clinical hypocalcemia. Recent work suggests that risk factors for SCH also include having moderate reductions in blood Ca in the week prior to parturition and lameness in the periparturient period (Neves et al., 2017). It is likely that risk factors that cause further reductions in DMI in the periparturient period may increase susceptibility to SCH since this will impact the efficacy of preventative strategies as well as reducing the contribution of dietary Ca to maintaining blood Ca status.

MONITORING HYPOCALCEMIA

Blood Calcium Fractions

Identification of SCH is complicated by the differentiation of blood Ca into three separate pools. Total blood calcium (**tCa**) circulates in the body in the following forms; bound to proteins (mainly albumin), bound to anions (such as bicarbonate, citrate, and acetate), or in the free ionized form. Ionized calcium (**iCa**) is the metabolically active portion of Ca in the blood (Rosol et al., 1995). The proportion of Ca that is in the free ionized form can be influenced by the concentration of protein in the blood as well as acid base balance (Joyce et al., 1997). Total Ca has primarily been used to assess Ca status in periparturient cows because of ease of sample

collection and analysis. Current methods for iCa measurement are expensive and require special

collection and handling techniques to acquire reliable data. Alterations in the pH of the sample

due to air exposure can create falsely low measurements of iCa because competition with H^+ for

protein binding sites is decreased and more iCa is present in the protein bound form (Lincoln and

Lane, 1990). For this reason, anaerobic sample collection methods are recommended as well as

measuring Ca concentration as close to sample collection as possible to avoid continued

alterations in sample pH with storage time (Boink et al., 1991). Further, the use of some

anticoagulants can artificially reduce iCa measurements due to binding of iCa. This can be

avoided with the use of Ca-balanced heparin syringes, but adds further cost to the analysis

(Boink et al., 1991; Tappin et al., 2008).

Despite the complications associated with accurate measurement of iCa, it is understood

that iCa may be a more sensitive measure of Ca status in the immediate postpartum period of the

dairy cow. Physiological functions requiring Ca such as activation of immune cells (Kimura et

al., 2006; Martinez et al., 2014) and gut motility (Daniel, 1983) utilize iCa. Comparisons of total

and ionized Ca in blood of dairy cows in the day after calving demonstrated less variation in iCa

measurements (Szenci et al., 1994). Based on this observation, Szenci et al. (1994) concluded

that this fraction was likely more indicative of the Ca status of the cow. Ballantine and Herbein

(1991) measured changes in the proportion of tCa that was in the ionized form throughout the

period from 4 wk before to 10 wk after parturition and showed that the proportion that was

ionized increases after calving. Similar results were found by Joyce et al. (1997) and the ratio of

iCa to tCa increased from approximately 0.50 prepartum to 0.54 at parturition.   This alteration in

the relationship between iCa and tCa in the immediate postpartum period may be a result of

increased Ca metabolism, changes in acid-base balance, altered protein concentrations in blood,

or other factors. In cows that have large drops in blood tCa, an elevated proportion of iCa may compensate and prevent those cows from having compromised physiology, which would not be distinguishable if only tCa were measured. These data indicate that measurement of tCa may include variation that is unrelated to functional Ca status, however, data associating iCa and tCa with health and productive outcomes are needed to determine the relative sensitivity and specificity of these measures.

Thresholds and Strategies for Blood Calcium Measurement

Recently proposed thresholds for identification of SCH are near < 2.15 mmol/L (Martinez et al., 2012). This threshold was established based on the risk of cows being diagnosed with metritis in a study population selected for higher risk of metritis and matched with cows at a lower risk of metritis. Blood Ca measurements were made between 0 and 3 DIM and the lowest measurement was used as the indicator of Ca status. Similar thresholds have been proposed by other authors when measured within 2 h of parturition (Wilhelm et al., 2017) or even across the first 7 DIM (Chapinal et al., 2011; Chapinal et al., 2012). Blood Ca measurements taken across this highly variable time should be interpreted cautiously. The timing of measurement of blood Ca relative to parturition can significantly alter the magnitude of the potential consequences of the disorder. Chapinal et al. (2012) associated SCH diagnosed at wk 1, 2 and 3 after parturition with increasing severity of decreased milk yield. Subclinical hypocalcemia diagnosed at different times has also been shown to have different predisposing factors. Neves et al. (2017) investigated risk factors associated with SCH diagnosed within 4 h of parturition and at 2 DIM. Plasma Ca concentrations ≤ 2.4 mmol/L in the week prior to parturition, as well as higher parity, were risk factors for SCH at parturition in that study depending on time of SCH diagnosis. Prepartum Ca status was not associated with SCH at 2

DIM but lameness, SCH (≤2.1 mmol/L) at parturition, and the occurrence of retained placenta (**RP**) were important predictors of SCH at 2 DIM. The difference in risk factors may at least partially be due to the fact that cows classified as SCH at 2 DIM may be suffering from other transition disorders that are preventing a recovery in blood Ca.

Caixeta et al. (2017) introduced the concept of chronic SCH, defined as cows with blood Ca concentration ≤ 2.15 mmol/L for the first 3 DIM and found that the outcomes of interest, return to cyclicity and odds of pregnancy at first service, were most affected in cows with chronic SCH in comparison to those with low blood Ca for less than 3 d. A drop in blood Ca close to parturition, within a reasonable range, could be a reflection of appropriate adaptation to lactation and a single measurement of Ca status may just be capturing that response. The concept of chronic SCH is a more definitive way of separating animals that are clearly struggling to adapt after that initial response. The final caution about timing of SCH identification relative to parturition is that low blood Ca could be a secondary response to a robust immune response to disease (Waldron et al., 2003) or a drop in DMI associated with other transition disorders, thus making cause and effect between these disorders nearly impossible to establish and timing of blood sampling critical. Further epidemiological studies of this disorder are needed to identify the most appropriate sample timing, accurate thresholds based on time relative to calving and effect of parity on these factors. This information is vital to providing guidelines for assessing the impact of SCH on herds and assessing the efficacy of management and preventative strategies in place.

PERIPARTURIENT HEALTH AND THE INTERACTION WITH HYPOCALCEMIA

Intracellular Ca signaling is involved in many physiological systems throughout the body and is dependent on a stable concentration of Ca in the extracellular fluid (Hall, 2011). A

consequence of the ubiquity in cell signaling mediated by Ca throughout the body is that perturbations in systemic Ca status are implicated for loss of function across many different systems. Vital metabolic adaptations to lactation and aspects of periparturient immune function will be discussed as well as the role that hypocalcemia plays in those processes.

Negative Energy Balance

The classic example of homeorhetic adaptation in the dairy cow is the acclimation to the dramatically increased energy demands of lactogenesis (Bauman and Currie, 1980). As described by Bell (1995) the energetic demands for milk production at 4 DIM, as compared to the gravid uterus at 250 d of gestation, are increased by a factor of approximately 2.7 for glucose, 2.0 for amino acids and 4.5 for fatty acids. The periparturient period is also associated with a depression in DMI and thus, decreased intake of nutrients that could be used to support the energy demands of lactation (Ingvartsen and Andersen, 2000; Hayirli et al., 2003). When corrected for body weight, intake can decline approximately 30% in the 3 wk prior to calving (Bertics et al., 1992; Hayirli et al., 2002) and recovery of DMI postpartum is not as rapid as the increase in milk production. Circulating concentrations of both glucose and insulin are low postpartum, indicative of a relative deficit in nutrient availability (Bauman et al., 1988; Mann et al., 2016).

The inability of the energy consumed from the diet to meet the requirements for maintenance, milk production, and growth in the case of younger cows, is referred to as negative energy balance (**NEB**). Compensation for NEB is accomplished by mobilization of body reserves including fatty acids mobilized from adipose tissue and amino acids from muscle tissue (Drackley et al., 2001). The metabolic capacity of the liver is greatly elevated in the immediate postpartum period as evidenced by greater oxygen consumption (Reynolds et al., 2003) and in

addition to substrates provided by the diet and ruminal fermentation, the liver utilizes the carbon of amino acids, lactate and to a smaller extent, glycerol, for glucose production (Drackley et al., 2001; Reynolds et al., 2003). As part of the homeorhetic adaptation to support milk production, the use of glucose by extramammary tissues is reduced to spare glucose for the production of lactose. This effect is mediated by somatotropin, which is elevated in the period around parturition (Bauman et al., 1988; Bell, 1995). Somatotropin also mediates an increase in the oxidation of fatty acids in peripheral tissues to compensate for reduced utilization of glucose (Bauman et al., 1988).

The fatty acids mobilized from adipose tissue are taken up by the liver in proportion to their concentration in blood (Reynolds et al., 1988; Pullen et al., 1989). Fatty acids have three fates within the liver, they can be completely oxidized within the mitochondria of hepatocytes to CO_2 and therefore used as a fuel for the liver, they can be partially oxidized and produce ketone bodies including β-hydroxybutyrate (**BHB**), acetoacetate and acetone with can be released into the blood, or they can be re-esterified to triglycerides to be exported as very low density lipoproteins (Drackley et al., 2001). Hepatocytes of dairy cows have a low capacity for exporting triglycerides as very low density lipoproteins, especially in early lactation (Pullen et al., 1989). As a result, cows that experience more severe negative energy balance have greater lipid accumulation in hepatocytes in the transition period (Bertics et al., 1992).

Some degree of NEB is a normal adaptation to the initiation of lactation (Bauman and Currie, 1980) but excessive adipose tissue mobilization or production of ketone bodies can have detrimental effects on the health and productivity of cows. Clinical ketosis and fatty liver can dramatically reduce the productive life of dairy cows (Grohn et al., 1998). Subclinically elevated fatty acids and ketone bodies have been identified as risk factors for similar consequences.

Elevated blood concentrations of NEFA and BHBA have repeatedly been associated with increased risk of health disorders (Ospina et al., 2010a; Chapinal et al., 2011), early lactation culling (Roberts et al., 2012), and compromised productive and reproductive performance (Ospina et al., 2010b; Chapinal et al., 2012).

Hypocalcemia and Metabolic Health

Hypocalcemia has been associated with disorders relating to excessive NEB and compromised DMI in the early postpartum period. Cows with parturient paresis had 8.9 times greater odds of being diagnosed with clinical ketosis and tended to have 3.4 times greater odds of left displacement of the abomasum in a field trial of 33 farms in New York state (Curtis et al., 1983). Similarly, decreases in blood Ca below 2.2 mmol/L in the week following parturition were associated with 2.7 times greater odds of displacement of the abomasum in a more recent study conducted with cows from 55 herds across the U.S. and Canada (Chapinal et al., 2011). Studies assessing SCH at or immediately following calving have shown increases in blood concentrations of NEFA (Reinhardt et al., 2011; Martinez et al., 2012; Chamberlin et al., 2013) and BHB postpartum (Martinez et al., 2012), as well as increased liver triglyceride content (Chamberlin et al., 2013). Decreased blood Ca is associated with a decline in gut motility (Daniel, 1983; Martinez et al., 2014) and this is likely an important component of the interaction between hypocalcemia and metabolic health. Facilitating better maintenance of blood Ca at parturition would theoretically remove one limitation to increasing DMI postpartum to meet energetic demands. The association between gut motility, Ca status, and DMI likely play a large part in the connection between hypocalcemia and excessive NEB, but other potential roles of Ca in energy metabolism will be discussed.

Intracellular signaling dependent on Ca is known to play an important role in release of insulin from the pancreas in response to glucose (Grodsky and Bennett, 1966). When SCH is induced in cows by infusion of ethylene glycol tetraacetic acid (**EGTA**), plasma insulin concentrations are decreased and plasma glucose and NEFA are increased (Martinez et al., 2014). Baseline blood glucose concentrations and insulin concentrations, as well as insulin responses to intravenous glucose challenges are lower in postpartum dairy cows compared to in the prepartum period (Grunberg et al., 2011; Mann et al., 2016). Although the energetic challenges at parturition are likely the overlying factor in this metabolic profile, hypocalcemia may play a role in impairing blood insulin responses. Intravenous administration of Ca has been demonstrated to increase insulin secretion and decreased blood glucose in cows that were hypocalcemic postpartum (Blum et al., 1973). It is plausible that hypocalcemia exacerbates mobilization of NEFA in the early postpartum period by decreasing blood insulin concentrations and release of insulin in response to glucose, contributing to the decreased antilipolytic signal to adipose tissue.

Other mechanisms may contribute to the elevated blood NEFA concentrations observed in hypocalcemic cows. In *in vitro* studies with human adipocytes, it has been shown that stimulation of the Ca sensing receptor has an antilipolytic effect (Cifuentes and Rojas, 2008). Theoretically, low concentrations of Ca in blood could diminish the antilipolytic stimulation from the Ca sensing receptor at the level of the adipocyte, contributing to greater adipose tissue mobilization at this time. Experimentally induced SCH has been demonstrated to result in increased blood NEFA concentrations (Martinez et al., 2014). However, from this experiment it is unclear if the increase in NEFA is a result of decreased blood insulin concentrations, reduced stimulation of the Ca sensing receptor on adipocytes, or some other mechanism. Investigation of

this theory in the transition cow is needed to determine if it plays a role in adipose tissue mobilization in the transition period.

Periparturient Immune Function

The transition period is characterized by a state of immune dysfunction (Kehrli et al., 1989; Kimura et al., 1999) and a large percentage of infectious disease is observed during the early lactation period. It has been demonstrated that functional capacity of both innate and acquired immune cells is dramatically reduced during this time, beginning prior to calving and recovering weeks after parturition (Kehrli and Goff, 1989; Kehrli et al., 1989). A large emphasis is placed on the functional capacity of innate immune cells in the transition period because this piece of the immune system is essential for expelling the placenta after parturition (Kimura et al., 2002) and clearing the uterus of the pathogens introduced during parturition to prevent uterine disease (Hammon et al., 2006). Interactions between the innate and acquired immune system likely contribute to the compromised innate immune responses. Mallard et al. (1997) identified groups of cows that had compromised antibody response to antigen in the periparturient period. Phagocytes of the innate immune system depend on opsonization of pathogens by antibody and components of the complement system to efficiently phagocytose those invading pathogens (Abbas et al., 2014).

The endocrine and metabolic changes around parturition can both be implicated for the suppression in immune cell function that occurs at this time. In a series of seminal studies, cows that had been mastectomized were managed alongside intact cows through the transition period (Kimura et al., 1999; Goff et al., 2002; Nonnecke et al., 2003). The resulting comparison demonstrated the relative contribution of endocrine changes associated with parturition and the metabolic demands of lactation to periparturient immune dysfunction. Mastectomization did not

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alter the decline in neutrophil function measured prior to parturition and in both groups a decline was observed in expression of L-selectin, an endothelial adhesion molecule that initiates diapedesis to sites of infection, as well as myeloperoxidase activity, an index of neutrophil killing ability (Kimura et al., 1999). This indicates that endocrine factors associated with pregnancy and parturition impact neutrophil functions and adhesion molecule expression. Indeed, parturition is associated with a spike in cortisol concentrations (Goff et al., 2002) and glucocorticoids such as cortisol and dexamethasone are known to decrease expression of L-selectin and cause a subsequent neutrophilia which is observed at parturition in dairy cows (Burton et al., 1995; Kimura et al., 1999; Weber et al., 2001). Estrogens, which are increased prior to parturition (Goff et al., 2002), have also been demonstrated to suppress function of immune cells *in vitro* and may contribute to the decline in immune cell function prior to parturition (Wyle and Kent, 1977).

In the mastectomy model, myeloperoxidase activity of neutrophils recovered quickly in mastectomized cows but remained compromised in intact cows for the 3 wk postpartum (Kimura et al., 1999). Glucose has been shown to be an important energy substrate for innate immune cells upon activation (Newsholme et al., 1986). More severe NEB in periparturient dairy cows has been associated with compromised immune cell function (Schwarm et al., 2013), decreased neutrophil glycogen concentrations and uterine disease (Galvao et al., 2010). In another study, cows that ultimately were diagnosed with uterine disease had reduced DMI and elevated concentrations of NEFA and BHB in the peripartum period and those cows had decreased functional capacity of neutrophils beginning in the wk prepartum and continuing through 3 wk postpartum (Hammon et al., 2006). The energetic demands of immune cell functions likely at least partially underpin the immune dysfunction observed in the transition period leading to

increased risk of infectious disease in cows that experiences excessive NEB in the early

postpartum period (Duffield et al., 2009; Dubuc et al., 2010).

Hypocalcemia and Immune Function

Associations between hypocalcemia and increased risk of infectious disease have been

demonstrated in epidemiological studies (Curtis et al., 1983; Martinez et al., 2012; Wilhelm et

al., 2017). Cows with hypocalcemia have been shown to have reduced immune cell function in

the immediate postpartum period (Kimura et al., 2006; Martinez et al., 2012). Ducusin et al.

(2003) measured lower intracellular Ca concentrations in polymorphonuclear leukocytes (**PMN**)

of cows experiencing parturient paresis. *In vitro*, those PMNs were less capable of

phagocytosing pathogens and supplementation of the media with Ca *in vitro* increased

intracellular stores and restored phagocytic function. Kimura et al. (2006) further explored this

relationship and found that stores of Ca within the endoplasmic reticulum were reduced in

hypocalcemic cows, with decreased intracellular Ca evident before hypocalcemia was measured

in blood samples. This condition led to lower Ca release from these stores upon stimulation,

compromising the functional capacity of those cells. Martinez et al. (2014) demonstrated similar

deficits in the release of intracellular Ca stores of neutrophils during experimental induction of

SCH. These data suggest that hypocalcemia is partially causal in the immune dysfunction of

periparturient cows and that immune cell function may be compromised prior to an observed

drop in systemic Ca status.

Cows with SCH have been demonstrated to have reduced reproductive performance

(Chapinal et al., 2012; Martinez et al., 2012; Caixeta et al., 2017) using different thresholds and

timing for SCH diagnosis. The risk of compromised immune cell function, and subsequently

increased risk of clinical and subclinical uterine disease (Martinez et al., 2012; Wilhelm et al., 2017), likely play a large part in compromising fertility in those cows.

NUTRITIONAL PREVENTION OF HYPOCALCEMIA

Manipulation of Acid-Base Balance with Prepartum Rations

The prevention of hypocalcemia has largely focused on dietary methods designed to enhance the cow's own regulatory mechanisms for increasing Ca turnover around parturition. Whereas the calciotropic effects of PTH and 1,25-dihydroxyvitamin D maintain blood Ca within normal ranges during steady physiological states, the challenge presented at the initiation of lactation results in a demand upon this system that requires maximal function. Early work demonstrated that cows in the prepartum period had less pronounced response to administration of exogenous PTH than cows in early lactation (Martig and Mayer, 1973). One factor that has been recognized as an inhibitor of PTH action is a state of mild metabolic alkalosis which is a common when cows are fed high K prepartum rations without anions supplemented beyond requirements. It is hypothesized that in the alkalotic state, the slightly higher blood pH changes the PTH receptor-G-protein complex conformation on tissues vital to Ca homeostasis including the kidney and bone cells, resulting in decreased ability of the PTH protein hormone to bind and signal that receptor (Krapf et al., 1992; Goff et al., 2014). Evidence for this pseudohypoparathyroidism can be found in studies conducted in humans. Krapf et al. (1992) observed a hypocalcemia in human subjects with experimentally induced metabolic alkalosis due to respiration in a CO_2 chamber. Upon reestablishing acid-base balance, an increase in nephrogenous cyclic adenosine monophosphate [**cAMP**; an indicator of PTH signaling at the kidney (Broadus et al., 1977)] excretion was observed despite no changes in circulating PTH and normocalcemia was established. Further evidence for improved Ca metabolism in induced

metabolic acidosis is found from studies in thyroparathyroidectomized rats (Beck and Webster, 1976). Metabolic acidosis was induced by infusion with NH₄Cl and in another group, metabolic alkalosis was induced by infusion with NaHCO_s. Injection of PTH resulted in no change in blood Ca concentration for rats in metabolic alkalosis, but a significant blood Ca response was observed in the rats in metabolic acidosis. In the same study, when the intestine and kidneys were removed from the rats, metabolic acidosis increased basal blood Ca concentrations as well as improving the response of blood Ca to PTH injection, indicating that metabolic acidosis also improves the contribution of bone resorption to blood Ca maintenance.

Metabolic acidosis can be induced in dairy cows through manipulation of the prepartum rations of by minimizing the concentration of cations in the ration and adding supplemental anions. In application, the balance of these ions is referred to as the dietary cation-anion difference (**DCAD**). The simplified strong ion model for determining plasma pH provides an understanding of the metabolic changes that occur when feeding a low or negative DCAD to induce a metabolic acidosis. The simplified strong ion model, as described by Constable (2003), states that plasma pH is determined by three independent factors; plasma CO_2 tension (**PCO_2**), strong ion difference (**SID**), and concentration of individual nonvolatile plasma buffers (**Atot**). Strong ion difference can be calculated as the difference between strong cations and strong anions in milliequivalent units in plasma and *Atot* primarily represents the concentration of albumin, globulins and inorganic phosphate in plasma. The simplified equation for plasma pH according to this model is as follows:

$$pH = pK_1' + \log \frac{SID - Atot/(1 + 10^{pK_a - pH})}{S\, P_{CO_2}}$$

Where *pK_1'*, *pK_a* and *S* are the apparent dissociation constant for carbonic acid, the effective dissociation constant for nonvolatile plasma buffers and the solubility of *CO_2* in plasma,

respectively. Using this model, when a net negative balance of strong ions are absorbed from the ration of prepartum dairy cows, a reduction in the *SID* will ultimately decrease the plasma pH by imposing a strong ion metabolic acidosis. The blood profile of cows fed a negative DCAD diet reflects varying degrees of a metabolic acidosis. Blood bicarbonate and blood CO_2 levels are decreased, and H^+ concentrations are increased (Goff and Horst, 1997; Goff et al., 2014). Similar profiles were shown in pregnant does fed diets containing excess anions (Fredeen et al., 1988). In this study, the lower plasma CO_2 was accompanied by higher O_2 suggesting respiratory compensation for the increased H^+ concentration in addition to the urinary excretion of H^+.

The major contributors to the calculation of the *SID* are the strong cations Na^+, K^+, Mg^{2+}, Ca^{2+} and NH_4^+ and the strong anions Cl^- and SO_4^{2-}. Anions can be added to the ration in several forms including $CaCl_2$, $CaSO_4$, $MgCl_2$, $MgSO_4$, NH_4Cl and $(NH_4)SO_4$, strong cation salts, or HCl and H_2SO_4, strong anion acids. More recently, commercial anion sources combine some anions with carriers that improve the palatability of the ingredients. Collectively, these are referred to as "anionic supplements" because the anions are absorbed to a greater extent than the strong cations, reducing the *SID* of the plasma (DeGaris and Lean, 2008). In application for ration balancing, the calculation of DCAD is simplified to include the cations Na^+ and K^+ and the anions Cl^- and SO_4^{2-} [DCAD in mEq/100 g of diet DM $= (K^+ + Na^+) - (Cl^- + SO_4^{2-})$]. Other equations including more strong ions, or correction factors to account for varying absorption efficiencies, have been considered in relation to their ability to predict milk fever incidence (Lean et al., 2006) as well as the urine pH response to these diets (Charbonneau et al., 2006). The inclusion of other ions in the calculation of DCAD, such as Ca^{2+} and Mg^{2+}, confound the ability of these formulas to associate with health outcomes due to the complex relationship

between the dietary supply of these macrominerals and pathophysiology of hypocalcemia (Charbonneau et al., 2006) which will be discussed in a later section.

From a practical standpoint, urine pH can be used to assess the degree of metabolic acidosis induced through inclusion of anionic salts in the prepartum ration (Jardon, 1995). The blood pH of mammals must be maintained within a narrow range to allow proper physiological function and as a result multiple mechanisms exist to correct acid-base disturbances. Indeed the change in blood pH induced by feeding negative DCAD rations is of a small magnitude (Goff et al., 2014). Primary physiological mechanism of the cow will attempt to correct this change in blood pH. An increase in respiration rate will occur which attempts to first correct the plasma pH by decreasing the plasma PCO_2. Decreased plasma CO_2 can be observed in goats fed negative DCAD rations (Fredeen et al., 1988). The excretion of excess anions and protons (H^+) in the urine is quantitatively more important in conditions of chronic metabolic acidosis induced in prepartum dairy cow rations (Constable et al., 2009). The majority of H^+ excretion in urine is conducted through the ammonia and ammonium buffering system. Because H^+ concentration of the urine is a relatively low proportion of the total acid excretion, a more accurate reflection of the degree of metabolic acidosis imposed is the sum of total acid and ammonium minus bicarbonate, referred to as net acid excretion (**NAE**). Constable et al. (2009) investigated the relationship between urine pH and net base excretion (**NBE**; the negative of NAE) and observed a strong relationship for urine pH values above 6.2. Accounting for ammonium excretion significantly improved the relationship between the two parameters at lower urine pH values (Constable et al., 2009), emphasizing the importance of the ammonium buffering system in chronic metabolic acidosis imposed with negative DCAD feeding.

Despite these nuances, the urine pH measurement allows for a practical and simple cow side test to determine the degree of metabolic acidosis that has been achieved. Meta-analyses have shown that the relationship between calculated dietary DCAD and urine pH, as well as incidence of clinical milk fever, is strong (Charbonneau et al., 2006; Lean et al., 2006). Charbonneau et al. (2006) also suggested that decreasing the DCAD resulted in linear decreases in DMI. Because of this relationship between DMI and ration DCAD, recommended target urine pH values for Holstein cows were proposed to be approximately 6.2 to 6.8 with a lower target for Jersey cows due to the increase risk for hypocalcemia in that breed (Goff, 2008). Others previously proposed lower targets such as 6.0 to 6.5 (Byers, 1994) which is more reflective of aggressive DCAD approaches that are implemented today. Based on the development of commercial anion products with improved palatability, studies have now demonstrated that rations with DCAD resulting in urine pH around 6.0 can be fed without compromising DMI of those cows (Moore et al., 2000; DeGroot et al., 2010; Weich et al., 2013).

Work conducted in dairy cows provides evidence for alleviation of the insensitivity to PTH in low DCAD feeding situations. In a study by Goff et al. (1991), cows fed an anion supplemented diet had improved Ca status postpartum compared to cows fed a cationic diet. Cows on the anionic diet prior to calving had higher plasma hydroxyproline and higher concentrations of plasma 1,25-dihydroxyvitamin D produced per a unit of PTH around calving. This suggests that upon increases in Ca demand, cows fed anionic diets prepartum had greater responsiveness of both bone and kidney to PTH stimulation. Joyce et al. (1997) similarly detected a stronger relationship between the concentrations of serum 1,25-dihydroxyvitamin D and the decline in serum tCa in cows fed anionic diets. Goff et al. (2014) provided more direct evidence supporting this theory in a study in which late gestation cows were fed a diet with

either a high DCAD (+18.8 mEq/100 g DM) or a low DCAD (-18.1 mEq/100 g DM). The cows were subsequently injected with synthetic PTH. Responsiveness to PTH was enhanced in cows fed the low DCAD diets as evidenced by more rapid increases in blood Ca and increased production of 1,25-dihydroxyvitamin D. Rodriguez et al. (2016) further hypothesized that metabolic acidosis in prepartum rations would increase the expression of PTH receptors on the kidney based on their findings in steers fed to create a metabolic acidosis. This increased efficiency of PTH signaling results in a Ca homeostatic system that is primed to respond to the increased demand for Ca at calving. Studies over the past several decades have shown changes in Ca metabolism in transition dairy cows when prepartum rations with a low or negative DCAD were fed as well as improved Ca status (Ender et al., 1971; Dishington, 1975; Block, 1984).

The impacts of lowering DCAD can be further described to include increases in urine Ca flux. Renal reabsorption of Ca is directly affected by this change in urinary pH that occurs in low or negative DCAD feeding situations (Stacy and Wilson, 1970). Urine Ca excretion has repeatedly been shown to increase when dietary DCAD is decreased (Gaynor et al., 1989; Takagi and Block, 1991; Joyce et al., 1997; Grunberg et al., 2011). Even in cows not supplemented with anions, and urine pH within the normal range, a significant inverse relationship can be seen between urine pH within the 2 d before calving and serum Ca postpartum (Seifi et al., 2004). Since this degree of urine pH change is not consistent with altered blood pH, it suggests a relationship between excretion of Ca and elevated Ca turnover which would help maintain Ca status around parturition. In negative DCAD feeding situations, high Ca excretion in urine must be compensated by either increased intestinal absorption, bone mobilization, or both. Since prepartum blood Ca concentrations typically do not change when cows are fed a low DCAD, it

suggests that this elevated turnover fully compensates for the urinary excretion (Goff et al., 1991; Ramos-Nieves et al., 2009).

Effects of prepartum low or negative DCAD feeding on prepartum DMI have been variable in the literature. It is likely that the inclusion rate of supplemental anionic salts, primarily determined by the starting cation content and desired DCAD, as well as the source of dietary anions, will determine the effects on intake. As discussed previously, a meta-analysis of several early DCAD studies suggested a linear decrease in DMI with decreasing DCAD (Charbonneau et al., 2006). It is hypothesized that more recent adoption of commercial anion sources with improved palatability lessen the impact of negative DCAD diets on DMI. In contrast, DeGroot et al. (2010) found no effect of feeding a negative DCAD on DMI despite feeding commercial anion supplements or a mixture of salts. Interestingly, Goff and Horst (1997) found that in prepartum rations with equal chloride and sulfate concentrations, the addition of K increased DMI, suggesting that anion inclusion rate was not the driver of reduced DMI but instead perhaps the induced metabolic acidosis in the low K ration. When anions were supplemented to a low-K ration to reach a DCAD of -10 mEq/100 g DM in the study by Ramos-Nieves et al. (2009), average DMI was lower in the low DCAD group in the early treatment period but as the groups approached parturition intake was similar. This is consistent with results seen by Moore et al. (2000) in multiparous cows fed a negative DCAD (-15 mEq/100 g DM). Regardless, several studies have now been conducted in which the negative DCAD ration resulted in urine pH near 6 to 6.5 and minimal to no effects on prepartum DMI or adipose tissue mobilization were observed (Moore et al., 2000; Ramos-Nieves et al., 2009; DeGroot et al., 2010; Weich et al., 2013; Weiss et al., 2015)

Postpartum DMI and milk production responses to negative DCAD feeding in the prepartum period have also been variable in the literature. The reason for variation in these responses is likely due to heterogeneity in the effects on prepartum DMI and blood Ca status. Milk production responses to low DCAD diets were first shown by Block (1984) in a two lactation switchover study where cows fed a negative DCAD (-12.9 vs. +33.1 mEq/100 g DM) had no clinical milk fever and 47.4% of cows fed the high DCAD diet had clinical milk fever. In the subsequent lactation, 305 day milk production was 6.8% higher for cows fed the negative DCAD. More recently, DeGroot et al. (2010) showed that DMI and milk production were higher in the first 21 d postpartum for multiparous cows fed a negative DCAD (approximately -11 mEq/100 g DM) from three different anion sources as compared to a control diet (+22 mEq/100 g DM) prepartum. When negative DCAD diets (-16 mEq/100 g DM) were fed for either 21 or 42 days prior to calving, compared to a positive DCAD control ration, milk production was greater for the first 8 wk of lactation regardless of the duration of negative DCAD feeding (Weich et al., 2013). In another study, low DCAD fed cows with higher blood iCa and tCa around calving had higher DMI but no difference in milk production, although yield was numerically higher for the 4 wk early lactation observation period (Joyce et al., 1997). Ramos-Nieves et al. (2009) found no difference in DMI postpartum or milk production between cows fed a low-K control ration (+10 mEq/100 g DM) and an anion supplemented low-K ration (-15 mEq/100 g DM) prepartum. In this study, blood Ca tended to be increased only in the 24 h after parturition which may have been insufficient to impact health and performance postpartum. Moore et al. (2000) detected differences in blood iCa concentration and milk production was numerically increased but not statistically significant, the statistical power of that study may have been insufficient to detect those differences. Dry matter intake was not reported in that study but

liver triglyceride accumulation was lower in cows fed lower DCAD rations (0 mEq or -15 mEq/100 g DM) suggesting improved energetic status. Despite overall heterogeneity in responses, Lean et al. (2014) observed an increase in milk production of 1.15 kg/d for the first 65 DIM in a meta-analysis of 15 studies with 34 DCAD comparisons.

As discussed previously, older cows are recognized to be at higher risk for hypocalcemia and have been the target of most research investigating preventative strategies. However, some degree of SCH occurs in primiparous cows (Reinhardt et al., 2011; Caixeta et al., 2015) and many dairy farms will house heifers entering their first lactation with mature dry cows prior to parturition; thus understanding the impact that low DCAD diets have on primiparous cows is imperative to successful management. To the author's knowledge, two studies have included primiparous cows in negative DCAD feeding investigations. Moore et al. (2000) found that primiparous cows are negatively impacted by diets with a low or negative dietary DCAD and have reduced DMI prepartum and compromised peripartum metabolic health while their multiparous counterparts did not have decreased DMI. In contrast, DeGroot et al. (2010) showed no effect of negative DCAD diets on DMI in the prepartum period and primiparous cows in that studied had increased postpartum DMI as well as decreased postpartum blood β-HBA and NEFA concentrations (DeGroot et al., 2010). The contrasting results in the literature urge the continued investigation of the impact that negative DCAD rations have on primiparous cows.

Low Calcium Supply in Prepartum Rations

Feeding a low Ca ration in the prepartum period is an effective strategy for priming the cow's homeostatic mechanisms and has been demonstrated to decrease risk of milk fever and improve Ca status overall (Boda and Cole, 1954; Goings et al., 1974; Kichura et al., 1982; Shappell et al., 1987). Decreased dietary Ca availability causes a minor drop in blood Ca shortly

after commencing the Ca deficient diet, inducing a PTH response (Shappell et al., 1987) which ultimately increases indicators of bone resorption (urinary hydroxyproline) and absorption efficiency from the ration through stimulation of 1,25-dihydroxyvitamin D production in the kidney (Green et al., 1981; Goff, 2006). Studies using radiolabeled ^{45}Ca have illustrated that as ration Ca concentration decreases, absorption efficiency increases (van't Klooster, 1976).

To accomplish increased Ca turnover with this approach, the Ca supplied in the ration must be below the cow's requirements. Requirements for macrominerals are determined according to a factorial approach (NRC, 2001). In the prepartum period the requirement is determined based on maintenance, estimated by endogenous fecal losses determined in radioisotope studies and calculated to be 0.0154 g/kg body weight for a nonlactating cow (Visek et al., 1953; Hansard et al., 1957), as well as the Ca required for the fetus as determined in harvest studies by House and Bell (1993). For a 680 kg Holstein cow at 270 d of gestation, the requirement for absorbable Ca is 21.5 g/d (0.45% of DM) in an example ration (NRC, 2001). In studies mentioned previously, improvements in postpartum Ca status were seen when dietary Ca intake was less than 10 g/d in the prepartum period (Boda and Cole, 1954; Goings et al., 1974). Achieving this level of dietary Ca can be a practical challenge based on the current forages and feed sources typically available for balancing prepartum rations.

In order to actualize a low Ca approach, efforts have been made to feed products capable of binding Ca such as rumen protected rice bran (high content of phytic acid) and zeolite A (a sodium aluminum silicate) to create a functionally low Ca diet. Feeding of zeolite A has been shown to increase circulating prepartum 1,25-dihydroxyvitamin D and subsequently improve postpartum Ca status (Thilsing-Hansen et al., 2002; Thilsing et al., 2007; Grabherr et al., 2009). Thilsing-Hansen et al. (2002) also observed lower blood P concentrations in cows fed zeolite A

and this is hypothesized to be an important mechanism for zeolite A in improving Ca status. In the study by Grabherr et al. (2009), a moderate dose of zeolite A was found to beneficially effect Ca metabolism in older cows, without the negative impacts on DMI and energy balance that were seen at higher doses. Feeding rumen protected rice bran as a source of phytic acid for Ca binding has been shown, indirectly, to alter Ca metabolism through increased apparent Ca absorption efficiency, as evidenced by excessive Ca excretion after being switched to a ration containing no rice bran (Martin-Tereso et al., 2011). In this study the ration containing rice bran with no supplemental Ca resulted in lower Ca intake in those cows (36.1 vs. 44.7 g/d) and results cannot be attributed to Ca binding alone. In a later study, improved postpartum Ca status was shown in rice bran fed cows when Ca consumption was equal to the control group (Martin-Tereso et al., 2014). Studies with rice bran show no negative effects on DMI during the treatment period (Martin-Tereso et al., 2011), which is advantageous in comparison to the risks associated with zeolite A feeding, and improved postpartum DMI after feeding rice bran suggest the potential for improved health and performance of transition cows (Martin-Tereso et al., 2014). Most of the work conducted with rice bran and zeolite A have been conducted with shorter feeding durations that would be implemented in the U.S. system and in lower producing cows. Additionally, minimal data on health and production outcomes in the early postpartum period after feeding these products is available. More studies are needed to determine mechanisms of action of Ca binding sources, the potential consequences for macromineral and trace mineral status, and the efficacy in the U.S. feeding system and in higher producing cows based on longer prepartum feeding durations and with the feeds available.

Emerging Strategies for Nutritional Prevention of Hypocalcemia

Preliminary characterization of the typical patterns of serotonin concentrations in dairy cows 1 d after calving show a positive correlation with blood Ca concentration (Laporta et al., 2013a) and the temporal trends of serotonin in the periparturient period are similar to blood Ca (Moore et al., 2015). These observations suggest that improving serotonin status in the transition period could have potential for improving Ca status. Feeding 5-HTP to mice has been demonstrated to increase bone osteoclast numbers and increase markers of bone resorption in the transition to lactation as well as increasing the concentrations of Ca in milk and serum (Laporta et al., 2013b). Infusion of 5-HTP to late lactation dairy cows has been demonstrated to increase PTHrP concentrations, decrease urinary excretion of Ca, increase milk Ca and decrease blood Ca concentration. In this study, blood was sampled intensively during the infusion period only and the authors hypothesized that there may be a biphasic response to 5-HTP administration where increases in blood Ca occur only after the initial decline (Laporta et al., 2015). It is also plausible that the response of dairy cows to 5-HTP administration would differ during a time of Ca challenge. Indeed, when 5-HTP was infused in dairy cows in the week leading up to calving, the expression of key Ca transport proteins in mammary epithelial cells, CaSR and PMCA2, were increased and blood Ca concentrations were increased in the 2 wk after parturition (Weaver et al., 2015). Infusion of 5-HTP in this study was also associated with reductions in milk production and DMI. Serotonin has been identified as a feedback inhibitor of lactation in dairy cows (Hernandez et al., 2008) and this may be the cause of reduced performance in the study by Weaver et al. (2015). Further studies have confirmed the positive effects of 5-HTP infusion on blood Ca concentration in transition cows (Hernandez et al., 2017). Serotonin and the administration of 5-HTP clearly alter Ca metabolism in dairy cows but a more thorough

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understanding of the impact on performance, as well as modes of administration, are needed before this is a plausible approach to managing hypocalcemia.

INTERACTIONS IN MACROMINERAL AND VITAMIN NUTRITION

Dietary Calcium Supply in Conjunction with Negative DCAD

Despite the potential efficacy of Ca binding feed additives in prepartum rations on postpartum Ca status, the predominant nutritional prevention strategy for hypocalcemia is the inclusion of anion sources. According to the 2014 Dairy NAHMS Study, 28% of farms feed anion supplements to cows in the close up period (USDA, 2014). Limited experiments have been conducted assessing the appropriate feeding rate of Ca in conjunction with low DCAD rations. In a meta-analysis conducted by Lean et al. (2006), the relationship between dietary Ca concentration and clinical hypocalcemia risk was quadratic, with decreased risk at very low and very high concentrations. The overall prevalence of clinical hypocalcemia across all of the studies included in the meta-analysis was very low and the ability to make definitive recommendations of this dataset is questionable. Further, with the current focus shifted to SCH as the main outcome of interest for preventative strategies, the dietary risk factors may be different than in the case of clinical hypocalcemia. In contrast to the results of that meta-analysis, Goff (2006) suggested that when Ca is fed at or above recommended feeding levels, there is little influence on the severity of hypocalcemia that occurs at parturition. This was supported by the work of Kronqvist et al. (2011) in which cows fed prepartum rations containing Ca at 0.49, 0.93 or 1.36% of DM with a positive DCAD showed no differences in Ca status or markers of Ca homeostasis regulation. Other studies feeding Ca above the requirement show no effect of varying Ca levels on hypocalcemia postpartum (Goff and Horst, 1997).

Due to increases in Ca flux when feeding low DCAD diets, it is hypothesized that greater improvements in postpartum Ca status will occur when higher Ca feeding rates are implemented in conjunction with low DCAD rations. Early work in non-pregnant dry and lactating cows assessed the correlation between ration DCAD and Ca digestibility and found a significant negative correlation between the two parameters when controlling for diet Ca concentration (Lomba et al., 1978), suggesting that either the systemic effects of decreasing ration DCAD alter the regulation of absorption efficiency or there is some direct relationship between higher dietary anion concentration and absorption of Ca. Similarly, Ender et al. (1971) showed that Ca balance, total Ca consumption minus Ca lost to urine, feces and milk, was improved in cows fed low DCAD rations prepartum. A slight advantage in postpartum Ca balance was found when the low DCAD was fed in conjunction with high dietary Ca (approximately 150 vs. 40 g/d). However, dietary Ca treatments were continued into the postpartum period, confounding the interaction between prepartum dietary Ca and level of DCAD.

The practice of feeding higher dietary Ca in conjunction with low DCAD is supported by the work of Oetzel et al. (1988). Those authors showed no overall effect of prepartum dietary Ca (53 g vs. 105 g/d) but prevalence of parturient hypocalcemia (plasma iCa < 1.0 mmol/L) was reduced for cows fed high Ca in conjunction with a negative DCAD (+18 mEq/100g DM vs. -7.5 mEq/100 g DM). Only 12 cows were used per treatment in this study and results should be interpreted with caution. Beede et al. (2001) found improved Ca status postpartum when feeding a negative DCAD compared to a positive DCAD as expected (-4 vs. +18 mEq/100 g DM) but there was no influence of varying dietary Ca levels (0.47, 0.98, 1.52, or 1.95% of DM). In that study, there were negative impacts of higher Ca concentrations (1.52 and 1.95% of DM) on DMI and these authors concluded that feeding 0.98% dietary Ca in conjunction with a low DCAD

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resulted in optimal transition performance and health. Interestingly, this dietary Ca level would fall within the high risk category identified by Lean et al. (2006).

More recent work assessing the impact of low DCAD rations on Ca homeostasis with varying dietary Ca concentrations suggest no effect when dietary Ca is varied from 0.99% to 1.50% of DM (Chan et al., 2006). The statistical power of this experiment is questionable considering that more than half of the 21 cow study population was entering their first lactation. Primiparous cows have reduced risk of hypocalcemia which could diminish the potential response to low DCAD rations (Moore et al., 2000). This may have inhibited Chan et al. (2006) from detecting important effects that could be observed in multiparous cows. Liesegang et al. (2007) also attempted to investigate this relationship but were unable to show an interaction between dietary Ca and level of DCAD for markers of bone metabolism in periparturient cows. The degree of metabolic acidosis induced in this study is questionable since urine pH was well above the recommended range in the low DCAD group. Moore et al. (2000) investigating decreasing ration DCAD (+15, 0 and -15 mEq/100g of DM) while simultaneously increasing ration Ca concentration (0.44, 0.97 and 1.50% of DM) and observed increased blood Ca concentration. The interaction between Ca and DCAD cannot be assessed from that study since there was no repetition of Ca feeding rate within different DCAD levels. The literature lacks consensus on the appropriate dietary Ca level to be fed in conjunction with low DCAD feeding prepartum and more direct comparisons with aggressive DCAD feeding strategies are needed.

Minimal work has been done to investigate the appropriate feeding rate for Ca postpartum. The requirement for absorbed Ca during lactation, as determined by the NRC (2001), is calculated based on the requirement for maintenance, determined to be 0.031 g/kg of live body weight for lactating cows (Martz et al., 1990), as well as the requirement for Ca

secreted into the milk which is determined to be 1.22 g/kg of milk for the Holstein and 1.45 g/kg for the Jersey. The example ration for a fresh cow (680 kg of body weight, producing 25 kg of milk) provides 52.1 g of absorbable Ca which equates to 0.74% of DM based on the model predicted DMI (NRC, 2001). Taylor et al. (2009) fed three levels of dietary Ca postpartum (0.45, 0.75, or 1.1% of DM) starting immediately postpartum and continuing through 140 DIM. Those authors observed no effect on blood Ca concentration, milk production or phosphorus balance. Apparent Ca balance was higher for cows fed the highest concentration of dietary Ca, but the lack of effect on blood Ca draws question to the value of increased Ca balance. In this experiment, only 6 cows per treatment were tested and approximately half of each group was in their first lactation.

The appropriate inclusion rate for Ca in the postpartum ration is complicated by the ability to increase the absorption efficiency of Ca from the diet when dietary Ca is low (van't Klooster, 1976). Additionally, Ca absorption coefficients differ based on the dietary source and studies often formulate and report based on total dietary supply as opposed to absorbable Ca. True bioavailability of Ca from a given source is challenging to determine because that source must be fed when the cow is being provided less Ca than the requirement. The NRC (2001) has based absorption coefficients for Ca on early work that assessed multiple feed ingredients in these conditions. Inorganic sources of Ca are considered to have the greatest absorption efficiency and are only considered to be limited by the solubility in the gastrointestinal tract. For this reason the NRC (2001) has made estimates of availability ranging from 70 to 95% for some of the common supplemental Ca sources. Absorption for Ca chloride is assigned the highest coefficient with Ca carbonate considered less available at 75% bioavailable and limestone slightly lower at 70%. These conclusions are based on variable results from experiments in dairy

cows and steers in multiple physiological stages (Hansard and Plumlee, 1954; Hansard et al., 1957; Goetsch and Owens, 1985). The complex changes associated with the transition period and alterations to Ca metabolism induced by negative DCAD diets likely change the applicability of these values.

Role of Magnesium in Calcium Homeostasis

Magnesium acts as an important cofactor in numerous enzymatic reactions in the body and therefore, maintenance of adequate intracellular and extracellular Mg is required for normal function of these physiological processes (Goff, 2006). Magnesium plays an important role in the signaling of the PTH mediated Ca homeostasis system. The role that Mg deficiency plays in this system has been investigated in mice fed Ca deficient diets (Forbes and Parker, 1980). Administration of PTH extracts to these mice did not increase urinary cAMP excretion (a marker of PTH signaling in renal cells) unless Mg status was restored. Mg deficient mice in this study responded similarly to hypocalcemia by resorbing bone Ca suggesting that the bone cell response PTH is less affected than the kidney by hypomagnesemia. In contrast, experiments in cows fed Mg deficient diets prepartum suggest a lack of bone Ca mobilization compared to cows fed Mg sufficient rations prepartum (van Mosel et al., 1991). Higher parity cows in this study that were fed low Mg rations had increased incidence of parturient paresis.

The importance of restoring adequate Mg status for proper Ca homeostasis has been demonstrated with studies in hypomagnesemic, hypocalcemic human patients. The administration of intravenous Mg in those patients with low serum Mg, Ca and PTH concentrations caused a rapid increase in serum PTH that was not seen in normal patients injected with Mg (Rude et al., 1978). Another study in humans illustrated that hypocalcemia can occur secondary to hypomagnesemia, and Mg administration to those patients is necessary for

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recovery of blood Ca. In some patients, increases in 1,25-dihydroxyvitamin D occurred after Ca

administration (Rude et al., 1985). Impairment of vitamin D activation likely plays a role in

diminishing Ca homeostatic responses in hypocalcemia that is precipitated by hypomagnesemia.

Magnesium Metabolism and Periparturient Dietary Supply

In ruminants, the primary site of dietary Mg absorption has been shown to be the rumen

with negligible absorption occurring beyond the rumen (Care et al., 1984). In fact, postruminal

infusion of Mg to hypomagnesemic sheep is unable to restore Mg status, indicating that ruminal

absorption is essential for homeostasis (Tomas and Potter, 1976). Experiments in sheep in with

either excessive or deficient Mg status, imposed through intravenous infusion of Mg or deficient

dietary Mg supply, have demonstrated the lack of hormonal regulation of Mg absorption

(Martens and Stossel, 1988). This indicates that in conditions of low dietary Mg, increased

absorption efficiency cannot be employed to maintain Mg status. Absorption of dietary Mg can

occur through two transcellular mechanisms, both a secondary active, potential difference driven

channel and a passive chemical gradient driven mechanism (Care et al., 1984; Leonhard-Marek

and Martens, 1996; Schweigel et al., 2000). The first is dependent on a potential difference on

the luminal side of rumen epithelial cells created by the basolateral Na^+/K^+ ATP-ase as well as

low intracellular Mg concentrations maintained by the Na^+/Mg^{2+} exchanger on the basolateral

membrane. The electromotive force is generated by both the chemical gradient for Mg and the

electrical gradient which creates a negative charge in the cytosol of the epithelial cell and the

magnitude of which is dependent on ruminal K concentrations (Leonhard-Marek and Martens,

1996). Another transcellular mechanism exists that occurs independently of the membrane

potential difference and which has not been fully characterized but likely involves the exchange

of Mg and hydrogen ions or cotransport with anions such as short chain fatty acids, bicarbonate

and chloride (Schweigel et al., 2000). The activity of this transport process can be increased by intraruminal Mg and anion concentration. Through this process, magnesium absorption can increase with increased dietary magnesium concentrations (Chicco et al., 1972; Van Ravenswaay et al., 1989; Jittakhot et al., 2004a).

Dietary Mg that is absorbed in excess of requirements will be excreted by the kidney (Chicco et al., 1972; Jittakhot et al., 2004b). The amount of Mg that is reabsorbed by the kidney depends on the Mg concentration of the filtrate. The transepithelial transport of filtered Mg in the kidney has been shown to be facilitated primarily by the transient receptor potential melastatin 6 (TRPM6) in mice (Groenestege et al., 2006). When blood Mg is above 0.74 mmol/L in dairy cows, the kidney is unable to absorb the excess Mg and it is therefore excreted. If blood Mg is below this threshold, the kidney will absorb Mg in the filtrate very efficiently (Goff, 2006). This renal threshold for Mg can be increased by PTH which is secreted in response to hypocalcemia, and consequently plasma Mg is elevated in response to PTH stimulation (Goff et al., 1986; Goff et al., 2014). Increases in blood Mg concurrent with the drop in blood Ca at parturition have been demonstrated in several studies (Blum et al., 1973; Shappell et al., 1987; Riond et al., 1995; Kronqvist et al., 2011). This interaction confounds interpretation of blood Mg concentrations within the day or two around parturition.

The Mg requirement of dairy cows proposed by the NRC (2001) includes the following; maintenance requirement of 3 mg/kg of body weight [endogenous fecal loss only since obligate urinary loss is considered to be minimal (Martz et al., 1990)], a growth requirement of 0.45 g/kg of body weight gain, a gestational requirement of 0.33 g/d in late gestation, and a production requirement of 0.12-0.15 g/kg milk. An example fresh cow ration by NRC (2001) was formulated to 0.27% of DM as Mg (680 kg of body weight producing 25 kg of milk) with a note

that this value is contingent on proper active Mg absorption. For the prepartum cow at 270 d of gestation, to meet her tissue demands, the diet is formulated at 0.12% of DM as Mg. This would be considered exceptionally low by current standards due to variation in dietary K and variation in the availability of Mg in inorganic supplemental sources.

The availability of dietary Mg is largely dependent on the concentration of soluble Mg in the rumen. Important factors in determining the amount of soluble Mg include the dietary Mg concentration (Chicco et al., 1972; Jittakhot et al., 2004b), the particle size of inorganic Mg sources (Jesse et al., 1981), potential solubility as a result of calcination temperature during manufacturing (Van Ravenswaay et al., 1989), the rumen pH (Gaebel et al., 1987), the chemical structure of the inorganic Mg source (Rahnema and Fontenot, 1983) and interactions with other nutrients (Behar, 1975). Dietary K is recognized to be an important factor in determining absorption of dietary Mg because of interference with the active Mg absorption mechanisms in the rumen epithelial cells. Sodium is necessary for the cotransport into epithelial cells and therefore, adequate sodium is necessary to facilitate proper uptake. High dietary K can inhibit the active absorption of Mg via depolarization of the epithelial membrane decreasing the electromotive force that drives the sodium coupled secondary active transport (Care et al., 1984). Practically, dietary levels of K that would be commonly fed in dairy rations could inhibit the active transport of Mg. This is illustrated by Greene et al. (1983a) who tested four levels of dietary K (ranging from 0.6 to 4.8% of DM) and their interaction with dietary Mg level (0.1 or 0.2% of DM) in lambs. A linear decrease in apparent Mg absorption with increasing dietary K concentration was observed and serum Mg concentrations were similarly decreased. In this study, feeding higher Mg increased total apparent Mg absorption and increased serum Mg concentrations, suggesting that the influence of high dietary K can be attenuated with increased

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ruminal Mg supply. Similar results were found in wethers fed higher Mg concentrations ranging from 0.13 to 0.37% of DM (Ram et al., 1998). Similarly in dairy cows, Jittakhot et al. (2004b) fed either approximately 0.55 or 0.90% of DM as Mg in combination with 2.0, 4.8 or 7.5% of DM as K. Magnesium absorption was decreased by increasing K concentrations, and this affect was attenuated by increasing dietary Mg supply. Weiss (2004) compiled data from several studies and suggested that for each additional unit of dietary K above 1% of DM, 18 g of additional Mg must be supplied to maintain the same absorbed Mg.

It is recommended to feed Mg in prepartum rations at a concentration of 0.35-0.40% of DM to promote optimal Ca metabolism at parturition, regardless of level of DCAD (Goff, 2006). This recommendation is based on the work of Ram et al. (1998), showing that dietary Mg concentrations of 0.35% of DM were sufficient to overcome high K concentrations within the range of current rations. This practice is supported by a meta-analysis which considered macromineral feeding levels, varying forms of DCAD calculation and days relative to parturition that the close up ration was fed as predictors of clinical hypocalcemia. One of the stronger associations that was found was a protective effect of increasing dietary Mg concentration. When dietary Mg was increased from 0.3 to 0.4% of DM, risk of clinical milk fever was reduced by 62% (Lean et al., 2006). Feeding higher rates of dietary Mg prepartum has become common practice.

In the early postpartum period, many cows have blood Mg profiles indicative of a lactational drain of Mg (Ramos-Nieves et al., 2009). Survey studies have demonstrated an average of 0.7 g/kg of Mg in colostrum (Kehoe et al., 2007). For production of 10 kg of colostrum, approximately 7 g of Mg is required, representing a several fold increase in Mg outflow. Interestingly, the drop in blood Mg appears to be delayed compared to that of Ca and

occurs between 2 and 7 DIM (Green et al., 1981; Shappell et al., 1987; Kronqvist et al., 2011). In studies with repeated measurements of blood Ca, the time to recovery of blood Ca has been demonstrated to be up to 10 d or more (Ramos-Nieves et al., 2009). In a recent epidemiological survey study, hypocalcemia at 1, 2 and 3 wk postpartum was associated with compromised health postpartum health and performance with increasing severity based on the time from parturition at identification of hypocalcemia (Chapinal et al., 2011; Chapinal et al., 2012). Since blood Ca curves from the time of parturition were not characterized in that study, it is unclear if the hypocalcemia observed was primarily associated with periparturient changes or the consequence of poor health. Regardless, these data warrant the investigation of strategies for aiding recovery of early postpartum blood Ca concentrations. Providing higher dietary Mg concentrations in postpartum rations may improve the homeostatic response, although this has not been directly investigated. In addition to the potential for compromised postpartum Mg concentrations to inhibit Ca homeostasis, direct consequences of low Mg on health and performance may exists due to the expansive role of Mg in different physiological processes. The consequences of early postpartum hypomagnesemia have been minimally studied and investigation at the epidemiological level is important for further understanding.

Phosphorus Metabolism and the Role in Calcium Homeostasis

Dietary P absorption occurs mainly in the small intestine of ruminants, with minimal absorption occurring in more proximal portions of the gastrointestinal tract. Phosphorus is typically absorbed proportionally to the amount that is available in the small intestine unless dietary supply is deficient (Knowlton and Herbein, 2002). Mechanisms for absorption of dietary P include both a passive process, which predominates with adequate or excessive dietary P supply (Horst, 1986), and an active process which is mediated by 1,25-dihydroxyvitamin D in

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response to low blood P concentrations (Horst et al., 1978a). This active mechanism is the same as that for active Ca absorption but plasma P is maintained secondary to Ca. This is demonstrated in the study by Kichura et al. (1982) in which dietary P supply was restricted and transient reductions in blood Ca were induced and subsequently corrected while blood P concentrations remained compromised. Moreira et al. (2009) demonstrated that decreasing dietary P reduced fecal Ca excretion, suggesting greater absorption efficiency of Ca with lower ration P. Aspects of Ca metabolism can also trigger release of bone P and urinary excretion of P. Upon stimulation of bone Ca resorption, P is also released through the breakdown of hydroxyapatite. It has been demonstrated in mice *in vivo* and *in vitro* that 1,25-dihydroxyvitamin D stimulation of bone chondrocytes results in production of the hormone FGF23 by osteoblasts (Masuyama et al., 2006), a hormone that mediates P metabolism. Renal P excretion is potently stimulated by FGF23 (Shimada et al., 2004). The main route of excretion of P is through saliva and P is an important component of ruminal buffering, however, it appears that urinary excretion is an important mechanism for correcting excess P. Infusion of PTH in pregnant cows has been demonstrated to stimulate high levels of urinary P loss (Goff et al., 1986), and this is likely at least partially mediated through FGF23 production by osteoblasts. The relationships between P and Ca metabolism have not been fully elucidated and data is especially lacking in periparturient cows. A large amount of variation in blood P concentrations occurs in the early postpartum period and the consequences of the apparent hypophosphatemia are unclear (Ramos-Nieves et al., 2009).

Phosphorus Requirements and Prepartum Dietary Supply

The requirements for dietary P as calculated by the NRC (2001) are determined by the factorial approach. The maintenance requirement is determined as a proportion of DMI because

of the role of P in saliva as a buffer and assumes that the salivary excretion of P will be mostly determined by intake. Using this approach, the maintenance requirement for nonlactating and lactating cows is set at 1 g/kg of DMI based on the work of Spiekers et al. (1992) who quantified fecal P excretion at different levels of intake when consuming a diet sufficient, but not excessive, in P. Phosphorus requirements for development of the conceptus range from 1.9 to 5.4 g/d from 190 to 280 days of gestation, and requirements prior to that period are minimal (House and Bell, 1993). The lactational demand is equal to the P content of milk and is approximated at 0.9 g/kg of milk (NRC, 2001). The NRC (2001) example ration for a fresh cow (680 kg of body weight, producing 25 kg of milk) provides 37.3 g of absorbable phosphorus which equates to 0.38% of DM based on the model predicted DMI. For the prepartum cow, at 270 days of gestation, the dietary phosphorus concentration is 0.23% of DM, providing 20.3 g of absorbable phosphorus to meet the requirement.

Because of the interrelationships between Ca and P metabolism, concentrations of P in prepartum rations and the relationship with Ca homeostasis have been investigated in the past in the context of the ratio between Ca and P as well as the total dietary supply of P independent of Ca concentration. Kichura et al. (1982) investigated the interactions of low and high Ca diets (9.5 g/d vs. 86 g/d) with low and high P diets (10 g/d vs. 82 g/d). While the low Ca diets overall showed the greatest increases in Ca status, with evidence of increased upregulation of intestinal Ca absorption and bone resorption, the negative effect of the high Ca diet was somewhat attenuated by feeding low P. Cows fed the low P diet had lower blood P concentrations during the entire feeding period, as well as a tendency for higher concentrations of 1,25-dihydroxyvitamin D, suggesting that the low P status may have triggered an increase in intestinal absorption efficiency which would positively influence Ca status. Similarly, Barton et al. (1987)

investigated the effects of feeding 0.7, 1 or 3 times the P requirement according to the 1978 Nutrient Requirements for Dairy Cattle and found that low P feeding increased blood Ca postpartum. Peterson et al. (2005) compared three levels of dietary P prepartum and found that feeding approximately 0.21% of DM as P (35 g of P intake) for 4 wk prior to calving decreased blood P in the feeding period compared to groups fed 0.31 or 0.44% of DM. The protective effect of low P feeding was not observed in this study, potentially because ration P concentration was not low enough. In contrast, cows fed P at 0.44% of DM had decreased blood Ca for 2 d after calving. This study indicated that 0.44% (70 g of intake in this study) may represent an upper limit for prepartum P in regards to Ca homeostasis (Peterson et al., 2005).

Dietary Vitamin D Supply and Calcium Homeostasis

Vitamin D clearly plays an important role in mediating aspects of Ca homeostasis and strategies for utilization of different forms of Vitamin D, both nutritionally and parenterally, have been investigated over the years. Parenteral approaches have largely been abandoned due to the challenges associated with dosage and timing relative to parturition as well as the possibly detrimental effects of rebounding Ca status after clearance of these treatments (Goff et al., 1988; Goff and Horst, 1990). Because of these reasons, the focus will be the current recommendations and emerging strategies for Vitamin D supplementation in the peripartum period. Due to current management strategies, many dairy cows are not sufficiently exposed to sunlight to rely on irradiation of 7-dehydrocholesterol to vitamin D_3 in the cow, therefore vitamin D is provided in the ration, and endogenous production of vitamin D_3 is not considered as a contributor to the requirement in the most recent NRC (2001). Both vitamin D_3 and vitamin D_2 are recommended as feed additives and the NRC (2001) does not alter recommendations (30 IU/kg of BW) based on the form of vitamin D used. For a 680 kg Holstein cow, the example prepartum and

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postpartum rations provided about 21,000 IU/d (35 kg of milk production). Nelson and

Merriman (2014) suggested that feeding between 30,000 and 50,000 IU/d of vitamin D_3 resulted

in blood concentrations of 25-hydroxyvitamin D within the suspected "normal" ranges. Although

more work evaluating approaches for assessing vitamin D status are needed.

More recent work conducted in prepartum dairy cows has investigated the potential use

of supplemental vitamin D in the form of 25-hydroxyvitamin D to enhance Ca metabolism at

parturition. This form of vitamin D represents the primary form in circulation of the cow and

activation to the hormonally active form only requires one additional hydroxylation at the kidney

(Nelson and Merriman, 2014). Wilkens et al. (2012) fed a positive or negative DCAD (+14.4 vs.

-16.8 mEq/100 g DM) with or without an oral dose of 25-hydroxyvitamin D from 270 d of

gestation until 10 DIM. Cows fed both a negative DCAD with the 25-hydroxyvitamin D

supplement responded with higher blood Ca postpartum compared to cows fed a negative DCAD

alone. The authors hypothesized that this benefit was due to the alleviated tissue insensitivity to

PTH stimulation by the induced metabolic acidosis, allowing those cows to benefit from greater

activation of the additional 25-hydroxyvitamin D, and this was supported by a lack of response

in cows supplemented with 25-hydroxyvitamin D with a positive DCAD. Weiss et al. (2015)

conducted a similar study but did not find the synergistic effects between negative DCAD and

25-hydroxyviatmin D supplementation and actually saw negative impacts on Ca status. In

another study presented in abstract, feeding 25-hydroxyvitamin D in comparison to vitamin D,

with or without a negative DCAD (+13 vs -13 mEq/100 g DM) resulted in reduced incidence of

retained placenta and metritis and significant increases in milk production in the first 42 DIM

regardless of dietary DCAD level (Martinez et al., 2015). The reason for variation in response to

supplemental 25-hydroxyvitamin D is unclear but strategic use of these products may aid in hypocalcemia management.

<div align="center">

TREATMENT OF HYPOCALCEMIA

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Supplementation of Calcium to Early Postpartum Cows

Nutritional strategies for prevention of hypocalcemia, such as feeding a negative prepartum DCAD, can prepare cows to respond to the increased Ca demand at parturition, resulting in increases in blood Ca concentration overall (Moore et al., 2000). However, despite implementation of these preventative practices, some cows still experience drops in blood Ca below previously identified risk thresholds (DeGroot et al., 2010; Neves et al., 2017). While it is arguable that some drop in blood Ca is likely an appropriate adaptation to lactation, providing non-dietary supplemental Ca postpartum may alleviate excessive drops in blood Ca. Expected responses to appropriate supplementation may include decreased risk of infectious disease, as a result of maintaining the integrity of Ca signaling in immune cells (Ducusin et al., 2003), and decreased risk of metabolic health disorders due to maintaining gut motility (Martinez et al., 2014). As a result of these two mechanisms, the potential for increased milk production and reproductive performance could arise. Options for supplemental Ca that have been explored in the past include intravenous and subcutaneous injections as well as oral drenches, pastes and boluses. In order for Ca supplementation to be effective, blood Ca must be increased but increases that interfere with the cow's adaptation to lactational demands should be avoided. Blanc et al. (2014) demonstrated that supplementation of hypocalcemic cows with intravenous Ca resulted in a dramatic spike in blood Ca concentration which was followed by a depression of blood Ca below that of unsupplemented cows. This suggests that the signal from excessive Ca supplied to the bloodstream downregulated Ca homeostasis and likely elicited a calcitonin

response to remove the excess Ca from the system. In the same study, providing supplemental Ca in the form of a two dose oral Ca bolus regimen provided a moderate but sustained response in blood Ca. Similar data is unavailable for subcutaneous Ca injection, although it is plausible that the response would be similar to that of intravenous Ca. Miltenburg et al. (2016) demonstrated that blood Ca concentration was increased following a subcutaneous Ca supplementation strategy, but did not characterize the detailed temporal changes over the day following parturition.

Because of the moderate effects on blood Ca, oral Ca boluses have received huge attention and favor within the industry in recent years. Martinez et al. (2016a) investigated different doses of oral Ca boluses and determined that providing either 43 or 86 g of Ca was capable of increasing blood iCa concentration. The response to 43 g was sustained for less than 2 h while the response to 86 g was sustained for at least 4 h. In the same study, a larger population of cows were administered either two 86 g doses in the 2 d following parturition or the same treatment plus three additional days of 43 g doses. Administration of these treatments reduced the prevalence of SCH in all cows during the treatment period. For primiparous cows, the termination of Ca supplementation was associated with an increase in SCH prevalence compared to controls. Multiparous cows returned to the same blood Ca concentration as controls. This data suggests that although more conservative doses of oral Ca boluses can provide sustained and moderate increase in blood Ca concentration, excessive supplementation may be detrimental.

Targeted Calcium Supplementation Strategies

In contrast to other metabolic disorders such as hyperketonemia (Ospina et al., 2013), tools that enable cowside determination of blood Ca concentration are not yet available.

Technology for this application is being developed (Neves et al., 2015), although at this time, blood Ca determination is not a practical strategy for identification of cows in need of supplementation. As discussed previously, age is an important risk factor for hypocalcemia and many farms implement a targeted supplementation approach for cows of higher parity. According to the 2014 Dairy NAHMS study, 69% of farms reported using supplemental Ca sources in a prophylactic approach as opposed to strictly for treatment of clinical disease (USDA, 2014). In an effort to improve the strategies for supplementation, Oetzel and Miller (2012) administered oral Ca boluses to multiparous cows calving at 2 farms and investigated periparturient risk factors that were important for determining responses. One important finding from this study was that there were no overall responses to bolus supplementation. This is in contrast to nutritional hypocalcemia prevention strategies for which group level increases in DMI and milk production in early lactation have been detected (DeGroot et al., 2010; Weich et al., 2013).

Oetzel and Miller (2012) did observe that cows in certain risk categories responded positively to bolus administration with decreased risk of health disorders in cows that were lame at parturition and increased milk production in cows with high production potential. Minimal to no group level responses have been confirmed in other studies using either supplemental Ca injections (Amanlou et al., 2016; Miltenburg et al., 2016) or oral Ca boluses (Martinez et al., 2016a; Martinez et al., 2016b). Recent research attempted to use oral Ca boluses as supportive therapy in the treatment of metritis and found no benefit to this approach (Pinedo et al., 2017). Concerning findings from Martinez et al. (2016b) included detrimental effects of bolus administration on some groups of cows. Primiparous cows had negatively affected reproductive performance when administered boluses in early lactation. Multiparous cows that were at a low

risk of metritis because of an easy, single calving and no retained placenta were subsequently at greater risk of metritis when boluses were administered. In the same study, multiparous cows with lower production potential had decreased milk production when boluses were administered. The quantity of boluses administered in that study were higher than recommended and may have precipitated the negative outcomes observed, however, it does raise the concern of supplementing cows that are not at increased risk of downstream consequences due to other periparturient risk factors. Further work should be done to investigate the most beneficial strategies for Ca supplementation postpartum.

RESEARCH OBJECTIVES

Hypocalcemia in the periparturient dairy cow has complex pathophysiology and significant consequences for dairy cow health, performance and longevity as well as herd profitability. With the shifted focus to subclinical disease, reevaluation of management strategies and development of new approaches are required to understand application in the context of SCH. The objectives of this dissertation were to 1) investigate strategies and tools for SCH monitoring, 2) optimize application of negative DCAD feeding for the prevention of hypocalcemia, 3) evaluate new approaches to macromineral nutrition in the transition period to support the recovery of blood Ca postpartum, and 3) identify opportunities for strategic use of supplemental Ca in the early postpartum period.

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CHAPTER 3

SHORT COMMMUNICATION: RELATIONSHIP BETWEEN METHODS FOR MEASUREMENT OF SERUM ELECTROLYTES, AND THE RELATIONSHIP BETWEEN IONIZED AND TOTAL CALCIUM AND NEUTROPHIL OXIDATIVE BURST ACTIVITY IN EARLY POSTPARTUM DAIRY COWS

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ABSTRACT

The objectives of this study were to 1) compare a test for serum measurement of total Ca (**tCa**), Mg and P (VetTest Chemistry Analyzer, IDEXX Laboratories, Inc., Westbrook, ME) to reference methods (spectrophotometric assays on a Beckman Coulter 640e automated clinical chemistry analyzer, Beckman Coulter, Brea, CA), 2) determine the relationship between ionized Ca (**iCa**) and reference method tCa in the immediate postpartum period, and 3) assess the relative value of these blood Ca indices as predictors of neutrophil oxidative burst activity. Samples were collected from multiparous Holstein cows (n = 33) over the first 5 d in milk (DIM). A total of 183 samples for objective 1 and 181 samples for objective 2 were available. Neutrophil oxidative burst activity was assessed once between 2 and 5 DIM (n = 29). Linear regression demonstrated strong relationships between serum tCa, Mg and P concentrations measured by the VetTest compared to the reference method. Bland Altman analysis indicated that the VetTest values were higher than the reference method by 0.22 mmol/L for tCa, 0.12 mmol/L for Mg, and 0.16 mmol/L for P. Compared to hypocalcemia categorized at ≤ 2.0 mmol/L or ≤ 2.125 mmol/L with the reference method tCa, thresholds for the VetTest measured tCa of ≤ 2.23 mmol/L (sensitivity = 87%, specificity = 89%) or ≤ 2.30 mmol/L (sensitivity = 86%, specificity = 96%) could be used. The relationship between whole blood iCa and reference method serum tCa differed by sampling timepoint after calving. Compared to identification of hypocalcemia with serum tCa measurements from the reference method (thresholds of ≤ 2.0 and 2.125 mmol/L), a whole blood iCa threshold of ≤ 1.17 mmol/L resulted in the highest combined sensitivities (94% and 82%) and specificities (80% and 94%) at either threshold. Ionized Ca measurements were more consistently related to outcomes of neutrophil oxidative burst activity measured *in vitro*. The VetTest measurements of serum tCa reliably identified hypocalcemia

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when thresholds were adjusted to account for the bias of the test. The variation in the relationship between iCa and reference method tCa in the days following parturition suggest that these measures cannot be used interchangeably as indicators of Ca status. The more consistent associations between iCa and *in vitro* measures of neutrophil function, compared to tCa, indicated that this may be a more sensitive predictor of functional outcomes associated with postpartum Ca status.

SHORT COMMUNICATION

Hypocalcemia is a disorder resulting from the rapid increase in demand for Ca that occurs as dairy cows transition from late gestation to lactation (Ramberg et al., 1970). Although blood Ca concentrations are regulated homeostatically, many cows experience a lag in adaptation to this increased demand, resulting in decreased blood Ca concentrations that can take several days to return to normal (Ramos-Nieves et al., 2009). Recent research has demonstrated that approximately 25% of primiparous cows and 50% or more of multiparous cows are affected by hypocalcemia (Reinhardt et al., 2011; Caixeta et al., 2015). The consequences of the disorder span from increased disease and early lactation culling to lost milk production and decreased reproductive performance (Chapinal et al., 2011; Chapinal et al., 2012; Martinez et al., 2012; Roberts et al., 2012).

As a result of the associations between hypocalcemia and productive and health outcomes of economic importance, interest in the ability to monitor the Ca status of cows at the herd and individual animal level has increased. In the majority of cases, cows do not display clinical signs of the disorder and hypocalcemia must be identified through the measurement of blood Ca (Reinhardt et al., 2011; Caixeta et al., 2015). Calcium circulates in blood in three forms; ionized Ca (iCa) and Ca bound to either proteins or anions (Rosol et al., 1995). Blood total Ca (tCa) is

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most often measured to assess the Ca status of a cow due to ease of sample handling, storage and analysis. Recent epidemiological studies have established blood Ca thresholds for identification of hypocalcemia based on the tCa concentration after parturition (Chapinal et al., 2011; Martinez et al., 2012). On-farm application of blood tCa measurement has been implemented minimally because of the lack of studies comparing tests that are easy to use and cost effective compared with methods typically employed in veterinary diagnostic laboratories.

It has been hypothesized that blood iCa concentration more accurately reflects the functional Ca status of the cow because this is the form of Ca readily available to cells to perform intracellular signaling necessary for such functions as contraction of muscles and activation of immune cells (Kimura et al., 2006). In addition, tCa is affected by blood protein levels (Rosol et al., 1995; Seifi et al., 2005). Ionized Ca is challenging to accurately measure because exposure of samples to air as well as the use of anticoagulants can change the proportion of Ca that is ionized in the sample (Boink et al., 1991). Although research has established thresholds for identification of hypocalcemia based on tCa, some work has extrapolated this to categorize Ca status using iCa measurements based on the assumption that iCa constitutes approximately 50% of tCa, which has been determined in clinically normal cows (Lincoln and Lane, 1990). Some evidence suggests that the relationship between tCa and iCa may be different around the time of parturition with a higher proportion of Ca in the ionized form (Ballantine and Herbein, 1991; Joyce et al., 1997). If iCa more accurately reflects functional Ca status of the cow, and the relationship to tCa is not consistent with previous assumptions in this period, this would suggest that thresholds using iCa for identification of hypocalcemia should be established independently to account for these dynamics immediately after calving.

The first objective of this study was to compare serum electrolytes measured by a commercially available test with potential on farm application to measurements using reference methods at a veterinary diagnostic laboratory. The second objective of the study was to assess the relationship between iCa and reference method tCa measured in the immediate postpartum period and third, assess the relative value of iCa and tCa for explaining variation in neutrophil oxidative burst activity. Neutrophils are a major cell activated in the innate immune response and are an important component of expelling the placenta after parturition (Kimura et al., 2002) and uterine disease risk has been associated with compromised neutrophil function in the transition period (Hammon et al., 2006). Oxidative burst activity of neutrophils is an index of killing capacity, and reductions in this function have been associated with induced subclinical hypocalcemia (Martinez et al., 2014), as well as spontaneous subclinical hypocalcemia occurring after parturition (Martinez et al., 2012).

Cows enrolled in this observational study represented a convenience sample of Holstein cows calving from June to July of 2013 (n = 17) and January to February of 2014 (n = 16) at the Cornell University dairy research farms (Harford, NY). Eligibility criteria for enrollment included parity (entering second or greater lactation), apparently healthy prior to parturition, and absence of *Staphylococcus aureus* mastitis in past lactations. The population included 15 cows entering their second lactation and 18 cows entering their third or greater lactation. All animal procedures were approved by the Cornell University Institutional Animal Care and Use Committee prior to the start of the study.

Samples of rations fed during the prepartum period were collected weekly, and a composite sample from each enrollment period was analyzed at a commercial laboratory (Cumberland Valley Analytical Services, Hagerstown, MD). Prepartum ration dietary cation-

anion difference [DCAD mEq/100 g DM = $(Na^+ + K^+) - (Cl^- + S^{2-})$] for cows calving between June and July of 2013 was +6.4 mEq/100 g DM, and for cows calving between January and February of 2014 was -6.1 mEq/100 g DM. This reflects some degree of additional anion inclusion in these rations for prevention of hypocalcemia and while they represent different degrees of implementation of a preventative approach, this range of ration DCAD would be considered representative of preventative strategies implemented in the field.

Blood samples were collected from all cows twice in the 24 h after calving (the next two samplings after calving with the morning sampling occurring between 0600 h and 0730 h and the evening sampling occurring between 1630 h and 1700 h), referred to as 0.3 and 0.6 DIM in the results, and once daily for the following 4 d at the morning sampling. Sampling was discontinued if cows were identified as clinically hypocalcemic by farm personnel and required treatment with intravenous Ca (n = 3). Samples collected prior to treatment remained in the dataset. A total of 183 samples for comparison of serum tCa, Mg and P methods were available, and 181 samples for the comparison of iCa and reference method tCa, due to sample clotting issues in two samples prior to measurement of whole blood iCa. Samples were collected from the coccygeal vessels using 20 G needles and 6 mL syringes (Covidien, Minneapolis, MN) containing no anticoagulant. Whole blood was dispensed into cartridges (CHEM8+, Abbott Point of Care, Inc., Princeton, NJ) according to manufacturer instructions for analysis of iCa within 3 min of sample collection using an iSTAT Portable Clinical Analyzer (iSTAT; Abbott Point of Care, Inc., Princeton, NJ) which uses ion selective electrode potentiometry. The whole blood was then dispensed into 16 mm x 100 mm glass tubes, capped, allowed to clot at room temperature and centrifuged at 2,000 x g for 20 min at 25°C. Serum was harvested and a portion of the serum was analyzed for tCa, Mg and P concentration using the VetTest Chemistry Analyzer (VetTest;

IDEXX Laboratories, Inc., Westbrook, ME). Quality control tests were run and samples were analyzed according to manufacturer instructions. The remaining serum was aliquoted into 1.7 mL microfuge tubes and stored at -20°C. Serum was shipped overnight on dry ice to the Michigan State Diagnostic Center for Population and Animal Health (Lansing, MI) for determination of total Ca, Mg and P using an automated clinical chemistry analyzer (Beckman Coulter 640e, Beckman Coulter, Inc., Brea, CA) with reagents provided by the company. The analysis conducted by the autoanalyzer was considered the reference method. For total Ca, the reaction between Ca in the sample and arsenazo III results in a purple complex, the absorbance is measured bichromatically at 660/800 nm, and the increase in absorbance is directly proportional to the total Ca in the sample. For Mg, the reaction between Mg in the sample and xylidyl blue results in a purple complex, interference by Ca is controlled by inclusion of glycoletherdiamine-N,N,N,N-tetraacetic acid, the absorbance is measured bichromatically at 520/800 nm, and the increase in absorbance is proportional to the Mg concentration. Phosphorus is determined by the reaction between inorganic phosphate in the sample and molybdate which form a heteropolyacid complex, the absorbance is measured bichromatically at 340/380 nm, and the increase in absorbance due to the formation of this complex is proportional to the inorganic phosphorus in the sample. According to the manufacturers, the results of these analyses are not significantly affected by hemolysis up to 500 mg/dL for Ca, 150 mg/dL for Mg and 350 mg/dL for P. The laboratory generated intra assay coefficients of variation (CV) are 0.8% for Ca, 2.7% for P, and 4.0% for Mg (n = 20). According to the manufacturer, total CV (measured by repeated analysis over several days) at low and high concentrations are 1.3% and 0.8% for Ca, 2.1% and 0.9% for P, and 1.4 and 2.1% for Mg. A visual hemolysis assessments was conducted at the diagnostic laboratory and no samples had severe hemolysis. The assays for the VetTest machine are based

on the reaction with arsenazo III for Ca, formazan dye for Mg, and ammonium molybdate and p-methylaminophenol sulfate for P. Filters are used that approximately the wavelengths for reading the reaction at 680 nm for Ca with a wavelength offset based on manufacturer calibration, 650 nm for Mg and 650 nm for P. The manufacturers indicate no significant effects of hemolysis for the VetTest, however, effect of hemolysis on these assays was not assessed in the current study.

An additional blood sample was collected from all cows that did not receive intravenous Ca treatment (n = 30) at one sampling between 2 and 5 DIM for flow cytometric determination of neutrophil oxidative burst activity using a commercial kit (Phagoburst, Glycotope GmbH, Berlin, Germany) as described by Yasui et al. (2015). Data from one cow was excluded because of laboratory error. The final neutrophil function dataset included 16 and 13 cows from the first and second enrollment periods, respectively, with 15 second lactation cows and 14 third and greater lactation cows. Samples were collected using a 20 G vacutainer needle (Becton Dickinson, Franklin Lakes, NJ) and 10 mL vacutainer tube containing sodium heparin (158 USP, Becton Dickinson, Franklin Lakes, NJ), placed on ice, transported to the laboratory, and analyzed within 2 h. Flow cytometric analysis of oxidative burst activity was conducted using a BD FACSCalibur flow cytometer with a 488-µm argon laser (Becton Dickinson Biosciences, Franklin Lakes, NJ). Data analysis was conducted using Cell Quest Analysis software (Becton Dickinson Biosciences, Franklin Lakes, NJ). Data were collected from a minimum of 10,000 leukocytes per assay tube. Scatterplots of forward light scatter and side light scatter were used to separate cell populations based on cell size and granularity to gate the subpopulation of neutrophils and to remove the population of cells with spontaneous fluorescence that was apparent in negative control tubes. Data from the tube stimulated with preopsonized, non-labeled

Escherichia coli and the tube stimulated with phorbol 12-myristate 13-acetate (PMA; high stimulus) were compared to the negative control tube (wash solution added in place of stimulant). To control for day to day variation, histogram plots of fluorescence activity for gated neutrophils in negative control tubes were used to set margins for each cow so that less than 5% of the neutrophil population was positive. These histogram margins were then applied to the data acquired from stimulated tubes for that cow to determine the number of neutrophils with fluorescence activity higher than the negative control tube (positive percent) and the geometric mean fluorescence intensity (GMFI) of those neutrophils which represents the mean oxidative burst activity of activated neutrophils.

Analysis of the association between methods for blood electrolyte measurement was conducted using the MIXED procedure of SAS (version 9.4, SAS Institute, Cary, NC). To determine the association between the test method (VetTest) and the reference method for total serum electrolytes, linear regression was conducted using the reference measurement as the fixed effect with cow as the subject of repeated measures to account for the covariance of measurements taken from the same cow. Four covariance structures were tested (compound symmetry, heterogeneous compound symmetry, antedependence and unstructured) with selection based on the lowest Akaike's Information Criterion. The estimate for the slope of the regression lines and the associated *P*-values are presented. To determine if the relationship between iCa and the reference method tCa varied by sampling timepoint, multivariate regression was conducted with whole blood iCa as the outcome, and the reference method serum tCa, sampling timepoint, and the interaction of serum tCa and sampling timepoint included as the fixed effects, accounting for cow as the subject of repeated measures with covariance structure selection as described above. Because the interaction between sampling timepoint and reference method tCa was

significant ($P < 0.0001$), a stratified analysis by sampling timepoint was conducted. The

regression slopes and associated P-values of the simple linear regression analysis at each

timepoint are presented. Agreement between the VetTest measurements of total Ca, Mg and P

with the reference method were assessed using Bland Altman plots in the software MedCalc

(MedCalc Software, Ostend, Belgium), and the mean differences of the methods (reference

method value minus the VetTest value) across the range of values is presented. Receiver

operator characteristic (ROC) analysis was used to determine the thresholds for serum tCa

measured by the VetTest and whole blood iCa that resulted in the highest combined sensitivity

and specificity for identifying hypocalcemia according to the reference method categorization at

two different serum tCa thresholds (≤ 2.0 mmol/L and ≤ 2.125 mmol/L). Significance was

declared at $P \leq 0.05$.

The relative value of whole blood iCa versus serum tCa measured by the reference

method as predictors of neutrophil oxidative burst activity were tested using multivariate models

in PROC MIXED. Outcomes included positive percent and GMFI for *E. coli*-stimulated

neutrophils and PMA-stimulated neutrophils. A data transformation was conducted for PMA-

stimulated positive percent prior to analysis to account for a left-skewed distribution

[transformed value = ln(101-positive percent)]. For each outcome, four separate models were

analyzed that included one of the following indices of Ca status: minimum whole blood iCa or

reference method serum tCa (measured on or before the day of neutrophil assessment), and the

whole blood iCa or reference method serum tCa measured on the day of neutrophil assessment.

Confounders that were included in all models included parity (second lactation vs. third lactation

and greater) and season (summer 2013 vs. winter 2014). The slope estimates from the

multivariable models for the effect of Ca status indices with $P \leq 0.05$ are reported and discussed.

The linear regression demonstrated strong relationships between blood electrolytes measured by the VetTest compared to the reference method for serum tCa (slope = 1.08 ± 0.02, $P < 0.0001$), serum Mg (slope = 0.89 ± 0.02, $P < 0.0001$) and serum P (slope = 0.99 ± 0.01, $P < 0.0001$), and these relationships are displayed in Figure 3-1. The Bland Altman plots of the comparison between the two methods are presented in Figure 3-2. The Bland Altman plots are necessary to determine the mean bias when comparing a test method to a reference method. The negative mean bias for the reference method value minus the VetTest value indicated that the VetTest read higher by 0.22 mmol/L (95% CI = 0.02 to 0.42) for Ca, 0.12 mmol/L (95% CI = -0.01 to 0.24) for Mg and 0.16 mmol/L (95% CI = -0.10 to 0.41) for P. Results of the ROC analysis for identification of hypocalcemia using the VetTest compared to the reference method are presented in Table 3-1. Compared to the reference method using a serum tCa threshold of $\leq$ 2.0 mmol/L, a threshold of $\leq$ 2.23 mmol/L for serum tCa measured by the VetTest resulted in a sensitivity and specificity of 87% and 89%, respectively. The VetTest performed similarly well but with higher specificity at a higher reference method threshold of $\leq$ 2.125 mmol/L, at which a threshold for serum tCa measured by the VetTest of $\leq$ 2.30 mmol/L performed with a sensitivity and specificity of 86% and 96%, respectively.

The linear regression between whole blood iCa and reference method serum tCa measured at each of the sampling timepoints is presented in Figure **3-**3. A linear relationship between these two blood Ca fractions was observed at each timepoint ($P \leq 0.0004$), and the equations for the regression lines are presented in Figure **3-**3. The results of the ROC analysis for hypocalcemia identification using whole blood iCa compared to the reference method serum tCa measurement are presented in Table 3-1. Compared to the reference method categorization of Ca status at either threshold ($\leq$ 2.0 mmol/L or $\leq$ 2.125 mmol/L), a whole blood iCa threshold

of ≤ 1.17 mmol/L resulted in the highest combined sensitivity and specificity. Compared to the lower reference method threshold, the sensitivity and specificity was 94% and 84%, respectively. At the higher reference method threshold, the sensitivity and specificity were 82% and 94%, respectively. If a threshold of ≤ 1.0 mmol/L of iCa was compared to a reference method serum tCa threshold of ≤ 2.0 mmol/L, the sensitivity and specificity for identifying hypocalcemia were 39% and 98%, respectively, resulting in a high proportion of false negative tests. This threshold has been used in previous research for hypocalcemia identification (Chamberlin et al., 2013), likely based on the relationship between tCa and iCa demonstrated in health cows (Lincoln and Lane, 1990). Our data suggests that assumed relationships between iCa and tCa do not reliably identify the same population of cows.

The effects of Ca status indices on measures of neutrophil oxidative burst activity are presented in Table 3-2. Neither minimum iCa nor minimum reference method tCa were associated with any of the measures of neutrophil function ($P \geq 0.21$); therefore, only associations for iCa and reference method tCa measured on the day of neutrophil assessment are presented. Neither Ca status index on the day of neutrophil assessment was associated with PMA-stimulated neutrophil positive percent ($P \geq 0.36$). Both iCa ($P = 0.001$) and reference method tCa ($P = 0.02$) measured on the day of neutrophil assessment were positively associated with PMA-stimulated neutrophil GMFI. Ionized Ca measured on the day of neutrophil assessment was positively associated with *E. coli*-stimulated neutrophil positive percent ($P = 0.004$) and GMFI ($P = 0.02$) but there was no association of reference method tCa with *E. coli*-stimulated positive percent ($P = 0.14$) or GMFI ($P = 0.48$).

The ability to monitor Ca status at the herd and cow level can be a powerful management tool enabling farm personnel to regularly monitor efficacy of preventative strategies and identify

cows in need of intervention. The VetTest was identified as a potential on-farm tool because of its ease of use and relatively low cost of analysis compared to a diagnostic lab. This study demonstrated that accounting for the VetTest mean bias in the serum Ca threshold resulted in sensitivities and specificities for identification of hypocalcemia of greater than 85%. At this time, thresholds for identification of hypomagnesemia and hypophosphatemia based on associations with downstream outcomes are not available. As we learn more about early postpartum mineral metabolism, measurement of these parameters may be a valuable diagnostic tool. The measurement of Mg and P by the VetTest were higher than measurements by the reference method which suggests that a correction factor should be used if this tool were implemented in the field for diagnostic purposes. The variation in mean bias for individual VetTest machines was not assessed in the current study but is a potential factor that should be accounted for when implementing this test. Users should determine the mean bias of the particular machine being used.

The relationship between whole blood iCa and reference method serum tCa differed by sampling timepoint over the first 5 DIM. The variation in these measurements at DIM 3, 4, and 5 was low and the regression at these timepoints should be interpreted with caution. This relationship should be considered when identifying hypocalcemia using blood iCa concentrations, considering that thresholds based on negative downstream consequences have been established only using blood tCa concentrations. Typically, iCa thresholds have been extrapolated based on previously established relationships that suggested tCa is composed of 50% iCa (Lincoln and Lane, 1990); however, this relationship was demonstrated in clinically healthy cows. Other researchers have identified that this relationship varies in the immediate postpartum period (Ballantine and Herbein, 1991; Joyce et al., 1997) when iCa constitutes a

greater proportion of tCa. Despite the varying relationship between iCa and reference method tCa by day postpartum in the current study, adjusting the iCa threshold resulted in sensitivities and specificities for diagnosing hypocalcemia according to a reference method tCa categorization greater than 80%.

Using an *in vitro* whole blood assay for neutrophil oxidative burst activity, measurements of Ca status on the day of neutrophil assessment were more highly associated with the outcomes of the assay than minimum iCa or reference method tCa prior to neutrophil assessment. This is likely a reflection of the importance of influx of Ca from the extracellular fluid that occurs during activation of neutrophils (Martinez et al., 2014) and therefore the blood Ca concentration on the day of neutrophil assessment has greater influence on the outcome measured *in vitro* than any lingering effects of low blood Ca right after parturition on neutrophil Ca status. While neither of the parameters measured *in vitro* in this study captures the full capacity of the immune response, they provide insight into the ability of the circulating neutrophils to respond to different stimuli (positive percent) and the intensity of the stimulated response (GMFI), which demonstrates the functional capacity of those cells in relation to Ca status. Previous researchers have reported that cows with subclinical hypocalcemia have increased risk for uterine disease (Martinez et al., 2012). Those cows were also demonstrated to have reduced neutrophil oxidative burst activity and this is hypothesized to be the causal link between the two disorders. The relationship between Ca status and neutrophil function was detected in our study using the reference method tCa as a predictor for one measure of neutrophil oxidative burst activity, however, iCa measured on the day of neutrophil assessment was more consistently associated with neutrophil outcomes. This work warrants further investigation into using iCa as an indicator of functional outcomes requiring Ca with the potential for more sensitive blood Ca

thresholds compared to those established based on tCa. Further work should be done to measure

associations between these indicators of Ca status and health and production outcomes in

epidemiological datasets.

Table 3- 1. Receiver operator characteristic analysis of two different methods for measuring blood Ca status compared to hypocalcemia identification at two thresholds by a reference method method[1] for serum total Ca concentration determination

Method[2]	Threshold (mmol/L)[3]	Area under the curve (95% CI)	P-value	Sensitivity (95% CI)	Specificity (95% CI)
VetTest serum total Ca					
Reference method threshold ≤ 2.0 mmol/L	≤ 2.23	0.95 (0.91-0.98)	<0.0001	87.4 (78.5-93.5)	88.8 (80.8-94.3)
Reference method threshold ≤ 2.125 mmol/L	≤ 2.30	0.97 (0.94-0.99)	<0.0001	85.7 (78.1-91.5)	95.5 (87.3-99.1)
iSTAT whole blood ionized Ca					
Reference method threshold ≤ 2.0 mmol/L	≤ 1.17	0.93 (0.88-0.96)	<0.0001	94.3 (87.1-98.1)	80.2 (70.8-87.6)
Reference method threshold ≤ 2.125 mmol/L	≤ 1.17	0.93 (0.88-0.96)	<0.0001	82.2 (74.1-88.6)	93.9 (85.0-98.3)

[1]Beckman Coulter 640e automated chemistry analyzer with reagents provided by Beckman Coulter, Inc. (Brea, CA)

[2]VetTest (VetTest Chemistry Analyzer, IDEXX Laboratories, Inc., Westbrook, ME), iSTAT (iSTAT Portable Clinical Analyzer, Abbot Point of Care, Inc., Princeton, NJ)

[3]Threshold for the test method with the highest combined sensitivity and specificity for categorizing hypocalcemia according to the reference method at the specified threshold

Table 3- 2. Association between indices of Ca status and indices of neutrophil oxidative burst activity assessed by in vitro stimulation of neutrophils in whole blood with E. coli or phorbol 12-myristate 13-acetate (PMA). Data were collected from 29 multiparous Holstein cows between 2 and 5 DIM.

| | | Calcium Status Index | | | |
| | | Assay day iCa[1] | | Assay day tCa[2] | |
Outcome	Mean (± SD)	Slope Estimate[3]	P-value	Slope Estimate[3]	P-value
E. coli-stimulated					
Positive %[4]	71.7 ± 15.9	95.7 ± 30.0	0.004	-	0.14
GMFI[5]	9.8 ± 3.6	13.2 ± 5.2	0.02	-	0.48
PMA-stimulated					
Positive %[4]	92.5 (87.9 – 95.5)[6]	-	0.36	-	0.66
GMFI[4]	41.3 ± 18.9	101.0 ± 27.3	0.001	44.8 ± 18.0	0.02

[1]Whole blood ionized Ca measured on the day of neutrophil assessment
[2]Serum total Ca measured on the day of neutrophil assessment by the reference method
[3]Estimate from the multivariate regression model for the effect of the Ca status index on the neutrophil function outcome accounting for the effects of season and parity (second lactation vs. third and greater)
[4]Percent of neutrophils positive for oxidative burst activity
[5]Geometric mean fluorescence intensity, corresponds to mean oxidative burst activity
[6]Geometric mean and back transformed 95% confidence limits

Figure 3- 1. Linear regression between serum electrolyte concentrations measured with a test method (VetTest Chemistry Analyzer, IDEXX Laboratories, Inc. Westbrook, ME) and a reference method [Beckman Coulter 640e automated chemistry analyzer with Beckman Coulter reagents (Brea, CA)]. Samples (n = 183) were collected from 33 multiparous Holstein cows over the 5 d following parturition. Dashed line represents linear regression line and solid line represents the line of unity. Panel A = serum total Ca (tCa; mmol/L); slope = 1.08 ± 0.03, $P <$ 0.0001. Panel B = serum Mg (mmol/L); slope = 0.89 ± 0.02, $P <0.0001$. Panel C = serum P (mmol/L); slope = 0.99 ± 0.01, $P < 0.0001$.

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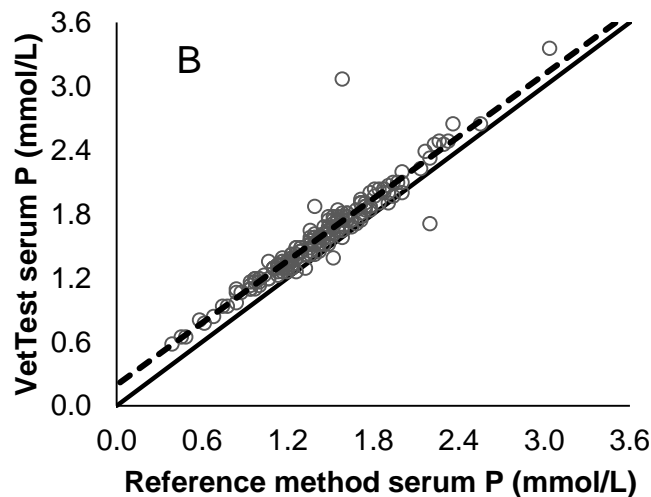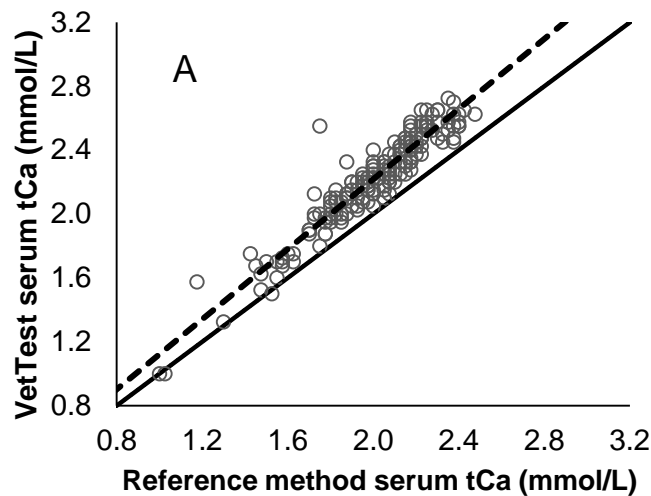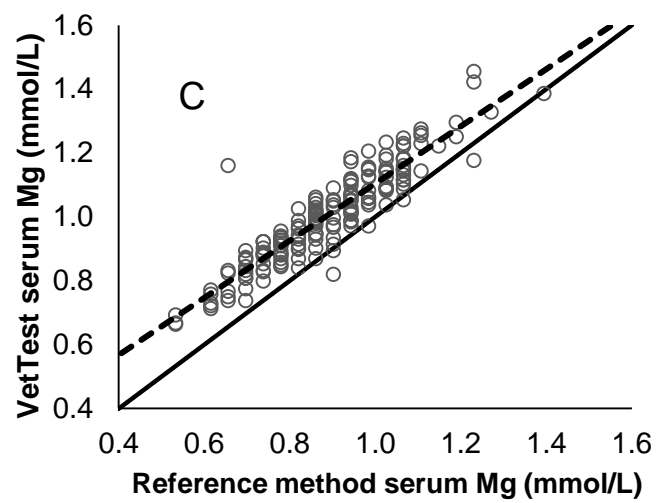
Figure 3- 2. Bland Altman plots comparing the difference between a reference method [Beckman Coulter 640e automated chemistry analyzer with Beckman Coulter reagents (Brea, CA)] and a test method (VetTest Chemistry Analyzer, IDEXX Laboratories, Inc., Westbrook, ME) to the mean of the two methods for measurement of serum total Ca (tCa; Panel A), serum Mg (Panel B) and serum P (Panel C). Y-axis = reference method value – VetTest value. Samples (n = 183) were collected from 33 multiparous Holstein cows over the 5 d following parturition. The solid line represents a mean difference of zero, the dashed line represents the observed mean difference, and the dotted lines represent the 95% CI of the mean difference.
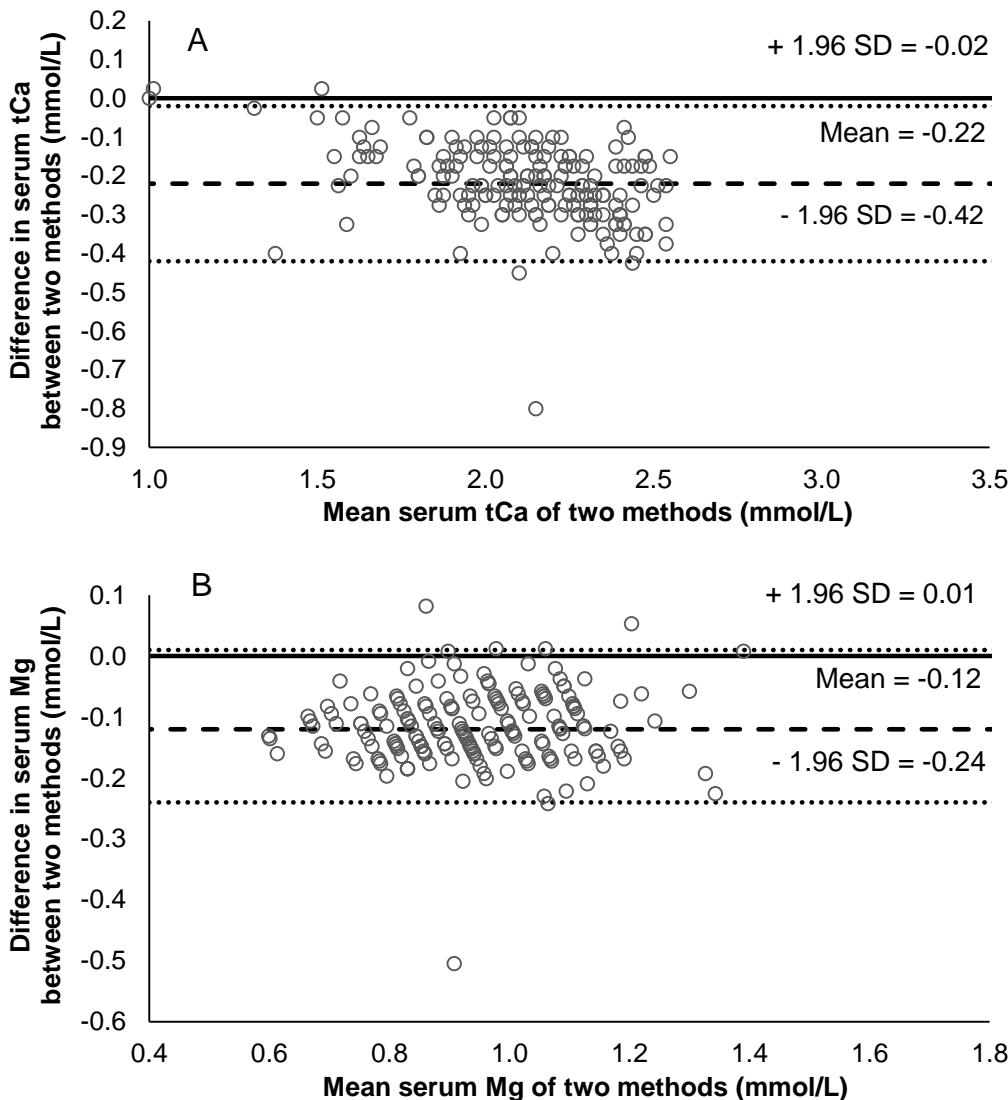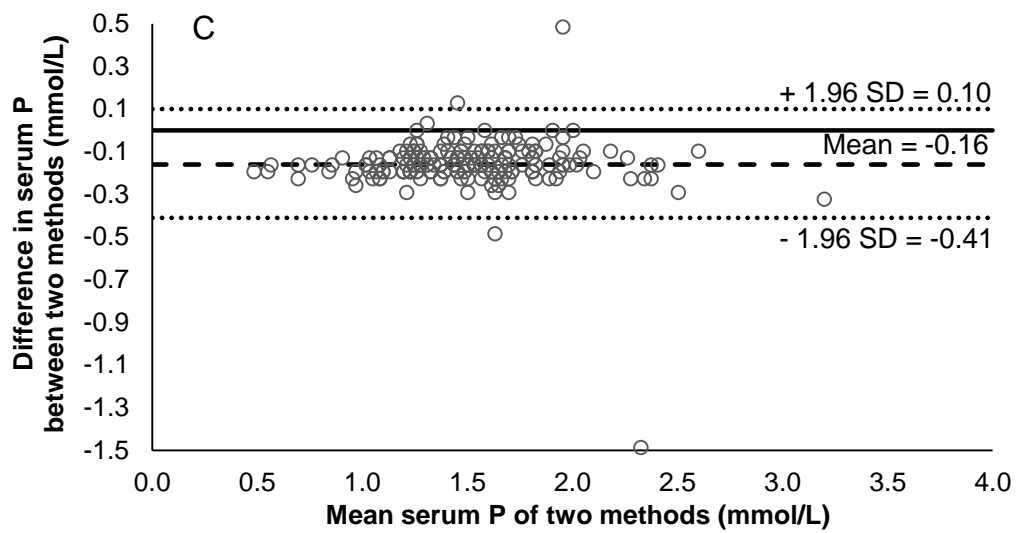
Figure 3- 3. Linear regression between ionized Ca (iCa) measured in whole blood (iSTAT Portable Clinical Analyzer, Abbot Point of Care, Inc., Princeton, NJ) and total Ca (tCa) measured in serum by a reference method [Beckman Coulter 640e automated chemistry analyzer with Beckman Coulter reagents (Brea, CA)] by sampling timepoint after parturition. Each plot represents one sampling timepoint as indicated. Samples (n = 181) were collected from 33 multiparous Holstein cows over the 5 d following parturition. The dashed line represents the linear regression line, and the equation of the regression lines are displayed with the *P*-value for the simple linear regression.

0.3 DIM

y = 0.57x - 0.02
$P < 0.0001$

0.6 DIM

y = 0.59x - 0.04
$P < 0.0001$

2 DIM

y = 0.54x + 0.04
$P < 0.0001$

3 DIM

y = 0.48x + 0.19
$P < 0.0001$

4 DIM

y = 0.35x + 0.46
$P < 0.0001$

5 DIM

y = 0.25x + 0.69
$P = 0.0004$

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CHAPTER 4

DIETARY CATION-ANION DIFFERENCE OF THE PREPARTUM DIET: PART I. EFFECTS ON DRY MATTER INTAKE, PERFORMANCE, ENERGY BALANCE AND PLASMA ENERGY METABOLITES IN MULTIPAROUS HOLSTEIN COWS

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ABSTRACT

The objectives of this study were to determine the effect of decreasing dietary cation-anion difference [$\textbf{DCAD} = (Na^+ + K^+) - (Cl^- + S^{2-})$] of the prepartum ration on dry matter intake (DMI), milk yield and aspects of energy metabolism in the transition period. Multiparous Holstein cows (n = 89) were enrolled in the study between d -38 and -31 relative to expected parturition and randomized to 1 of 3 prepartum rations in a completely randomized design (restricted to balance for previous 305-d mature equivalent milk production, parity and body condition score) at 24 d prior to expected parturition. Treatments were as follows: CON = low K ration with no anion supplementation (n = 30, DCAD = +18.3 mEq/100 g DM), MED = partial anion supplementation to a low K ration (n = 30, DCAD = +5.9 mEq/100 g DM) and LOW = anion supplementation to a low K ration to reach a targeted average urine pH between 5.5 and 6.0 (n = 29, DCAD = -7.4 mEq/100 g DM). Cows were fed a common postpartum ration and data collected through 63 d in milk. Urine pH was affected in a quadratic manner by decreasing prepartum DCAD such that cows fed LOW had much lower urine pH than CON and MED (8.22, 7.89, and 5.96 for CON, MED and LOW, respectively). A quadratic effect on prepartum DMI was observed such that cows fed MED (14.0 kg/d) had the highest intake, cows fed LOW (13.2 kg/d) had the lowest intake, and DMI of cows fed CON was intermediate (13.6 kg/d). Milk production in the first 3 wk postpartum was increased linearly for cows fed decreasing prepartum DCAD (CON = 40.8, MED = 42.4, and LOW = 43.9 kg/d) and DMI in this period also tended to linearly increase (CON = 20.2, MED = 20.9, and LOW = 21.3 kg/d) and was increased significantly when expressed as a percentage of body weight. Overall, effects on intake and milk yield analyzed over wk 1 to 9 postpartum were not significant. Measures of energy metabolism including plasma concentrations of glucose, non-esterified fatty acids and β-hydroxybutyrate

were similar among treatments in both the prepartum and postpartum periods. Ultimately, feeding decreasing DCAD prepartum resulted in increased postpartum DMI and milk production, specifically in the 3 wk after parturition, and those increases were greatest for cows fed the lowest prepartum DCAD.

INTRODUCTION

Dramatic changes in Ca demand from late gestation to the initiation of milk production can result in disorders of Ca homeostasis in the transition cow that have been recognized as detrimental to cow health and performance (Curtis et al., 1983). In recent years, the focus has shifted from clinical hypocalcemia, which affects only 2-5% of dairy cows (Chapinal et al., 2011; Reinhardt et al., 2011), to subclinical hypocalcemia (**SCH**) in the days following parturition. Subclinical hypocalcemia (serum Ca <2.0 mmol/L) is highly prevalent, affecting 25% of primiparous cows and 47% of multiparous cows (Reinhardt et al., 2011). Recent work has suggested that cows with higher productive potential may be at greater risk for experiencing SCH (Jawor et al., 2012; Oetzel and Miller, 2012). Subclinical decreases in blood Ca can result in depressions in gut motility and DMI (Daniel, 1983; Martinez et al., 2014), which could limit the ability of those cows to meet their productive potential or increase their risk of metabolic disease. Elevated markers of negative energy balance in blood including non-esterified fatty acids (**NEFA**) and BHB (Reinhardt et al., 2011; Martinez et al., 2012; Chamberlin et al., 2013) have been identified in cows with SCH and is likely related to depressions in DMI. Subclinical hypocalcemia impacts cow well-being and herd profitability due to increased risk of metabolic and infectious disease, loss of milk production, decreased reproductive success and increased risk of early lactation culling (Chapinal et al., 2012; Martinez et al., 2012; Roberts et al., 2012).

Formulating prepartum rations for a low or negative DCAD originated from efforts to prevent clinical hypocalcemia (Ender et al., 1971; Block, 1984). This strategy has been shown to minimize clinical disease (Block, 1984; Charbonneau et al., 2006) and improve blood Ca concentrations overall (Goff et al., 1991; Joyce et al., 1997; Moore et al., 2000) as a result of altering acid-base balance of the cow and inducing a metabolic acidosis in the prepartum period. The mechanisms of action by which low DCAD feeding improves blood Ca status likely include increased Ca flux prepartum (Grunberg et al., 2011) and increased sensitivity of the homeostatic system to respond to the Ca challenge at the onset of colostrum and milk production (Goff et al., 2014).

Supplementing additional anions to a low cation close up dry cow ration has been investigated previously and responses range from minimal differences in blood Ca and no production response (Ramos-Nieves et al., 2009) to positive responses in both postpartum intake and production (DeGroot et al., 2010; Weich et al., 2013). The magnitude of response likely depends on the degree and consistency of metabolic acidosis prepartum as well as maintenance of adequate prepartum intake. Moore et al. (2000) investigated the relative benefit of two different anion inclusion rates (0 and -15 mEq/100 g DM) compared to a low cation control ration (+15 mEq/100 g DM) and found that blood Ca responses were greatest for cows fed the lowest prepartum DCAD, however, statistical differences in performance were not detected. Additional research investigating the most appropriate strategy for anion supplementation is necessary to maximize both health and performance as well as profitability.

The primary objective of the current trial was to determine the effects of linearly decreasing the prepartum DCAD, starting with a low K control ration without the addition of an anion supplement, on DMI and milk production. We hypothesized that feeding lower DCAD

diets prepartum would result in increases in postpartum DMI and milk production as a result of improved blood Ca status. Our secondary objective was to investigate the influence of DCAD level on aspects of energy metabolism and hypothesized that postpartum plasma concentrations of NEFA and BHB would be decreased as a reflection of improved postpartum DMI.

MATERIALS AND METHODS

Study Population, Experimental Design, and Treatments

All animal handling and procedures were approved by the Cornell University Institutional Animal Care and Use Committee. Cows were enrolled in the study from March through August 2014. The study was conducted as a completely randomized design with randomization restricted to balance for parity group (2nd vs. 3rd +), previous lactation 305-d mature equivalent milk production and BCS at enrollment. A total of 98 multiparous Holstein cows were enrolled in the trial of which 89 were included in the final data set (CON; n = 30, MED; n = 30, LOW; n = 29). Reasons for removal from the trial included calving with less than 14 d on experimental diets (n=6) and euthanasia after parturition subsequent to dystocia or injury [n=3; 1 each from CON (2nd lactation), MED (3rd lactation) and LOW (4th lactation)]. Prepartum dietary treatment groups and analyzed DCAD [DCAD mEq/100 g DM = $(Na^+ + K^+) - (Cl^- + S^{2-})$] were as follows; low K ration with no supplemental anions (CON; K = 1.28% of DM, DCAD = +18.3 mEq/100 g DM), partial anion supplementation to a low K ration (MED; K = 1.26% of DM, DCAD = +5.9 mEq/100 g DM) and anion supplementation to a low K ration to reach a targeted average urine pH between 5.5 and 6.0 (LOW; K = 1.24% of DM, DCAD = -7.4 mEq/100 g DM).

Cows were moved to individual tie stalls and fed the CON ration starting between -38 and -31 d relative to expected parturition. At -24 d relative to expected parturition cows either

continued to be fed the CON ration or were fed MED or LOW until parturition, depending on treatment assignment. When calving was imminent, cows were moved to individual maternity pens. Within approximately 2 h, after parturition and collection of colostrum, cows were returned to individual tie stalls at which point all cows were fed a common postpartum diet until 63 DIM. Cows were observed daily throughout the trial for health disorders by farm personnel. To prevent the occurrence of recumbent cows, farm personnel were instructed to administer farm protocol treatment (500 mL 23% calcium gluconate intravenously, MWI Veterinary Supply Co., Boise, ID) when cows displayed early signs of clinical hypocalcemia including lethargy, muscle weakness or other nervous signs. All data from cows treated with intravenous Ca remained in the data set (n =3, 1, and 2 for CON, MED and LOW, respectively).

Diet Formulation, Composition and Dry Matter Intake

Diets were formulated using the Cornell Net Carbohydrate and Protein System (CNCPS version 6.1, Cornell University, Ithaca, NY). One batch of a base TMR was mixed for all prepartum treatment groups daily that contained wheat straw, brown mid-rib (**BMR**) corn silage and a concentrate mixture common to all prepartum diets. Before delivery to the cows, this base ration was further mixed with a small inclusion rate grain mix that was unique to each prepartum treatment group. Small inclusion rate grain mixes were formulated to supplement anions to the MED and LOW rations while maintaining similar concentrations of all other nutrients. Throughout the duration of the trial, average urine pH of cows that had been fed their treatment diets for at least 5 d were monitored to determine the need for adjustment of the inclusion rate of these mixes. The targeted average urine pH for cows fed LOW was between 5.5 and 6.0. Equal adjustments were made to the inclusion rate in all three prepartum diets when adjustments were necessary to meet the urine pH target of the cows fed LOW and were compensated by

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corresponding changes in inclusion rates of wheat straw and BMR corn silage. The inclusion rate of these mixes was adjusted 5 times over 5 months of dry cow feeding and ranged from 5.61 to 6.67% of DM. Average ingredient composition of the final rations, weighted by the number of cows calving during the feeding of that formulation, is presented in Table 4-1. Feed was delivered at approximately 0730 h for lactating cows and 0900 h for dry cows. Feed refused was collected just prior to feeding, weighed and recorded. The amount of feed delivered was adjusted each day to target a refusal rate of 10% to allow for ad libitum intake.

Samples of TMR, forages and grain mixes were collected weekly and DM determined by drying in a forced air oven at 40°C for 96 h. Forage and grain DM values were used to make weekly adjustments to as-fed inclusion rates of all ration ingredients. Dried samples were composited at 4-wk intervals and composites were ground through a 2-mm screen of a Wiley mill. Grain and forage samples were further composited over the length of the study. Four week composite samples of TMR and a single composite of forages and grain mixes were analyzed at a commercial laboratory (Cumberland Valley Analytical Services, Hagerstown, MD) via wet chemistry methods for DM at 135°C (Method 930.15, AOAC International, 2000), CP (Method 990.03, AOAC International, 2000), ADF (Method 973.18, AOAC International, 2000), NDF (Van Soest et al., 1991), starch (Hall, 2009), sugar (Dubois et al., 1956), ether extract (Method 2003.05, AOAC International, 2006), minerals (Method 985.01, AOAC International, 2000), chloride (silver nitrate titration after extraction with 0.5% nitric acid using a Brinkman Metrohm 848 Titrino Plus, Brinkmann Instruments Inc, Westbury, NY) and sulfur (according to Leco Organic Application Note "Sulfur and Carbon in Plant, Feed, Grain and Flower" Form 203-821-321, 5/08-REV1). Values for NE_L of TMR composite samples were calculated according to NRC (2001). Analyzed diet composition is presented in Table 4-2. Dry matters determined on

fresh TMR samples collected weekly were further adjusted for residual moisture in composite samples and the corrected DM were used to calculate DMI for the corresponding week.

Individual Animal Sampling, Analytical Methods and Calculations

Urine samples were collected once during the week prior to assignment to treatment and three times per week thereafter until parturition at 1700 h (approximately 8 h after feed delivery). Midstream samples were collected following manual stimulation of the space below the vulva into a paper cup. Urine pH was determined immediately after collection with a transportable glass electrode pH meter (model UP-5 pH meter, Denver Instrument, Denver CO). The pH meter was calibrated each day prior to sampling with a 3 point (pH 4, 7 and 10) calibration.

Blood samples were collected via coccygeal venipuncture between 0600 h and 0730 h once during the week prior to assignment to treatment (covariate sample), twice weekly thereafter until parturition (Monday and Friday), twice in the 24 h after parturition (the next two timepoints occurring after parturition with sampling occurring at approximately 0700 h and 1630 h), once daily though 5 DIM, and three times per week thereafter (Monday, Wednesday and Friday) through 56 DIM. Samples were collected using 10 mL sodium heparin evacuated tubes (158 USP, Becton Dickinson, Franklin Lakes, NJ) and 20 G vacutainer needles (Becton Dickinson, Franklin Lakes, NJ) and placed on ice immediately after collection. Plasma was harvested after centrifugation at 2,000 x g for 20 min at 4°C, snap frozen in liquid nitrogen and stored at -20°C until analysis. All samples collected from enrollment through 21 DIM were analyzed for BHB, NEFA and glucose. A commercial kit (β-Hydroxybutyrate; Catachem Inc., Oxford, CT) was adapted for analysis of plasma BHB concentrations in a 96-well plate. Briefly, 5 μL of standards, control sample and unknowns were pipetted in duplicate. A background reading at 340 nm was taken immediately after the addition of 250 μL of reagent A.

Subsequently, 50 µL of the catalyst reagent was added (Reagant B) and a second reading was taken after the plate was incubated for 2 min at 37°C with intermittent mixing. The change in absorbance for unknown samples was compared to the standard curve to determine BHB concentration. Analysis of plasma NEFA concentrations were conducted in triplicate using a commercial enzymatic kit (HR Series NEFA HR (2), Wako Pure Chemical Industries, Osaka, Japan). Commercial products (PGO Enzyme Preparation and o-dianisidine dihydrochloride) were used to determine glucose enzymatically in triplicate (glucose oxidase; protocol from Sigma Aldrich kit 510-A; St. Louis, MO). A Versamax tunable microplate reader (Molecular Devices, Sunnyvale, CA) was used for all spectrophotometric measurements of enzymatic assays. Coefficients of variation (both inter- and intra-assay) for all assays were maintained below 10%.

After parturition, all cows were milked three times daily at 0600, 1400 and 2200 h. Milk samples were collected at 3 consecutive milkings each week, mixed with a bronopol preservative and stored at 4°C until transportation to a commercial laboratory (DairyOne, Ithaca, NY) within 72 h of collection for analysis. Milk fat, protein, lactose, total solids and MUN were analyzed using mid-infrared techniques (Method 972.16, AOAC International, 2006) and SCC was determined by optical fluorescence (Method 978.26, AOAC International, 2006). Somatic cell scores were calculated from SCC [$SCS = \log_2(SCC/100{,}000)+3$]. Milk yield at the corresponding milking was used to weight milk composition and calculate yield of components. Weekly average yield of 3.5% FCM and ECM were calculated from weekly yield and composition as described by McCarthy et al. (2015).

Body weights were measured weekly and BCS were assigned weekly by two scorers according to Edmonson et al. (1989). The average of the two BCS were used for statistical

analysis. Weekly calculations of prepartum and postpartum net energy balance (**EBAL**) were determined according to NRC (2001) equations as described by McCarthy et al. (2015).

Statistical Analysis

Prepartum data analysis was restricted to the 21 d prior to actual parturition. Daily DMI and milk production were reduced to weekly means before statistical analysis. To account for the 2×/wk (prepartum blood) or 3×/wk (prepartum urine and postpartum blood) sampling schedule, aside from the daily sampling period, values were categorized to 3 or 4 d intervals. Multiple samples collected in the same interval were averaged prior to analysis. Prepartum and postpartum data were analyzed separately. Postpartum data for weekly measures were analyzed as wk 1 to 3 and wk 1 to 9 to determine effects of treatment that were primarily manifested in the early postpartum period. All statistical analyses were conducted with the statistical software SAS (version 9.4, SAS Institute Inc., Cary, NC). Chi-square tests for differences in distribution of lactation number were conducted with the FREQ procedure. One way ANOVA were conducted using the MIXED procedure to determine differences by treatment group in previous lactation 305-d mature equivalent milk production, BCS and BW at enrollment, and days on treatment diet. All measurements taken at multiple timepoints were subjected to repeated measures analysis using the REPEATED statement in the MIXED procedure of SAS (Littell et al., 1996). The fixed effects of time, treatment, and parity group (2nd vs. 3rd+ lactation) and all two way interactions were tested in the model. Parity interactions with $P > 0.10$ were removed from the model. Covariate measurements collected in the week prior to treatment assignment were included in all models. Previous lactation 305-d mature equivalent milk production was included as a covariate for milk production and composition analysis. The Kenward Rogers method was used for estimation of denominator degrees of freedom. Four covariance structures were tested

for each model and the model with the lowest Aikaike's Information Criterion was selected

(Littell et al., 1996). For data with equal intervals over time the following covariance structures

were tested; compound symmetry, heterogeneous compound symmetry, autoregressive order 1

and heterogeneous autoregressive order 1. For data with unequal intervals over time,

antedependence 1 and unstructured covariance structures were tested in place of the

autoregressive covariance structures. For all models, orthogonal contrast statements were

included to determine the overall linear or quadratic effects of decreasing DCAD. When $P \leq 0.10$

for interaction terms, the SLICE option was used in the LSMEANS statement to conduct an F-

test to determine at which levels of time or parity the treatment groups differed. Plots of

studentized residuals were inspected for normality and homogeneity of variance. The GROUP

option in the repeated statement was used to control for heterogeneity of variance due to

treatment when analyzing urine pH. When non-normality of residual variance was evident

(NEFA and BHB), data were log transformed and analysis repeated. Least squares means and

standard errors, or geometric mean and back transformed 95% confidence intervals for data that

were log transformed, are reported throughout. Significance was declared at $P \leq 0.05$ and trends

are discussed at $0.05 < P \leq 0.10$.

RESULTS

Study Population

The distribution of lactation number, previous lactation 305-d mature equivalent milk

yield, covariate BCS and BW, and days on treatment diet did not differ by treatment.

Descriptive statistics are provided in Table 4-3. Data from 6 cows with severe drops in intake or

milk production due to illness occurring on or after 30 DIM were removed from analysis from

the point of illness through the end of the observation period. All data collected prior to that

point were included in analysis. These exclusions were decided by a researcher blinded to treatment and included the following; five cows with clinical mastitis [one cow fed CON (3rd lactation), two cows fed MED (2nd and 4th lactation) and two cows fed LOW (2nd and 4th lactation)] and one cow with a digestive disorder (fed LOW, 3rd lactation). Four cows calved with twins but were distributed across treatments (CON, n=1; MED, n=2; LOW, n=1) and therefore remained in the dataset.

Diets and Urine pH

Analyzed composition of prepartum and postpartum composite TMR samples are presented in Table 4-2. Dietary K concentrations were similar across all three prepartum diets (CON = 1.28, MED = 1.26, and LOW = 1.24% of DM) and were representative of those from cows fed prepartum diets based upon corn silage and wheat straw as primary forages. Both dietary S (CON = 0.20, MED = 0.30, and LOW = 0.41% of DM) and Cl (CON = 0.27, MED = 0.47, and LOW = 0.69% of DM) concentration incrementally increased from CON and subsequently DCAD was incrementally decreased (CON = +18.3, MED = +5.9, and LOW = -7.4 mEq/100 g DM), supporting the use of orthogonal linear and quadratic contrasts for analysis of overall treatment effects. A quadratic effect of decreasing DCAD on urine pH was observed (CON = 8.22 ± 0.02, MED = 7.89 ± 0.03, and LOW = 5.96 ± 0.11, $P<0.0001$) and cows fed LOW had urine pH close to the targeted range throughout the treatment period (Table 4-4).

DMI, EBAL, BW and BCS

Prepartum results for DMI, EBAL, BW, and BCS are presented in Table 4-4. Prepartum intake responded in a quadratic manner to decreasing prepartum DCAD and overall intakes increased from CON to MED and decreased from MED to LOW (Figure 4-4; CON = 13.6, MED = 14.0, and LOW = 13.2 kg/d, $P = 0.01$). Similar quadratic effects on prepartum DMI as a

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percentage of BW (CON = 1.71, MED = 1.75, and LOW = 1.66%, quadratic P = 0.06) and

prepartum EBAL (CON = 4.3, MED = 5.0, and LOW = 3.7 Mcal/d, quadratic P = 0.01) were

observed. Prepartum BW was not affected by treatment. A trend for a treatment by time effect

was detected for prepartum BCS (P = 0.06) such that BCS differed in wk -2 (CON = 3.33, MED

= 3.41, and LOW = 3.36, P = 0.05); however, effects were comparatively small.

Postpartum DMI, EBAL, BW and BCS are presented in Table 4-5. Dry matter intake in

wk 1 to 3 tended to increase linearly with decreasing prepartum DCAD (Figure 4-1; CON = 20.2,

MED = 20.9, and LOW = 21.3 kg/d, P = 0.09) and was linearly increased when expressed as a

percentage of BW (CON = 2.88, MED = 2.98, and LOW = 3.07 % of BW, P = 0.04). Intake

from wk 1 to 9 was not different (CON = 23.5, MED = 24.4, and LOW = 24.0 kg/d, quadratic P

= 0.12) but a trend for a linear increase in DMI as a percentage of BW in wk 1 to 9 (CON = 3.38,

MED = 3.52, and LOW = 3.50% of BW, P = 0.06) was observed. There were no effects of

treatment on postpartum BW, BCS, or EBAL in wk 1 to 3 or wk 1 to 9.

Milk Production, Composition and Efficiency

Milk production, composition, and efficiency for wk 1 to 3 and wk 1 to 9 are presented in

Table 4-7. Decreasing prepartum DCAD resulted in a linear increase in milk yield from wk 1 to

3 (Figure 4-2; CON = 40.8, MED = 42.4, and LOW = 43.9 kg/d, P = 0.03). There were no

differences in milk fat content or fat yield in wk 1 to 3; however, there was a trend for a

treatment by week effect (P = 0.10) on milk fat content such that fat content was numerically

lowest in cows fed LOW in wk 1 and 2 but similar to CON and MED in wk 3. Milk protein

content decreased linearly with decreasing prepartum DCAD (CON = 3.54, MED = 3.49, and

LOW = 3.27%; P = 0.005). There was no overall effect on milk protein yield, however, a trend

for a treatment by week effect (P = 0.09) indicated numerically higher protein yield in wk 1 and

2 for cows fed LOW with similar yield to CON and MED in wk 3. There was no effect of treatment on milk lactose content; however, lactose yield increased linearly with decreasing prepartum DCAD ($P = 0.02$) and a treatment by week effect was observed ($P = 0.02$) such that lactose yield was different in wk 1 ($P = 0.03$) and 2 ($P = 0.04$). Ultimately, yield of ECM tended to increase linearly (CON = 46.2, MED = 48.1, and LOW = 49.6 kg/d, $P = 0.08$) for cows fed decreasing DCAD prepartum. There was no effect of treatment on milk production efficiency. Milk urea nitrogen decreased linearly with decreasing prepartum DCAD (CON = 10.32, MED = 9.72, and LOW = 9.44 mg/dL; $P = 0.04$).

From wk 1 to 9 there was no effect of treatment on milk yield (CON = 47.1, MED = 48.5, and LOW = 48.7 kg/d, linear $P = 0.18$), fat content or fat yield (Table 4-7). A linear decrease in protein content through wk 9 remained for cows fed decreasing DCAD prepartum (CON = 2.99, MED = 2.98, and LOW = 2.84%, $P = 0.02$) and a treatment by parity interaction was observed ($P = 0.04$) such that effects of treatment on protein content in second lactation cows significantly differed (CON = 3.02, MED = 3.06, LOW = 2.83%, $P = 0.003$) but not for older cows (CON = 2.95, MED = 2.90, and LOW = 2.85%, $P = 0.40$). Protein yield did not differ by treatment. A trend for a treatment by wk effect on milk lactose yield ($P = 0.07$) reflected differences in yield in weeks 1 and 2. Yield of ECM (CON = 48.3, MED = 49.6, and LOW = 49.6 kg/d, linear $P = 0.27$) and milk production efficiency were not different when analyzed over wk 1 to 9. A trend for a linear decrease in MUN remained over wk 1 to 9 (CON = 10.82, MED = 10.23, and LOW = 10.16 mg/dL, $P = 0.06$).

Plasma Metabolite Concentrations and Health Events

Prepartum and postpartum plasma concentrations of glucose, NEFA and BHB are presented in Table 4-8. There were no effects of treatment on any of the plasma energy

metabolites measured in the prepartum or postpartum period (Figure 4-3). Incidence of farm

personnel identified health disorders are presented in Table 4-7. This study did not have

sufficient sample size to test statistical differences in clinical disease occurrence.

Table 4- 1. Ingredient composition of the prepartum and postpartum diets (% of DM)

Ingredient (% of DM)	Prepartum Diet[1]			Postpartum Diet
	CON	MED	LOW	
Brown mid-rib corn silage	44.79	44.79	44.79	36.30
Wheat straw	28.06	28.06	28.06	7.85
Haylage	-	-	-	8.83
Amino Plus	7.98	7.98	7.98	7.06
Citrus pulp	3.25	3.25	3.25	3.92
Wheat midds	3.22	2.59	1.93	-
Ground corn grain	0.41	0.41	0.41	19.62
Corn gluten feed	-	-	-	3.92
Soybean hulls	2.26	2.26	2.26	-
Distillers grains, ethanol	2.19	1.31	0.39	1.96
Canola meal	2.16	2.16	2.16	5.89
Molasses	0.70	0.70	0.69	-
Urea	0.41	0.21	-	-
LysAAMet[3]	-	-	-	0.78
Megalac R[4]	-	-	-	0.39
Megamine L[4]	-	-	-	0.39
Alimet[5]	-	-	-	0.06
Animate[6]	-	1.95	3.99	-
Ca carbonate	3.18	3.09	3.00	-
Mono-dicalcium phosphate	0.47	0.47	0.47	-
Mg oxide	0.56	0.41	0.26	-
MIN-AD[7]	-	-	-	1.57
Sodium bicarbonate	-	-	-	0.78
Salt	0.24	0.24	0.24	0.39
Lactating mineral mix[8]	-	-	-	0.16
Selenium 0.06%	0.04	0.04	0.04	-
Dairy ADE mix[9]	0.04	0.04	0.04	-
1100 Dairy TM[10]	0.03	0.03	0.03	-
Vitamin E premix	0.02	0.02	0.02	0.06
Rumensin[11]	0.01	0.01	0.01	0.06

[1]Ration ingredient composition reflects a weighted average composition to account for changes in the inclusion rate (range = 5.61-6.67% of DM) of the small inclusion rate mix, compensated for by corn silage and wheat straw, throughout the trial. Dietary treatments: CON = low K ration with no anion supplementation (n = 30, DCAD = +18.3 mEq/100 g DM), MED = partial anion supplementation to a low K ration (n = 30, DCAD = +5.9 mEq/100 g DM) and LOW = anion supplementation to a low K ration to reach a targeted average urine pH between 5.5 and 6.0 (n = 29, DCAD = -7.4 mEq/100 g DM)

[2]Source of rumen bypass soybean meal (Ag Processing, Inc., Omaha, NE)

[3]Source of rumen protected DL-methionine and lysine hydrochloride (Perdue AgSolutions, LLC., Salisbury, MD)

[4]Megalac R = source of rumen bypass calcium salts of long chain fatty acids; Megamine L = source of rumen bypass L-Lysine monohydrochloride and calcium salts of long chain fatty acids (Church & Dwight Co., Inc., Ewing, NJ)

[5]Source of HMTBa which is a precursor for methionine (Novus International, Saint Charles, MO)

[6]Composed of 39.0% CP, 7.6% EE, 3.1% sugar, 5.8% starch, 23.5% NDF, 1.3% Ca, 0.5% P, 4.3% Mg, 5.5% S, 14.0% Cl (Phibro Animal Health, Corp., Quincy, IL)

[7]Source of supplemental Ca (21.5% of DM) and Mg (11.5% of DM) as dolomitic limestone (Papillon Agricultural Company, Inc., Easton, MD)

[8]Composed of 2.7% Ca, 12.6% K, 18.6% S, 25,560 ppm Zn, 7,154 ppm Cu, 21,958 ppm Mn, 214 ppm Se, 501 ppm Co, 331 ppm I, 3,704 KIU/kg vitamin A, 922 KIU/kg vitamin D, 12,496 KIU/kg vitamin E

[9]Composed of 19.9% Ca, 30,073 KIU/kg vitamin A, 5,783 KIU/kg vitamin D, 92,534 IU/kg vitamin E

[10]Composed of 18.4% S, 153,815 ppm Zn, 30,318 ppm Cu, 136,432 ppm Mn, 3,386 ppm Co, 3032 ppm I

[11]Prepartum Rumensin mix contained 200 g monensin/kg in a carrier of ground corn and mineral oil to deliver a targeted monensin feeding rate of 328 mg/d and postpartum Rumensin mix contained 29 g monensin/kg in a carrier of ground corn and mineral oil to deliver a targeted monensin feeding rate of 395 mg/d (Elanco Animal Health, Greenfield, IN)

Table 4- 2. Analyzed chemical composition and predicted nutrient composition of experimental prepartum diets and the common postpartum diet (mean ± S.D.)[1]

	Prepartum Diet[2]			
	CON	MED	LOW	Postpartum Diet
Analyzed Composition				
DM, %	45.3 ± 1.5	45.7 ± 1.7	45.5 ± 1.5	44.9 ± 2.2
CP, % of DM	13.0 ± 0.3	13.2 ± 0.4	13.2 ± 0.4	15.7 ± 0.2
ADF, % of DM	30.2 ± 0.6	30.5 ± 1.2	30.1 ± 1.2	20.6 ± 0.8
NDF, % of DM	44.3 ± 1.1	44.0 ± 2.0	43.2 ± 1.6	31.1 ± 0.9
Starch, % of DM	17.0 ± 0.4	16.0 ± 0.7	16.3 ± 0.8	26.0 ± 0.7
Sugar, % of DM	2.7 ± 1.2	3.3 ± 0.8	3.0 ± 1.0	3.1 ± 1.3
NFC, % of DM	33.6 ± 0.8	34.3 ± 2.3	35.0 ± 1.8	45.8 ± 1.1
Fat, % of DM	1.9 ± 0.5	1.9 ± 0.1	2.0 ± 0.1	3.0 ± 0.2
Ca, % of DM	1.54 ± 0.10	1.57 ± 0.13	1.57 ± 0.06	0.95 ± 0.03
P, % of DM	0.44 ± 0.00	0.43 ± 0.01	0.41 ± 0.01	0.41 ± 0.02
Mg, % of DM	0.47 ± 0.01	0.48 ± 0.02	0.50 ± 0.03	0.44 ± 0.02
K, % of DM	1.28 ± 0.06	1.26 ± 0.06	1.24 ± 0.07	1.37 ± 0.05
S, % of DM	0.20 ± 0.00	0.30 ± 0.02	0.41 ± 0.02	0.29 ± 0.01
Na, % of DM	0.13 ± 0.01	0.13 ± 0.01	0.14 ± 0.00	0.44 ± 0.02
Cl, % of DM	0.27 ± 0.02	0.47 ± 0.05	0.69 ± 0.04	0.40 ± 0.02
DCAD, mEq/100 g DM[3]	18.3 ± 0.7	5.9 ± 3.1	-7.4 ± 3.3	25.0 ± 1.4
Predicted Composition				
NE_L, Mcal/kg[4]	1.47 ± 0.03	1.48 ± 0.03	1.47 ± 0.04	1.61 ± 0.02
ME, Mcal/kg DM[5]	2.18	2.17	2.19	2.47
MP, g/kg DM[5]	92.6	91.9	91.0	114.7
Predicted Intake[5]				
MP, g/d	1259	1287	1201	2386
Vitamin A, KIU/d	163.2	168.0	158.4	120.6
Vitamin D, KIU/d	44.9	46.2	43.6	49.9
Vitamin E, IU/d	1886	1942	1831	6662

[1]Chemical composition was determined on 5 composites of the prepartum CON diet, 6 composites each of the MED and LOW prepartum diets and 8 composites of the postpartum diet. Dry matters are the mean of 18-21 weekly DM determinations on fresh prepartum ration samples and 30 weekly DM determinations for postpartum ration corrected for residual moisture in composite samples
[2]Dietary treatments: CON = low K ration with no anion supplementation (n = 30, DCAD = +18.3 mEq/100 g DM), MED = partial anion supplementation to a low K ration (n = 30, DCAD = +5.9 mEq/100 g DM) and LOW = anion supplementation to a low K ration to reach a targeted average urine pH between 5.5 and 6.0 (n = 29, DCAD = -7.4 mEq/100 g DM)
[3]DCAD = [(Na % of DM/0.023) + (K % of DM/0.039)] – [(S % of DM/0.016) + (Cl % of DM/0.0355)]
[4]Calculated from chemical composition (NRC, 2001)
[5]Predicted by CNCPS (v 6.1) based on composite forage analysis, average ingredient composition, and average DMI (prepartum CON =13.6, MED = 14.0, LOW = 13.2 kg/d, postpartum = 20.8 kg/d)

Table 4- 3. Descriptive statistics of the study population including the distribution of parity after parturition and the mean ± S. D. of the previous lactation 305-d mature equivalent milk production, BCS and BW at enrollment, and days on treatment diet

Variable	CON	MED	LOW	*P*-values
		Treatment[1]		
Entering parity				
2	17	16	15	0.73
3	5	8	9	
4+	8	6	5	
Previous 305 ME, kg	14,921 ± 1,843	14,882 ± 2,149	14,918 ± 1,552	1.00
BCS	3.38 ± 0.14	3.35 ± 0.16	3.34 ± 0.18	0.71
BW, kg	789 ± 67	796 ± 72	792 ± 71	0.94
Days on treatment	23.7 ± 5.2	22.7 ± 4.4	24.5 ± 4.4	0.35

[1]Treatments: CON = low K ration with no anion supplementation (n = 30, DCAD = +18.3 mEq/100 g DM), MED = partial anion supplementation to a low K ration (n = 30, DCAD = +5.9 mEq/100 g DM) and LOW = anion supplementation to a low K ration to reach a targeted average urine pH between 5.5 and 6.0 (n = 29, DCAD = -7.4 mEq/100 g DM)

Table 4- 4. Least squares means and standard errors for prepartum urine pH and weekly prepartum DMI, energy balance (EBAL), BW and BCS for cows fed decreasing DCAD levels prepartum

Variable[3]	Treatment[1]			SEM	P-values[2]		
	CON	MED	LOW		Linear[4]	Quadratic[4]	Trt×Wk
Urine pH[5]	8.22 ± 0.02	7.89 ± 0.03	5.96 ± 0.11		<0.0001	<0.0001	0.66
DMI, kg/d	13.6	14.0	13.2	0.2	0.16	0.01	0.54
DMI, % of BW	1.71	1.75	1.66	0.03	0.17	0.06	0.54
EBAL, Mcal/d[6]	4.4	5.0	3.7	0.3	0.13	0.02	0.59
BW, kg	806	805	803	3	0.41	0.99	0.48
BCS	3.37	3.40	3.36	0.02	0.71	0.13	0.06

[1]Treatments: CON = low K ration with no anion supplementation (n = 30, DCAD = +18.3 mEq/100 g DM), MED = partial anion supplementation to a low K ration (n = 30, DCAD = +5.9 mEq/100 g DM) and LOW = anion supplementation to a low K ration to reach a targeted average urine pH between 5.5 and 6.0 (n = 29, DCAD = -7.4 mEq/100 g DM)
[2]Interactions of parity group with treatment and week were tested but none had $P \leq 0.10$
[3]Weekly averages for DMI were calculated for the 3 wk prior to parturition after data were reduced to the 21 d prior to actual parturition, aside from urine pH, all other outcomes reflect weekly measures or calculations in the 3 wk prior to parturition
[4]Orthogonal contrasts were tested to determine the overall linear or quadratic effects of decreasing prepartum DCAD on outcomes
[5]Urine samples collected once in the week prior to treatment assignment and three times per week thereafter until parturition
[6]Calculated according to (NRC, 2001)

Table 4-5. Least squares means and standard errors for weekly postpartum DMI, energy balance (EBAL), BW and BCS for cows fed decreasing DCAD levels prepartum

| Variable[3] | Treatment[1] | | | SEM | P-values[2] | | |
	CON	MED	LOW		Linear[4]	Quadratic[4]	Trt×Wk
DMI, kg/d							
wk 1 to 3	20.2	20.9	21.3	0.5	0.09	0.80	0.32
wk 1 to 9	23.5	24.4	24.0	0.3	0.29	0.12	0.71
DMI, % of BW							
wk 1 to 3	2.88	2.98	3.07	0.06	0.04	0.90	0.45
wk 1 to 9	3.38	3.52	3.50	0.05	0.06	0.17	0.69
EBAL, Mcal/d[5]							
wk 1 to 3	-10.2	-10.0	-10.0	0.9	0.90	0.95	0.72
wk 1 to 9	-6.0	-5.3	-5.9	0.5	0.80	0.30	0.65
BW, kg							
wk 1 to 3	701	701	703	6	0.76	0.90	0.70
wk 1 to 9[6]	694	694	695	5	0.93	0.95	0.93
BCS							
wk 1 to 3	3.17	3.18	3.15	0.02	0.58	0.35	0.66
wk 1 to 9	3.05	3.06	3.02	0.02	0.39	0.32	0.94

[1]Treatments: CON= low K ration with no anion supplementation (n = 30, DCAD = +18.3 mEq/100 g DM), MED = partial anion supplementation to a low K ration (n = 30, DCAD = +5.9 mEq/100 g DM) and LOW = anion supplementation to a low K ration to reach a targeted average urine pH between 5.5 and 6.0 (n = 29, DCAD = -7.4 mEq/100 g DM)
[2]Interactions of parity group with treatment and week were tested and effects removed if $P > 0.10$, footnotes indicate interactions that remained in the model
[3]Weekly averages were calculated for DMI prior to analysis, all other outcomes represent weekly measures or calculations
[4]Orthogonal contrasts were tested to determine the overall linear or quadratic effects of decreasing prepartum DCAD on outcomes
[5]Calculated according to (NRC, 2001)
[6]$P < 0.05$ for parity by week interaction

Table 4-6. Least squares means and standard errors for weekly milk yield, composition, and milk production efficiency in wk 1 to 3 and wk 1 to 9 for cows fed decreasing DCAD levels prepartum

Variable[3]	Treatment[1]				P-values[2]		
	CON	MED	LOW	SEM	Linear[4]	Quadratic[4]	Trt×Wk
Milk yield, kg/d							
wk 1 to 3	40.8	42.4	43.9	1.0	0.03	1.00	0.46
wk 1 to 9	47.1	48.5	48.7	0.8	0.18	0.57	0.74
Fat, %							
wk 1 to 3	4.38	4.36	4.24	0.08	0.21	0.63	0.10
wk 1 to 9[6]	3.81	3.78	3.72	0.05	0.20	0.78	0.48
Fat, kg/d							
wk 1 to 3	1.74	1.81	1.87	0.06	0.13	0.99	0.58
wk 1 to 9	1.74	1.78	1.79	0.04	0.32	0.78	0.77
True protein, %							
wk 1 to 3	3.54	3.49	3.27	0.07	0.005	0.33	0.36
wk 1 to 9[5]	2.99	2.98	2.84	0.04	0.02	0.21	0.78
True protein, kg/d							
wk 1 to 3	1.36	1.42	1.42	0.04	0.21	0.57	0.09
wk 1 to 9	1.35	1.40	1.36	0.02	0.67	0.11	0.70
Lactose, %							
wk 1 to 3	4.64	4.67	4.69	0.03	0.25	0.94	0.38
wk 1 to 9	4.78	4.78	4.78	0.03	0.91	0.93	0.80
Lactose, kg/d							
wk 1 to 3	1.89	1.98	2.09	0.06	0.02	0.84	0.02
wk 1 to 9	2.24	2.31	2.33	0.04	0.13	0.66	0.07
Total solids, %							
wk 1 to 3	13.63	13.61	13.27	0.10	0.01	0.20	0.10
wk 1 to 9	12.51	12.51	12.28	0.07	0.02	0.18	0.72
Total solids, kg/d							
wk 1 to 3	5.42	5.65	5.86	0.17	0.06	0.96	0.13
wk 1 to 9[6]	5.76	5.94	5.93	0.10	0.23	0.47	0.39
ECM, kg/d[7]							
wk 1 to 3	46.2	48.1	49.6	1.3	0.08	0.88	0.42
wk 1 to 9[6]	48.3	49.6	49.6	0.8	0.27	0.51	0.76
ECM/DMI							
wk 1 to 3	2.31	2.34	2.38	0.07	0.52	0.99	0.79
wk 1 to 9[6]	2.08	2.06	2.11	0.03	0.47	0.46	0.56
MUN, mg/dL							
wk 1 to 3	10.32	9.72	9.44	0.30	0.04	0.67	0.17
wk 1 to 9[6]	10.82	10.23	10.16	0.25	0.06	0.38	0.44
SCS							
wk 1 to 3	2.62	3.26	2.73	0.25	0.75	0.06	0.27
wk 1 to 9	2.11	2.66	2.43	0.26	0.38	0.22	0.20

[1]Treatments: CON = low K ration with no anion supplementation (n = 30, DCAD = +18.3 mEq/100 g DM), MED = partial anion supplementation to a low K ration (n = 30, DCAD = +5.9 mEq/100 g DM) and LOW = anion supplementation to a low K ration to reach a targeted average urine pH between 5.5 and 6.0 (n = 29, DCAD = -7.4 mEq/100 g DM)

[2]Interactions of parity group with treatment and week were tested and removed from the model when $P > 0.10$, footnotes indicate interactions that remained in the model

[3]Weekly averages for milk yield were calculated prior to analysis, all other outcomes represent weekly measures or calculations
[4]Orthogonal contrasts were tested to determine the overall linear or quadratic effects of decreasing prepartum DCAD on outcomes
[5]$P < 0.05$ for treatment by parity interaction
[6]$P < 0.10$ for parity by wk interaction
[7]ECM = (0.327 × kg of wk average milk yield) + (12.95 × kg of fat) + (7.65 × kg of true protein)

Table 4-7. Incidence of health disorders as identified by farm personnel

Disorder[2]	Treatment[1]		
	CON	MED	LOW
Intravenous Ca treatment[3]	3	1	2
Retained placenta	5	5	1
Hyperketonemia	9	9	9
Mastitis	2	4	3
Metritis	2	2	3

[1]Treatments: CON = low K ration with no anion supplementation (n = 30, DCAD = +18.3 mEq/100 g DM), MED = partial anion supplementation to a low K ration (n = 30, DCAD = +5.9 mEq/100 g DM) and LOW = anion supplementation to a low K ration to reach a targeted average urine pH between 5.5 and 6.0 (n = 29, DCAD = -7.4 mEq/100 g DM)
[2]Identification of health disorders was as follows: intravenous Ca treatment = lethargy, muscle weakness or recumbency, treated with 500 mL 23% calcium gluconate intravenously (MWI Veterinary Supply Co., Boise, ID); retained placenta = retention of fetal membranes for > 24 h; hyperketonemia = urine ketones were measured daily for 10 d postpartum (Ketostix, Bayer Corporation, Elkhart, IN), cows with urine ketones measuring "small" or greater for two consecutive days were treated with 300 mL of oral propylene glycol for 5 d; mastitis = abnormal milk or an inflamed quarter; metritis = watery, fetid brown uterine discharge with or without systemic signs
[3]One cow in the control group that received intravenous Ca treatment was given this in conjunction with treatment of retained placenta, it is unclear if she was displaying clinical signs of hypocalcemia

Table 4-8. Least squares means (± standard error) or geometric means (back transformed 95% confidence limits) for prepartum and postpartum plasma glucose, non-esterified fatty acids (NEFA), and BHB for cows fed decreasing DCAD levels prepartum

Variable[3]	Treatment[1]			P-values[2]		
	CON	MED	LOW	Linear[4]	Quadratic[4]	Trt×Day
Glucose, mg/dL						
Prepartum	58.7 ± 0.5	58.7 ± 0.5	58.0 ± 0.5	0.35	0.58	0.29
Postpartum	49.5 ± 0.8	50.0 ± 0.8	48.5 ± 0.8	0.41	0.33	0.45
NEFA, µEq/L[5]						
Prepartum	179 (163-199)	185 (168-204)	196 (178-217)	0.20	0.83	0.57
Postpartum	541 (495-591)	602 (552-658)	585 (534-640)	0.23	0.21	0.20
BHB, mg/dL[5]						
Prepartum	5.1 (4.8-5.4)	4.7 (4.4-5.0)	4.7 (4.5-5.0)	0.11	0.24	0.13
Postpartum	7.8 (7.2-8.5)	7.9 (7.3-8.5)	8.2 (7.5-8.9)	0.47	0.80	0.16

[1]Treatments: CON = low K ration with no anion supplementation (n = 30, DCAD = +18.3 mEq/100 g DM), MED = partial anion supplementation to a low K ration (n = 30, DCAD = +5.9 mEq/100 g DM) and LOW = anion supplementation to a low K ration to reach a targeted average urine pH between 5.5 and 6.0 (n = 29, DCAD = -7.4 mEq/100 g DM)
[2]Interactions of parity group with treatment and week were tested but none had $P \leq 0.10$
[3]Blood samples collected once in the week prior to treatment assignment, twice per week during the treatment period, twice in the 24 h after parturition, daily through 5 DIM, and three times weekly through 21 DIM
[4]Orthogonal contrasts were tested to determine the overall linear or quadratic effects of decreasing prepartum DCAD on outcomes
[5]Geometric means and back transformed 95% confidence limits

Figure 4- 1. Least squares means and standard errors for weekly DMI (kg/d) for cows fed decreasing levels of DCAD beginning 24 d prior to expected parturition. Treatments: CON = low K ration with no anion supplementation (n = 30, DCAD = +18.3 mEq/100 g DM), MED = partial anion supplementation to a low K ration (n = 30, DCAD = +5.9 mEq/100 g DM) and LOW = anion supplementation to a low K ration to reach a targeted average urine pH between 5.5 and 6.0 (n = 29, DCAD = -7.4 mEq/100 g DM). An overall quadratic effect of decreasing prepartum DCAD was found for prepartum DMI ($P = 0.01$) with no treatment by wk effect ($P = 0.54$). There was a trend for a linear increase in DMI in wk 1 to 3 with decreasing DCAD ($P = 0.09$) with no treatment by wk effect ($P = 0.32$). No overall effect on DMI over wk 1 to 9 was observed (quadratic $P = 0.12$) as well as no treatment by wk effect ($P = 0.71$).
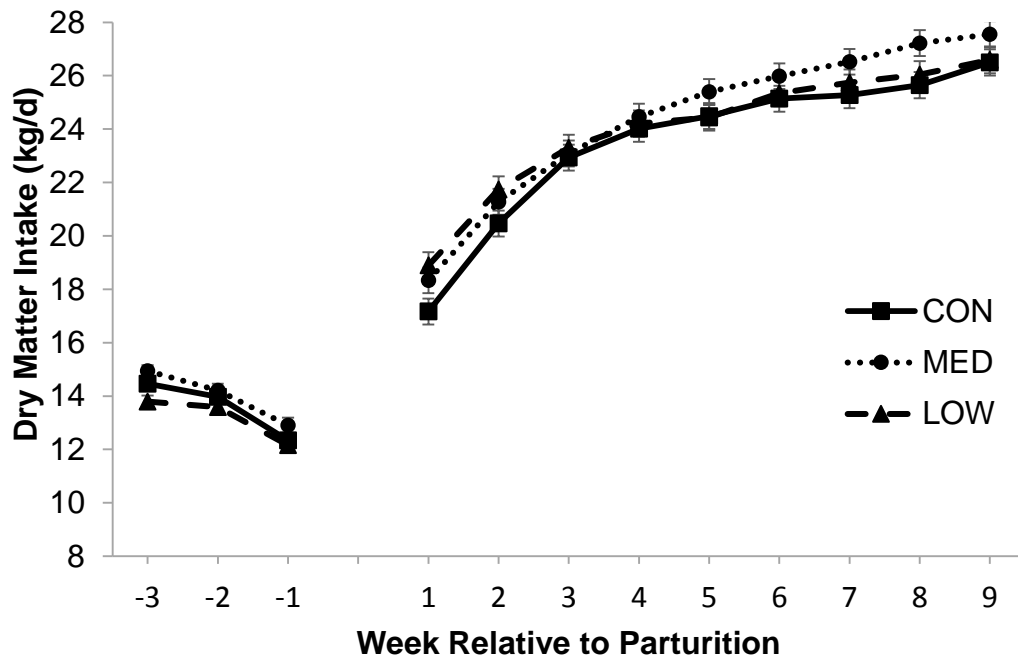
Figure 4- 2. Least squares means and standard errors for weekly average milk yield (kg/d) for cows fed decreasing levels of DCAD beginning 24 d prior to expected parturition. Treatments: CON = low K ration with no anion supplementation (n = 30, DCAD = +18.3 mEq/100 g DM), MED = partial anion supplementation to a low K ration (n = 30, DCAD = +5.9 mEq/100 g DM) and LOW = anion supplementation to a low K ration to reach a targeted average urine pH between 5.5 and 6.0 (n = 29, DCAD = -7.4 mEq/100 g DM).   An overall linear effect of decreasing prepartum DCAD was found for milk yield in wk 1 to 3 ($P = 0.03$) with no treatment by wk effect ($P = 0.46$). Effects on milk yield from wk 1 to 9 were not significant (linear $P = 0.18$) with no treatment by wk effect ($P = 0.74$).
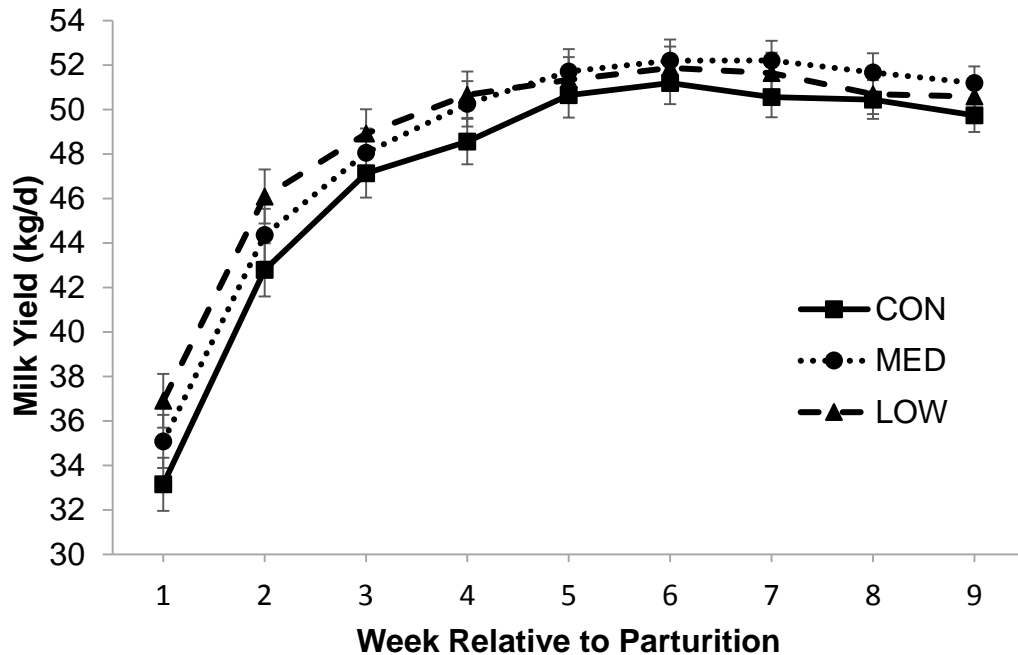
Figure 4- 3. Least squares means and standard errors for plasma glucose concentrations (Panel A; mg/dL), and geometric means and back transformed confidence limits for non-esterified fatty acids (NEFA; Panel B; μEq/L) and BHB (Panel C; mg/dL) for cows fed decreasing levels of DCAD beginning 24 d prior to expected parturition. Treatments: CON = low K ration with no anion supplementation (n = 30, DCAD = +18.3 mEq/100 g DM), MED = partial anion supplementation to a low K ration (n = 30, DCAD = +5.9 mEq/100 g DM) and LOW= anion supplementation to a low K ration to reach a targeted average urine pH between 5.5 and 6.0 (n = 29, DCAD = -7.4 mEq/100 g DM). No significant overall effects on prepartum glucose (linear $P = 0.35$), NEFA (linear $P = 0.20$), or BHB (linear $P = 0.11$) or postpartum glucose (quadratic $P = 0.33$), NEFA (quadratic $P = 0.21$), or BHB (linear $P = 0.47$) were observed. There were no interactions of treatment with time.

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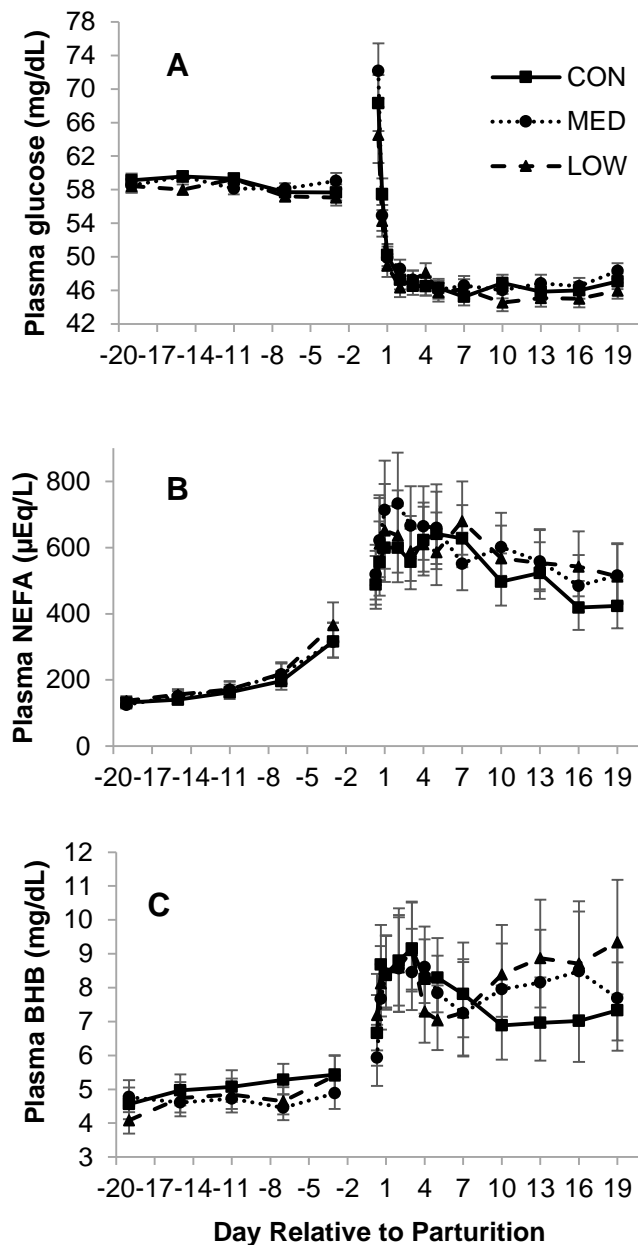
DISCUSSION

The primary hypothesis of this trial was that feeding decreasing DCAD prepartum would result in linear increases in blood Ca status postpartum and subsequently increase DMI and milk yield. Feeding decreasing DCAD in this trial resulted in linear increases in blood Ca in the postpartum period and is discussed more thoroughly in Chapter 5. Consistent with our hypotheses, milk production in the first 3 wk after parturition was increased and DMI also tended to be increased in this period for cows fed decreasing DCAD during the prepartum period. The association between blood Ca status and milk production in the literature is variable. Hypocalcemia after parturition has been associated with reductions in first test day milk production (Chapinal et al., 2012) which could be mediated by an increased susceptibility of hypocalcemic cows to infectious diseases and metabolic disorders (Chapinal et al., 2011; Martinez et al., 2012), limiting those cow's ability to reach their productive potential. In another study, hypocalcemic cows were matched with normocalcemic cows based on parity and occurrence of health disorders, which would control for detrimental effects of increased diseased susceptibility in cows with SCH, and milk production was higher in hypocalcemic cows in early lactation (Jawor et al., 2012). Further, Oetzel and Miller (2012) found that cows with a higher herd rank in previous lactation milk production benefited from oral Ca bolus supplementation shortly after parturition and responded with higher first test day milk yield. This suggests that higher producing cows may be more susceptible to hypocalcemia, potentially due to a greater Ca drain for colostrum and milk production. By alleviating hypocalcemia in cows with high production potential, a decrease in disease susceptibility may remove restraints that would otherwise inhibit that cow from meeting her potential.

The relationship between blood Ca status and DMI may be more direct, as induction of hypocalcemia has been shown to reduce abomasal and rumen motility (Daniel, 1983) as well as rumen contraction rate and DMI (Martinez et al., 2014). In this trial, facilitating a rapid recovery in blood Ca, and sustaining higher blood Ca concentrations for the first 14 DIM in cows fed LOW (Chapter 5), may have contributed to greater gut motility allowing for higher DMI after parturition. The additional intake observed in cows fed LOW likely supported the higher milk production, allowing them to meet their potential without negative effects on metabolic health.

Early work showed promising increases in milk production for cows fed a negative DCAD diet (Block, 1984), however, the control group had a high incidence of clinical hypocalcemia (47%). For trials in which the incidence of clinical disorders was not dramatically different between control and negative DCAD fed cows, the influence of DCAD feeding on postpartum performance has been variable. However, a meta-analysis conducted by Lean et al. (2014), which included 15 studies and 34 treatments, observed an overall effect on milk or fat-correct milk yield in multiparous cows of 1.15 L per day over the first 65 DIM. Those authors also noted the significant heterogeneity in response between studies. The opportunity for improved postpartum performance is likely partially dependent on maintenance of DMI prepartum for cows fed negative DCAD diets as well as positive effects on blood Ca status. For trials in which cows were fed diets to achieve average urine pH similar to that of cows fed LOW in the current trial, and in which prepartum DMI was not compromised, similar increases in postpartum performance were detected (DeGroot et al., 2010; Weich et al., 2013). Blood Ca responses in these trials were inconsistent. Numerical differences were observed in the study by DeGroot et al. (2010) in which cows were fed one of three negative DCAD diets (-10 to -12 mEq/100 g DM) compared to a positive DCAD control, however, the small sample size may

have limited their ability to detect statistical significance. In the study by Weich et al. (2013), cows fed a negative DCAD (-16 mEq/100 g DM) diet for 21 or 42 d prior to parturition both had improved performance compared to a positive DCAD fed group, however, only cows fed a negative DCAD diet for 42 d prepartum responded with higher postpartum blood Ca concentrations. In other work in which postpartum performance responses were not detected, study limitations could include insufficient alterations in acid-base balance and subsequently minimal blood Ca response (Ramos-Nieves et al., 2009), compromised prepartum DMI and exacerbated negative energy balance which may have limited the blood Ca response (Joyce et al., 1997), or lack of statistical power to detect differences in postpartum performance when blood Ca responses were evident and prepartum DMI was not compromised (Moore et al., 2000). The linear effects on postpartum performance in the current study suggested that increases in intake and milk production were greatest for the lowest DCAD diet, similar to the numerical differences observed in milk production by Moore et al. (2000), in which cows were fed similar treatment strategies to the current trial (DCAD = +15, 0 or -15 mEq/100 g DM) with the exception of increasing ration Ca concentrations with decreasing DCAD (Ca = 0.44, 0.97, 1.50% of DM).

The milk production response observed in wk 1 to 3 was not sustained throughout the 63 d observation period in this study. Numerically, cows fed LOW produced an average of 1.6 kg/d more over that period, but the difference was not statistically significant (linear $P = 0.18$). One potential reason for this could be that overall, this population of cows was healthy compared to industry standards. Hyperketonemia was identified by urine ketone monitoring in 30% of cows ($n = 27/89$), which is in the lower range of hyperketonemia incidence identified (26 – 56%) with intensive monitoring in the study by McArt et al. (2011). Prompt treatment of all hyperketonemic cows with oral propylene glycol in the current study likely mitigated negative downstream

energetic effects of the disorder. Zero displaced abomasums were diagnosed in this trial

compared to the average incidence of 3.6% in the cow-level cohort study on 55 herds by

Chapinal et al. (2011). The same study found average incidence of metritis of 16.7% compared

to 7.6% (n = 7/89) in the current trial. Retained placenta incidence was 12.4% (n=11/89) which

was higher than the average incidence of 7.4% observed by Chapinal et al. (2011).

Early work on low DCAD feeding consistently showed depressions in DMI during the

prepartum period for negative DCAD rations as summarized in the meta-analysis by

Charbonneau et al. (2006). More recently, the combination of minimizing basal ration dietary

cations to reduce the amount of supplemental anions that need to be included in the ration, as

well as the adoption of more palatable commercial anion sources, has resulted in several trials in

which anions were supplemented to reach urine pH values close to the range of the current trial

with no effect on prepartum intake (Moore et al., 2000; DeGroot et al., 2010; Weich et al., 2013;

Weiss et al., 2015), or minimal effects that did not result in evidence of impaired metabolic

health (Ramos-Nieves et al., 2009). The quadratic effect on prepartum intake in this trial

suggested that cows fed MED had the highest intake in the prepartum period with the lowest

intake in cows fed LOW. Absolute differences between groups were relatively small and there

were no effects of treatment on peripartum plasma concentrations of glucose, NEFA or BHB.

This suggests that any effect on DMI due to greater anion supplementation was not detrimental

to metabolic health.

Due to the association between the presence of SCH and increased concentrations of

blood NEFA (Chapinal et al., 2011; Martinez et al., 2012; Chamberlin et al., 2013) and BHB

(Martinez et al., 2012) in the early postpartum period, it was hypothesized that preventing

hypocalcemia by decreasing DCAD in the prepartum diet in this trial would improve metabolic

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health and decrease blood concentrations of NEFA and BHB. Our data suggest that cows fed

lower DCAD diets consumed more feed which supported more milk production, but did not

affect plasma glucose, NEFA or BHB. Other trials in which similar increases in postpartum

intake and milk production were found for cows fed a negative DCAD diet did not observe

differences in peripartum glucose and NEFA (DeGroot et al., 2010; Weich et al., 2013). In both

of those trials, postpartum BHB concentrations were lower in multiparous cows fed a negative

DCAD diet, however, this difference was not observed in the current trial.

Interesting effects of treatment on aspects of protein metabolism were observed in this

trial. Cows fed decreasing DCAD had linear decreases in both milk protein percentage and

MUN concentration throughout the 63 d observation period. One potential connection between

DCAD feeding and protein metabolism is the requirement for additional nitrogenous compounds

to support the excretion of acid as ammonium in the urine, especially when urine pH falls below

6.3 (Constable et al., 2009), which was the case in cows fed LOW. Glutamine, a non-essential

amino acid, particularly has an important role in ammoniagenesis and the excretion of acid in

urine during metabolic acidosis and this has been reviewed previously (Welbourne, 1987;

Patience, 1990). Glutamine also plays a role in ureagenesis and in vitro work using hepatocytes

from normal versus acidotic rats with radiolabeled Gln has illustrated that acidosis decreases Gln

uptake by hepatocytes and decreases the synthesis of urea in the liver from Gln (Nissim et al.,

1993). In experimentally induced acidosis in sheep, Gln transport through the kidney has been

shown to shift from net addition to the bloodstream to net uptake, while the liver decreases

uptake, suggesting prioritization to acid-base homeostasis (Heitmann and Bergman, 1980).

Additionally, uptake of Gln by the mammary gland is high relative to other essential and

nonessential amino acids (Hanigan et al., 1991; Mackle et al., 2000) and Gln constitutes a

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relatively large component of the milk protein casein (Barry, 1956). Glutamine has previously been described in the literature as "conditionally limiting" during early lactation (Meijer et al., 1993), and concentrations of Gln in plasma and muscle of dairy cows dramatically declines from late pregnancy to lactation, and persists until at least 15 wk after parturition (Meijer et al., 1995), which is a pattern unique to Gln compared to other non-essential amino acids. One hypothesis is that during the metabolic acidosis induced in negative DCAD feeding, Gln is prioritized to the kidney for acid excretion and as a result, the already limited amino acid is in short supply postpartum for ureagenesis in the liver and protein synthesis in the mammary gland, decreasing concentrations of both urea and protein in milk. Effects on milk protein percentage were also observed in the study by DeGroot et al. (2010) in which milk protein percentage was lower for one source of anions compared to two other anion sources, and average urine pH was 5.93 in that group compared to 6.5 or higher in the other groups. Other trials with similar experimental approaches either do not report milk protein percentage and MUN (Moore et al., 2000; DeGroot et al., 2010) or found no effects (Ramos-Nieves et al., 2009). Further work should be done to investigate the relationships between acid-base balance and amino acid metabolism in periparturient cows.

CONCLUSIONS

Decreasing the DCAD of the prepartum ration in this trial resulted in linear increases in DMI and milk production in the early postpartum period. Minimal effects on prepartum DMI were found and no effects on markers of energy metabolism. These results suggest that managing the prepartum DCAD to maintain an average urine pH between 5.5 and 6.0 will result in additional benefits in postpartum DMI and milk yield relative to either no supplementation (urine pH 8.22) or a lower level of anion inclusion (urine pH 7.89).

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CHAPTER 5

DIETARY CATION-ANION DIFFERENCE OF THE PREPARTUM DIET: PART II. EFFECTS ON PREPARTUM URINE PH AND URINE MINERAL EXCRETION AND PERIPARTUM PLASMA MINERAL CONCENTRATIONS IN MULTIPAROUS HOLSTEIN COWS

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ABSTRACT

The objectives of the current study were to determine the effects of linearly decreasing the prepartum dietary cation-anion difference [**DCAD** = $(Na^+ + K^+) - (Cl^- + S^{2-})$] on urine pH and aspects of mineral metabolism in the transition period. Multiparous Holstein cows (n = 89) were enrolled between -38 and -31 d relative to expected parturition and randomized to 1 of 3 prepartum rations (restricted to balance for previous 305-d mature equivalent milk production, parity and body condition score) at -24 d prior to expected parturition. Treatments were as follows: CON = low K ration with no anion supplementation (n = 30, DCAD = +18.3 mEq/100 g DM), MED = partial anion supplementation to a low K ration (n = 30, DCAD = +5.9 mEq/100 g DM) and LOW = anion supplementation to a low K ration to reach a targeted average urine pH between 5.5 and 6.0 (n = 29, DCAD = -7.4 mEq/100 g DM). Cows were fed a common postpartum ration and data collected through 63 d in milk. Urine pH (CON = 8.22 ± 0.02, MED = 7.89 ± 0.03, and LOW = 5.96 ± 0.11) and urine Ca:creatinine excretion ratio [geometric mean (back transformed 95% confidence limits); CON = 0.03 (0.02-0.04), MED = 0.07 (0.06-0.09), and LOW = 0.33 (0.27-0.40)] responded to a linear decrease in prepartum DCAD in a quadratic manner. This suggests that urinary compensation of the acid-base disturbance due to diet, as well as increases in urinary Ca flux, becomes more pronounced below a certain DCAD threshold. A linear relationship between urine pH and urine Ca:creatinine ratio also was observed (r = -0.81; *P* <0.0001). Estimated daily excretion of Ca in cows fed LOW was 8.2 g/d. Plasma Ca concentrations in the postpartum period (d 0 to 14; CON = 2.16, MED = 2.19, and LOW = 2.27 mmol/L) were increased linearly with decreasing prepartum DCAD. A treatment by parity (2nd vs. 3rd and greater) interaction for plasma Ca concentrations demonstrated that older cows had the greatest response to feeding the lower DCAD prepartum diet. Older cows fed LOW had a

reduced prevalence of hypocalcemia (plasma Ca < 2.125 mmol/L) at 0.6, 1 and 2 DIM. The results of this study demonstrate that feeding lower DCAD diets prepartum improves plasma Ca status in the immediate postpartum period and that lower DCAD feeding strategies in which an average urine pH of 5.5 to 6.0 is targeted result in greater improvements.

INTRODUCTION

An increase in Ca demand as dairy cows transition from late gestation, the relative nadir in Ca demand in the cow's life cycle (House and Bell, 1993), to early lactation when Ca demand dramatically increases (Ramberg et al., 1970; Kehoe et al., 2007), presents a metabolic challenge that results in subclinical disease in many cows. Subclinical hypocalcemia (**SCH**), a disorder in which blood Ca concentrations decrease but clinical signs of hypocalcemia are not displayed, affects at least 40-60% of older cows (Reinhardt et al., 2011; Caixeta et al., 2015; Martinez et al., 2016). More recent data suggest higher blood Ca thresholds (2.1 to 2.2 mmol/L) are relevant (Chapinal et al., 2012; Martinez et al., 2012), thus the population of cows affected by SCH is likely greater than these previous estimates. Due to negative associations of low blood Ca with DMI and energy metabolism (Daniel, 1983; Martinez et al., 2012; Chamberlin et al., 2013) and aspects of immune cell function (Kimura et al., 2006; Martinez et al., 2012), SCH is recognized as a costly disorder.

Feeding a negative DCAD in the prepartum period results in some degree of metabolic acidosis and, through alterations in acid-base balance, prevents excessive severity or duration of the decrease in blood Ca around parturition. Previously identified mechanisms for prevention of hypocalcemia with decreasing prepartum DCAD include increased Ca flux prepartum by increasing urinary Ca excretion (Fredeen et al., 1988; Grunberg et al., 2011) and restoring sensitivity of tissues to parathyroid hormone (**PTH**) stimulation which is impaired during

alkalotic states (Goff et al., 1991; Goff et al., 2014). These mechanisms may result in greater bone resorption or increased efficiency of intestinal Ca absorption (Fredeen et al., 1988; Takagi and Block, 1991; Goff and Horst, 1997) or both in parts of the transition period depending on the degree of metabolic acidosis imposed. Group level responses to manipulation of the prepartum DCAD have been demonstrated in both blood Ca concentrations (Joyce et al., 1997; Moore et al., 2000; Weich et al., 2013) and production outcomes (DeGroot et al., 2010; Weich et al., 2013).

Optimal prepartum DCAD level has not been established. A meta-analysis by Charbonneau et al. (2006) suggested that reducing DCAD resulted in linear decreases in DMI, discouraging more aggressive DCAD feeding strategies that would severely alter acid-base status. Since the publication of that meta-analysis, several studies have reduced urine pH to less than 6.5 without negatively impacting DMI (Moore et al., 2000; DeGroot et al., 2010; Weich et al., 2013; Weiss et al., 2015). Charbonneau et al. (2006) also described a curvilinear relationship between DCAD and urine pH, suggesting that some threshold in DCAD must be reached before significant alterations in acid-base balance occur. Evidence suggests that sensitivity of target tissues to PTH stimulation is impaired when cows experience some degree of metabolic alkalosis and restoration of PTH sensitivity has been demonstrated at a relatively low (< 6.0) urine pH (Goff et al., 2014). However, the critical threshold for acid-base balance at which PTH sensitivity is restored has not been identified. The excretion of Ca in urine has also been shown to increase as urine pH decreases (Grunberg et al., 2011; Weiss et al., 2015). Together, this indicates that more significant alterations in acid-base balance, as evidenced by lower urine pH, may have greater potential impacts on Ca metabolism.

Opportunity exists to determine the level of prepartum DCAD that results in the greatest improvements in blood Ca in the transition period to ultimately balance the health and

productivity of the cows with costs and management effort required for the level of DCAD implemented. The objectives of the current trial were to determine the relative effect of linearly decreasing the DCAD of the prepartum diet, starting with a low K control ration without the addition of an anionic supplement, on aspects of acid-base balance and mineral metabolism. We hypothesized that decreasing the prepartum DCAD would result in decreased prepartum urine pH, increased prepartum urinary Ca excretion, and increased plasma Ca concentrations postpartum.

MATERIALS AND METHODS

Study Population, Experimental Design, and Treatments

All animal handling and procedures were approved by the Cornell University Institutional Animal Care and Use Committee. Cows were enrolled in the study from March through August 2014. A more thorough description of the experimental design, study population and dietary treatments are presented in the companion paper (Leno et al., submitted). Briefly, the study was conducted as a completely randomized design with randomization restricted to balance for parity group (2nd vs. 3rd +), previous lactation 305-d mature equivalent milk production and BCS at enrollment. The final dataset included 89 multiparous Holstein cows (CON; n = 30, MED; n = 30, LOW; n = 29). Prepartum dietary treatment groups and analyzed ration DCAD [DCAD mEq/100 g DM = $(Na^+ + K^+) - (Cl^- + S^{2-})$] were as follows; low K ration with no supplemental anions (CON; K = 1.28% of DM, DCAD = +18.3 mEq/100 g DM), partial anion supplementation to a low K ration (MED; K = 1.26% of DM, DCAD = +5.9 mEq/100 g DM) and anion supplementation to a low K ration to reach a targeted average urine pH between 5.5 and 6.0 (LOW; K = 1.24% of DM, DCAD = -7.4 mEq/100 g DM). Cows were moved to individual tie stalls and fed the CON ration between -38 and -31 d relative to expected

parturition. At -24 d relative to expected parturition cows either continued to be fed the CON ration or were fed MED or LOW until parturition, depending on treatment assignment.

Ingredient and analyzed nutrient composition of the experimental diets are presented in Table 4-1 and Table 4-2, respectively. A complete description of diet formulation, mixing strategy, sampling and analytical methods is presented in Chapter 4. Briefly, one batch of a base TMR was mixed for all prepartum treatment groups daily that contained wheat straw, brown mid-rib corn silage and a grain premix. Before delivery to the cows, the base ration was mixed with a small inclusion rate grain mix which was unique to each prepartum treatment group. Small inclusion rate grain mixes were formulated to supplement anions to the MED and LOW rations while maintaining similar concentration of all other nutrients. Throughout the duration of the trial, average urine pH of cows that had been fed their treatment diets for at least 5 d were monitored to determine the need for adjustment of the inclusion rate of these mixes. The targeted average urine pH for cows fed LOW was between 5.5 and 6.0. Equal adjustments were made to the inclusion rate in all three prepartum diets when adjustments were need to meet the urine pH target of the cows fed LOW and were compensated by corresponding changes in inclusion rates of wheat straw and corn silage. The inclusion rate of these mixes was adjusted 5 times over 5 months of dry cow feeding and ranged from 5.61 to 6.67% of DM.

When parturition was imminent, cows were moved to individual maternity pens. Within approximately 2 h, after parturition and collection of colostrum, cows were returned to individual tie stalls at which point all cows were fed a common postpartum diet until 63 DIM. Cows were observed daily for health disorders by farm personnel. To prevent the occurrence of recumbent cows, farm personnel were instructed to administer farm protocol treatment (500 mL 23% calcium gluconate intravenously, MWI Veterinary Supply Co., Boise, ID) when cows displayed

early signs of clinical hypocalcemia including lethargy, muscle weakness or other nervous signs. All data from cows treated with intravenous Ca remained in the data set (n = 3, 1 and 2 for CON, MED and LOW, respectively). No cows received supplemental Ca in the form of boluses, pastes or gels. Four cows calved with twins but were distributed across treatments (CON, n = 1; MED, n = 2; LOW, n = 1) and therefore remained in the dataset.

Individual Animal Sampling, Analytical Methods and Calculations

Urine samples were collected once during the week prior to assignment to treatment and three times per week thereafter until parturition at 1700 h (approximately 8 h after feed delivery). Midstream samples were collected following manual stimulation of the space below the vulva into a paper cup. Urine pH was determined immediately after collection with a transportable glass electrode pH meter (model UP-5 pH meter, Denver Instrument, Denver CO). The pH meter was calibrated with a 3 point (pH 4, 7 and 10) calibration each day prior to sampling. Urine samples were transferred to 12 mL polypropylene tubes, centrifuged at $2,000 \times g$ for 5 min to remove debris, aliquoted into 5-mL polypropylene tubes and stored at -20°C until analysis. One sample from the week prior to treatment assignment and one sample each from wk -3 to -1 were analyzed for creatinine, Ca and Mg concentrations at the Cornell University Animal Health and Diagnostic Center (Ithaca, NY) on an automated analyzer (Hitachi Modular P800, Roche Diagnostics, Indianapolis, IN). To account for dilution in urine, the ratio of Ca and Mg to creatinine in urine samples was calculated. Total daily excretion of Ca and Mg was estimated based on a daily creatinine excretion rate of 29 mg/kg of BW (Valadares et al., 1999).

Blood samples were collected via coccygeal venipuncture between 0600 h and 0730 h once during the week prior to assignment to treatment, twice weekly thereafter until parturition (Monday and Friday), twice in the 24 h after parturition (the next two timepoints occurring after

parturition with sampling occurring at approximately 0700 h and 1630 h), once daily through 5 DIM, and three times per week thereafter (Monday, Wednesday and Friday) through 56 DIM. Samples were collected using 10 mL sodium heparin evacuated tubes (158 USP, Becton Dickinson, Franklin Lakes, NJ) and 20 G vacutainer needles (Becton Dickinson, Franklin Lakes, NJ) and placed on ice immediately after collection. Additional information on harvesting of plasma and sample storage is presented in Chapter 4. One sample from the wk prior to treatment assignment, one sample each from wk -3 to -1, and all samples collected from parturition through 14 DIM were analyzed for Ca, Mg, and P concentrations at the Cornell University Animal Health and Diagnostic Center (Ithaca, NY) using the analyzer and reagents described above.

Statistical Analysis

Data were reduced to the 21 d prior to actual parturition prior to analysis. When applicable, samples were categorized to 3 day intervals based on actual day relative to parturition. Since one sample each of plasma and urine per week prepartum was analyzed for minerals, those samples were categorized to weekly intervals based on actual day relative to parturition. Multiple samples collected in the same interval were averaged prior to analysis. Prepartum and postpartum data were analyzed separately.

All statistical analyses were conducted with the statistical software SAS (version 9.4, SAS Institute Inc., Cary, NC). The correlation between the Ca:creatinine ratio and urine pH was analyzed with PROC CORR to determine the Pearson's Correlation Coefficient. All measurements taken at multiple timepoints were subjected to repeated measures analysis using the REPEATED statement in the MIXED procedure of SAS (Littell et al., 1996). The fixed effects of time, treatment, and parity group (2nd vs. 3rd+ lactation) and all two way interactions

were tested in the model. Parity interactions with $P > 0.10$ were removed from the model. Covariate measurements collected in the week prior to treatment assignment were included in all models. The Kenward Rogers method was used for estimation of denominator degrees of freedom. Four covariance structures were tested for each model and the model with the lowest Aikaike's Information Criterion was selected (Littell et al., 1996). For data with equal intervals over time the following covariance structures were tested; compound symmetry, heterogeneous compound symmetry, autoregressive order 1 and heterogeneous autoregressive order 1. For data with unequal intervals over time, antedependence 1 and unstructured covariance structures were tested in place of the autoregressive covariance structures. For all models, orthogonal contrast statements were included to determine the overall linear or quadratic effects of decreasing DCAD. When $P \leq 0.10$ for interaction terms, the SLICE option was used in the LSMEANS statement to conduct an F-test to determine at which levels of time or parity the treatment groups differed. Plots of studentized residuals were inspected for normality and homogeneity of variance. When non-normality of residual variance was evident (urine Ca:creatinine and estimated daily Ca excretion), data were log transformed and analysis repeated. The GROUP option in the repeated statement was used to control for heterogeneity of variance due to treatment when analyzing urine pH. Prevalence of hypocalcemia was calculated at two different thresholds (plasma Ca < 2.0 mmol/L and plasma Ca < 2.125 mmol/L) at each sampling timepoint from parturition through 5 DIM was tested using Fisher's Exact Test. Prevalence of hypocalcemia was analyzed separately for 2nd lactation cows and older cows due to a treatment by parity interaction for postpartum plasma Ca concentration. Least squares means and standard errors, or geometric mean and back transformed 95% confidence limits for data that were log

transformed, are reported throughout. Significance was declared at $P \leq 0.05$ and trends are discussed at $0.05 < P \leq 0.10$.

RESULTS

Prepartum Urine pH and Mineral Excretion

Urine pH, ratios of urinary Ca and Mg to creatinine and estimated daily excretion of Mg and Ca are presented in Table 5-3. An overall quadratic effect of decreasing DCAD on urine pH was observed (CON = 8.22 ± 0.02, MED = 7.89 ± 0.03, and LOW = 5.96 ± 0.11; $P < 0.0001$) with the lowest urine pH in cows fed LOW. The lack of a treatment by day effect ($P = 0.66$) or day effect ($P = 0.11$) indicated that urine pH was consistent within treatment group across the 21 d prior to parturition. When analyzed by average day after initiation of the treatment diet (data not shown), urine pH for cows fed LOW reached 6.56 ± 0.16 on average d 2 on treatment diet, and was within the targeted range at 5.91 ± 0.16 on average d 5 on treatment diet (treatment by day $P = 0.01$).

A quadratic effect of decreasing DCAD on urine Ca:creatinine was observed ($P = 0.009$) such that the ratio was highest for cows fed LOW. A treatment by wk effect was also observed for urinary Ca:creatinine ratio ($P = 0.0001$). Cows fed LOW had the highest Ca:creatinine ratio throughout the prepartum period, followed by cows fed MED, and the pattern across weeks differed for treatment groups (Figure 5-1). A similar quadratic effect ($P = 0.003$) on estimated urinary Ca excretion, and treatment by wk effect ($P < 0.0001$) was observed. Estimated daily urinary excretion of Ca [geometric mean (back transformed 95% confidence limits)] was 0.7 (0.6-0.9), 1.7 (1.4-2.0) and 8.2 (6.8-10.0) g/d for cows fed CON, MED and LOW, respectively. Urine Ca:creatinine ratio was highly correlated with urine pH (r = -0.81, $P < 0.0001$; Figure 5-2).

Urine Mg:creatinine ratio and estimated daily urinary Mg excretion were not affected by treatment (quadratic $P = 0.24$ and $P = 0.24$, respectively). An effect of week ($P < 0.0001$) for both Mg:creatinine and estimated daily urinary excretion of Mg indicated that Mg excretion decreased as parturition approached (Figure 5-1).

Peripartum Plasma Mineral Concentrations

Prepartum and postpartum plasma concentrations of Ca, Mg and P are presented in Table 5-4. There were no overall effects on prepartum plasma Ca concentrations (linear $P = 0.32$). Postpartum plasma Ca concentration was increased linearly by decreasing prepartum DCAD (CON = 2.16, MED = 2.19, and LOW = 2.27 mmol/L, $P = 0.002$; Figure 5-3A) and there were no interactions of treatment with time for prepartum or postpartum plasma Ca ($P = 1.00$ and $P = 0.49$, respectively). There were interactions of treatment with parity group for both prepartum and postpartum plasma Ca. When tested for effects of treatment within each parity group prepartum plasma Ca did not significantly differ for either second lactation (CON = 2.36, MED = 2.44, and LOW = 2.41 mmol/L, $P = 0.13$) or older cows (CON = 2.38, MED = 2.33, and LOW = 2.40 mmol/L, $P = 0.23$) but the pattern for treatment means differed within parity groups. Overall postpartum plasma Ca did not differ by treatment for second lactation cows (CON = 2.18, MED = 2.25, and LOW = 2.26 mmol/L, $P = 0.17$) but differed for older cows (CON = 2.14, MED = 2.13, and LOW = 2.28 mmol/L, $P = 0.001$).

There were no overall effects of treatment on prepartum (linear $P = 0.16$) or postpartum (linear $P = 0.29$) plasma Mg concentrations; however, postpartum plasma Mg differed for treatments by day (Figure 5-3B, $P = 0.03$). Plasma Mg concentrations were different at the first 3 sampling timepoints after parturition (0.3, 0.6 and 1 d postpartum, $P < 0.05$) with the highest concentrations in cows fed CON and the lowest concentrations in cows fed LOW. No overall

effects of treatment on prepartum (quadratic $P = 0.55$) or postpartum (linear $P = 0.11$) plasma P concentrations were observed and no interactions of treatment and day (Figure 5-3C).

Effects of Parity Group on Mineral Metabolism

Aside from plasma Ca concentrations, there were no interactions between parity group and treatment on measures of mineral metabolism. Therefore, overall effects of parity group on urine mineral excretion and plasma concentrations of Mg and P will be described herein and statistics for parity effects can be found in Table 5-3 and Table 5-4, respectively. The ratio of urine Ca:creatinine differed by parity group ($P = 0.01$), as did estimated daily Ca excretion, and was higher for second lactation cows compared to older cows [second lactation = 2.4 g/d (2.1 – 2.8), older = 1.9 g/d (1.6 – 2.2); $P = 0.03$]. There were no effects of parity on Mg:creatinine excretion ratio ($P = 0.25$) nor estimated daily Mg excretion ($P = 0.67$).

Interactions of parity group with day were observed for both prepartum ($P = 0.02$) and postpartum plasma Mg ($P = 0.0004$, Figure 5-4A). Prepartum plasma Mg was higher for second lactation cows at wk -2 relative to parturition (second lactation = 0.93, older = 0.89 mmol/L, $P = 0.03$) but plasma Mg declined in second lactation cows as parturition approached and did not differ from older cows at wk -1. Postpartum plasma Mg was higher in older cows through 1 DIM, but subsequently dropped below that of second lactation cows. Plasma P was affected by parity, and cows entering their second lactation had higher prepartum plasma P than older cows (second lactation = 2.0, older = 1.9 mmol/L, $P = 0.02$). There was no interaction of parity and day for prepartum plasma P and therefore least squares means are not presented by day. An interaction of parity and day for postpartum plasma P ($P = 0.03$) was observed such that second lactation cows had higher plasma P through 1 DIM and older cows had higher plasma P from 2 through 5 DIM, after which second lactation cows had higher plasma P (Figure 5-4B).

Prevalence of Hypocalcemia and Clinical Health Disorders

Prevalence of hypocalcemia by sampling timepoint is presented separately for second lactation cows and older cows for two different plasma Ca thresholds [plasma Ca $\leq$ 2.0 mmol/L (Reinhardt et al., 2011); plasma Ca $\leq$ 2.125 mmol/L (Martinez et al., 2016a)] in Figure 5-5. There were no differences in hypocalcemia prevalence for second lactation cows by treatment for either threshold. Peak hypocalcemia prevalence for second lactation cows occurred at 0.6 DIM with both thresholds (plasma Ca $\leq$ 2.0 mmol/L = 64%; plasma Ca $\leq$ 2.125 mmol/L = 81%). Hypocalcemia prevalence for older cows, categorized using a threshold of 2.0 mmol/L, differed by treatment at 0.3 DIM [CON = 92% (n = 11/12), MED = 100% (n = 14/14), and LOW = 64% (n = 9/14), P = 0.02] and 1 DIM [CON = 77% (n = 10/13), MED = 79% (n = 11/14), and LOW = 29% (n = 4/14), P = 0.01]. Hypocalcemia prevalence categorized with the higher threshold of 2.125 mmol/L tended to differ for older cows at 0.6 DIM [CON = 92% (n = 12/13), MED = 100% (n = 14/14), and LOW = 71% (n = 10/14), P = 0.07] and significantly differed by treatment at 1 DIM [CON = 92% (n = 12/13), MED = 100% (n = 14/14), and LOW = 57% (n = 8/14), P = 0.006] and at 2 DIM [CON = 69% (n = 9/13), MED = 64% (n = 9/14), and LOW = 14% (n = 2/14), P = 0.007].

Incidence of clinical health disorders, as identified by farm personnel, is more thoroughly presented in the Chapter 4. Statistical analysis of this data was not conducted due to insufficient sample size. Intravenous Ca treatment occurred in 3 cows fed CON (one in 2nd lactation in conjunction with retained placenta treatment, unclear if signs of clinical hypocalcemia were present, and two in 4th lactation), 1 cow fed MED (5th lactation) and 2 cows fed LOW (one in 5th lactation and one in 2nd lactation calving with twins).

Table 5- 1. Ingredient composition of the prepartum and postpartum diets (% of DM)

Ingredient (% of DM)	Prepartum Diet[1]			Postpartum Diet
	CON	MED	LOW	
Brown mid-rib corn silage	44.79	44.79	44.79	36.30
Wheat straw	28.06	28.06	28.06	7.85
Haylage	-	-	-	8.83
Amino Plus	7.98	7.98	7.98	7.06
Citrus pulp	3.25	3.25	3.25	3.92
Wheat midds	3.22	2.59	1.93	-
Ground corn grain	0.41	0.41	0.41	19.62
Corn gluten feed	-	-	-	3.92
Soybean hulls	2.26	2.26	2.26	-
Distillers grains, ethanol	2.19	1.31	0.39	1.96
Canola meal	2.16	2.16	2.16	5.89
Molasses	0.70	0.70	0.69	-
Urea	0.41	0.21	-	-
LysAAMet[3]	-	-	-	0.78
Megalac R[4]	-	-	-	0.39
Megamine L[4]	-	-	-	0.39
Alimet[5]	-	-	-	0.06
Animate[6]	-	1.95	3.99	-
Ca carbonate	3.18	3.09	3.00	-
Mono-dicalcium phosphate	0.47	0.47	0.47	-
Mg oxide	0.56	0.41	0.26	-
MIN-AD[7]	-	-	-	1.57
Sodium bicarbonate	-	-	-	0.78
Salt	0.24	0.24	0.24	0.39
Lactating mineral mix[8]	-	-	-	0.16
Selenium 0.06%	0.04	0.04	0.04	-
Dairy ADE mix[9]	0.04	0.04	0.04	-
1100 Dairy TM[10]	0.03	0.03	0.03	-
Vitamin E premix	0.02	0.02	0.02	0.06
Rumensin[11]	0.01	0.01	0.01	0.06

[1]Ration ingredient composition reflects a weighted average composition to account for changes in the inclusion rate (range = 5.61-6.67% of DM) of the small inclusion rate mix, compensated for by corn silage and wheat straw, throughout the trial. Dietary treatments: CON = low K ration with no anion supplementation (n = 30, DCAD = +18.3 mEq/100 g DM), MED = partial anion supplementation to a low K ration (n = 30, DCAD = +5.9 mEq/100 g DM) and LOW = anion supplementation to a low K ration to reach a targeted average urine pH between 5.5 and 6.0 (n = 29, DCAD = -7.4 mEq/100 g DM)

[2]Source of rumen bypass soybean meal (Ag Processing, Inc., Omaha, NE)

[3]Source of rumen protected DL-methionine and lysine hydrochloride (Perdue AgSolutions, LLC., Salisbury, MD)

[4]Megalac R = source of rumen bypass calcium salts of long chain fatty acids; Megamine L = source of rumen bypass L-Lysine monohydrochloride and calcium salts of long chain fatty acids (Church & Dwight Co., Inc., Ewing, NJ)

[5]Source of HMTBa which is a precursor for methionine (Novus International, Saint Charles, MO)

[6]Composed of 39.0% CP, 7.6% EE, 3.1% sugar, 5.8% starch, 23.5% NDF, 1.3% Ca, 0.5% P, 4.3% Mg, 5.5% S, 14.0% Cl (Phibro Animal Health, Corp., Quincy, IL)

[7]Source of supplemental Ca (21.5% of DM) and Mg (11.5% of DM) as dolomitic limestone (Papillon Agricultural Company, Inc., Easton, MD)

[8]Composed of 2.7% Ca, 12.6% K, 18.6% S, 25,560 ppm Zn, 7,154 ppm Cu, 21,958 ppm Mn, 214 ppm Se, 501 ppm Co, 331 ppm I, 3,704 KIU/kg vitamin A, 922 KIU/kg vitamin D, 12,496 KIU/kg vitamin E

[9]Composed of 19.9% Ca, 30,073 KIU/kg vitamin A, 5,783 KIU/kg vitamin D, 92,534 IU/kg vitamin E

[10]Composed of 18.4% S, 153,815 ppm Zn, 30,318 ppm Cu, 136,432 ppm Mn, 3,386 ppm Co, 3032 ppm I

[11]Prepartum Rumensin mix contained 200 g monensin/kg in a carrier of ground corn and mineral oil to deliver a targeted monensin feeding rate of 328 mg/d and postpartum Rumensin mix contained 29 g monensin/kg in a carrier of ground corn and mineral oil to deliver a targeted monensin feeding rate of 395 mg/d (Elanco Animal Health, Greenfield, IN)

Table 5- 2. Chemical composition of experimental prepartum diets and the common postpartum diet (mean ± S.D.)[1]

Nutrient	Prepartum Diet[2]			Postpartum Diet
	CON	MED	LOW	
DM, %	45.3 ± 1.5	45.7 ± 1.7	45.5 ± 1.5	44.9 ± 2.2
CP, % of DM	13.0 ± 0.3	13.2 ± 0.4	13.2 ± 0.4	15.7 ± 0.2
ADF, % of DM	30.2 ± 0.6	30.5 ± 1.2	30.1 ± 1.2	20.6 ± 0.8
NDF, % of DM	44.3 ± 1.1	44.0 ± 2.0	43.2 ± 1.6	31.1 ± 0.9
Starch, % of DM	17.0 ± 0.4	16.0 ± 0.7	16.3 ± 0.8	26.0 ± 0.7
Sugar, % of DM	2.7 ± 1.2	3.3 ± 0.8	3.0 ± 1.0	3.1 ± 1.3
NFC, % of DM	33.6 ± 0.8	34.3 ± 2.3	35.0 ± 1.8	45.8 ± 1.1
Fat, % of DM	1.9 ± 0.5	1.9 ± 0.1	2.0 ± 0.1	3.0 ± 0.2
Ca, % of DM	1.54 ± 0.10	1.57 ± 0.13	1.57 ± 0.06	0.95 ± 0.03
P, % of DM	0.44 ± 0.00	0.43 ± 0.01	0.41 ± 0.01	0.41 ± 0.02
Mg, % of DM	0.47 ± 0.01	0.48 ± 0.02	0.50 ± 0.03	0.44 ± 0.02
K, % of DM	1.28 ± 0.06	1.26 ± 0.06	1.24 ± 0.07	1.37 ± 0.05
S, % of DM	0.20 ± 0.00	0.30 ± 0.02	0.41 ± 0.02	0.29 ± 0.01
Na, % of DM	0.13 ± 0.01	0.13 ± 0.01	0.14 ± 0.00	0.44 ± 0.02
Cl, % of DM	0.27 ± 0.02	0.47 ± 0.05	0.69 ± 0.04	0.40 ± 0.02
DCAD, mEq/100 g DM[3]	18.3 ± 0.7	5.9 ± 3.1	-7.4 ± 3.3	25.0 ± 1.4
NE_L, Mcal/kg[4]	1.47 ± 0.03	1.48 ± 0.03	1.47 ± 0.04	1.61 ± 0.02
ME, Mcal/kg DM[5]	2.18	2.17	2.19	2.47
Predicted MP, g/kg DM[5]	92.6	91.9	91.0	114.7
Predicted MP Intake, g/d[5]	1259	1287	1201	2386

[1]Chemical composition was determined on 5 composites of the prepartum CON diet, 6 composites each of the MED and LOW prepartum diets and 8 composites of the postpartum diet. Dry matters are the mean of 18-21 weekly DM determinations on fresh prepartum ration samples

[2]Dietary treatments: CON = low K ration with no anion supplementation (n = 30, DCAD = +18.3 mEq/100 g DM), MED = partial anion supplementation to a low K ration (n = 30, DCAD = +5.9 mEq/100 g DM) and LOW = anion supplementation to a low K ration to reach a targeted average urine pH between 5.5 and 6.0 (n = 29, DCAD = -7.4 mEq/100 g DM)

[3]DCAD = [(Na % of DM/0.023) + (K % of DM/0.039)] − [(S % of DM/0.016) + (Cl % of DM/0.0355)]

[4]Calculated from chemical composition according to (NRC, 2001)

[5]Predicted by CNCPS (v 6.1) based on composite forage analysis, average ingredient composition, and average intakes (prepartum CON =13.6, MED = 14.0, LOW = 13.2, and postpartum = 20.8 kg/d)

Table 5- 3. Least squares means (± standard error) or geometric means (back transformed 95% confidence limits) for prepartum urine pH and urine mineral excretion for cows fed decreasing DCAD levels prepartum

Variable[3]	Treatment[1]			P-values[2]				
	CON	MED	LOW	Linear[4]	Quadratic[4]	Parity	Day	Trt×Day
Urine pH	8.22 ± 0.02	7.89 ± 0.03	5.96 ± 0.11	<0.0001	<0.0001	0.97	0.11	0.66
Ca:creatinine[5]	0.03 (0.02-0.04)	0.07 (0.06-0.09)	0.33 (0.27-0.40)	<0.0001	0.009	0.01	0.58	0.0001
Ca, g/d[5,6]	0.7 (0.6-0.9)	1.7 (1.4-2.0)	8.2 (6.8-10.0)	<0.0001	0.003	0.03	0.13	<0.0001
Mg:creatinine	0.38 ± 0.02	0.36 ± 0.02	0.39 ± 0.02	0.49	0.24	0.31	<0.0001	0.15
Mg, g/d[6]	8.9 ± 0.4	8.4 ± 0.4	9.1 ± 0.4	0.65	0.24	0.71	<0.0001	0.30

[1]Treatments: CON = low K ration with no anion supplementation (n = 30, DCAD = +18.3 mEq/100 g DM), MED = partial anion supplementation to a low K ration (n = 30, DCAD = +5.9 mEq/100 g DM) and LOW = anion supplementation to a low K ration to reach a targeted average urine pH between 5.5 and 6.0 (n = 29, DCAD = -7.4 mEq/100 g DM)

[2]Interactions of parity group with treatment and wk were tested but none had $P \leq 0.10$

[3]Urine samples collected once in the week prior to treatment assignment and 3×/wk thereafter until parturition with urine minerals determined on one sample per week

[4]Orthogonal contrasts were tested to determine the overall linear or quadratic effects of decreasing prepartum DCAD on outcomes

[5]Geometric means and back transformed 95% confidence limits

[6]Calculated based on daily urine creatinine excretion rate of 29 mg/kg of BW (Valadares et al., 1999)

Table 5- 4. Least squares means and standard errors for prepartum and postpartum plasma mineral concentrations for cows fed decreasing DCAD levels prepartum

Variable	Treatment[1]				P-values[2]				
	CON	MED	LOW	SEM	Linear[3]	Quadratic[3]	Parity	Day	Trt×Day
Prepartum[4]									
Ca, mmol/L[7,8]	2.37	2.39	2.40	0.02	0.32	0.96	0.13	0.01	1.00
Mg, mmol/L[8]	0.92	0.90	0.90	0.01	0.16	0.71	0.47	0.07	0.78
P, mmol/L	1.99	1.96	1.97	0.03	0.71	0.55	0.01	0.006	0.83
Postpartum[5]									
Ca, mmol/L[6]	2.16	2.19	2.27	0.02	0.002	0.35	0.03	<0.0001	0.49
Mg, mmol/L[8]	0.87	0.88	0.85	0.02	0.29	0.46	0.99	<0.0001	0.03
P, mmol/L[8]	1.54	1.50	1.46	0.04	0.11	0.93	0.92	0.002	0.87

[1]Treatments: CON = low K ration with no anion supplementation (n = 30, DCAD = +18.3 mEq/100 g DM), MED = partial anion supplementation to a low K ration (n = 30, DCAD = +5.9 mEq/100 g DM) and LOW = anion supplementation to a low K ration to reach a targeted average urine pH between 5.5 and 6.0 (n = 29, DCAD = -7.4 mEq/100 g DM)
[2]Interactions of parity group with treatment and wk were tested and footnotes indicate significant effects
[3]Orthogonal contrasts were tested to determine the overall linear or quadratic effects of decreasing prepartum DCAD on outcomes
[4]Samples collected 1×/wk from the week prior to treatment assignment until parturition
[5]Samples collected 2×/24 h after parturition, daily through 5 DIM, and 3×/wk through 14 DIM
[6]Trt×Parity P-value ≤ 0.05
[7]Trt×Parity P-value ≤ 0.10
[8]Parity×Day P-value ≤ 0.05

Figure 5- 1. Geometric means and back transformed 95% confidence limits for urine Ca:creatinine ratio (A) and least squares means and standard error for urine Mg:creatinine ratio (B) for cows fed decreasing levels of DCAD beginning 24 d prior to expected parturition. Treatments: CON= low K ration with no anion supplementation (n = 30, DCAD = +18.3 mEq/100 g DM), MED = partial anion supplementation to a low K ration (n = 30, DCAD = +5.9 mEq/100 g DM) and LOW = anion supplementation to a low K ration to reach a targeted average urine pH between 5.5 and 6.0 (n = 29, DCAD = -7.4 mEq/100 g DM). An overall quadratic effect of decreasing prepartum DCAD on urine Ca:creatinine ratio was found ($P = 0.009$) with a treatment by day interaction ($P = 0.0001$). No effects of treatment on urine Mg:creatinine ratio were found (quadratic $P = 0.24$, treatment by wk $P = 0.15$) but an effect of wk was observed ($P < 0.0001$). Asterisks (*) indicate timepoints where treatment means significantly differ ($P \leq 0.05$).

A
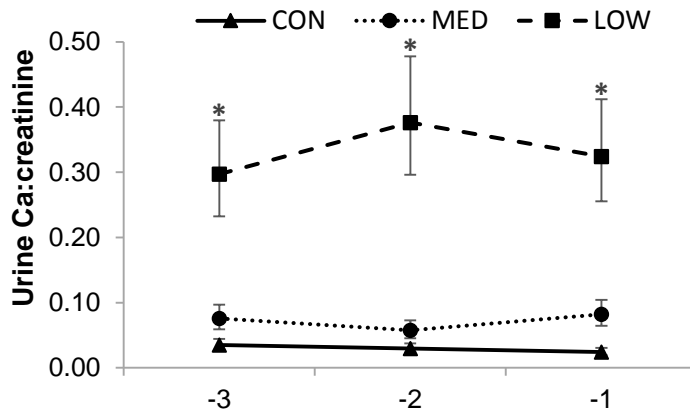
B
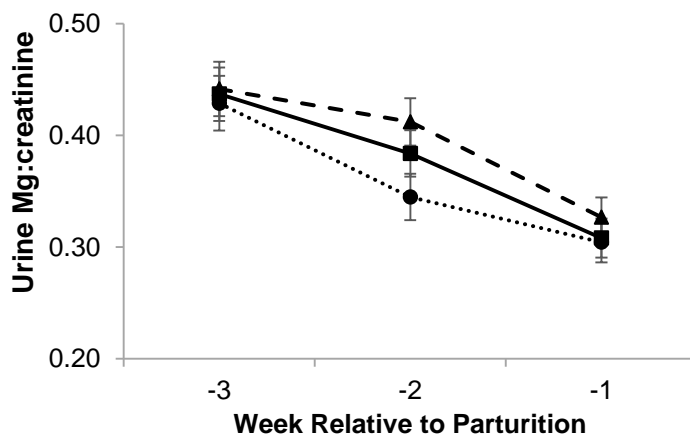
Figure 5- 2. The correlation between urine Ca:creatinine ratio and urine pH ($r = -0.81$, $P < 0.0001$) over the 3 weeks prior to parturition for cows fed decreasing levels of DCAD beginning 24 d prior to expected parturition.One urine sample collected each week was analyzed Ca and creatinine content and correlation represents up to 3 samples per cow. Treatments: CON= low K ration with no anion supplementation (n = 30, DCAD = +18.3 mEq/100 g DM), MED = partial anion supplementation to a low K ration (n = 30, DCAD = +5.9 mEq/100 g DM) and LOW = anion supplementation to a low K ration to reach a targeted average urine pH between 5.5 and 6.0 (n = 29, DCAD = -7.4 mEq/100 g DM).
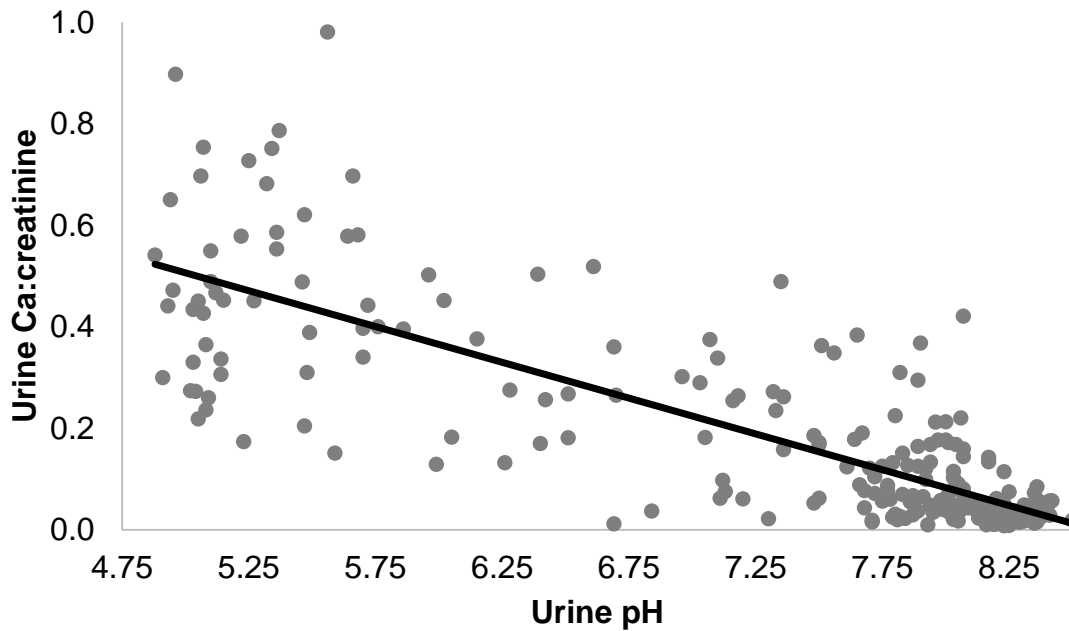
Figure 5- 3. Least squares means and standard errors for plasma Ca (A), Mg (B) and P (C) in the peripartum period (mmol/L) for cows fed decreasing levels of DCAD beginning 24 d prior to expected parturition. Treatments: CON= low K ration with no anion supplementation (n = 30, DCAD = +18.3 mEq/100 g DM), MED = partial anion supplementation to a low K ration (n = 30, DCAD = +5.9 mEq/100 g DM) and LOW = anion supplementation to a low K ration to reach a targeted average urine pH between 5.5 and 6.0 (n = 29, DCAD = -7.4 mEq/100 g DM). There were no overall effects on prepartum plasma Ca (linear $P = 0.32$) and no treatment by day interaction. Postpartum plasma Ca linearly increased with decreasing prepartum DCAD ($P = 0.002$) with no treatment by day interaction. No overall linear or quadratic effects of treatment were observed for prepartum or postpartum plasma Mg or P. A treatment by day interaction was observed for postpartum plasma Mg ($P = 0.03$). Asterisks (*) indicate timepoints where treatment means significantly differ ($P \leq 0.05$).

A
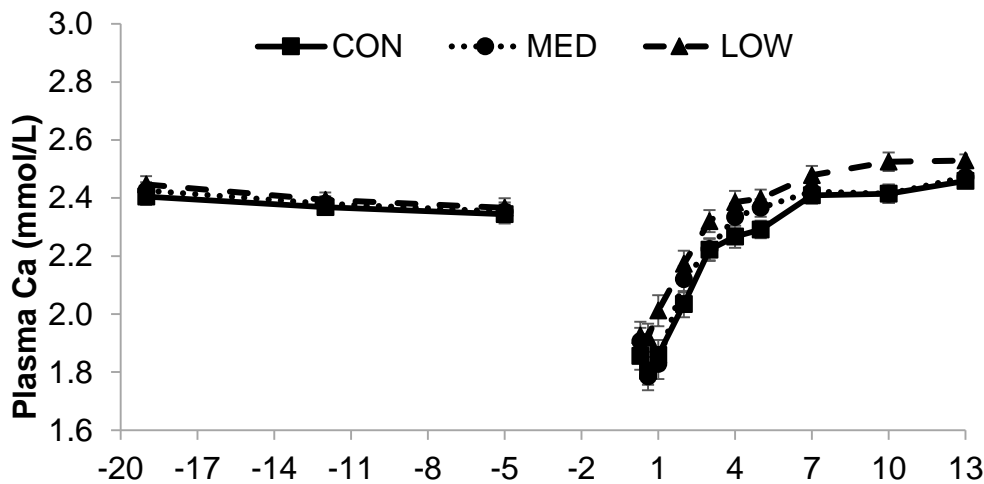
B
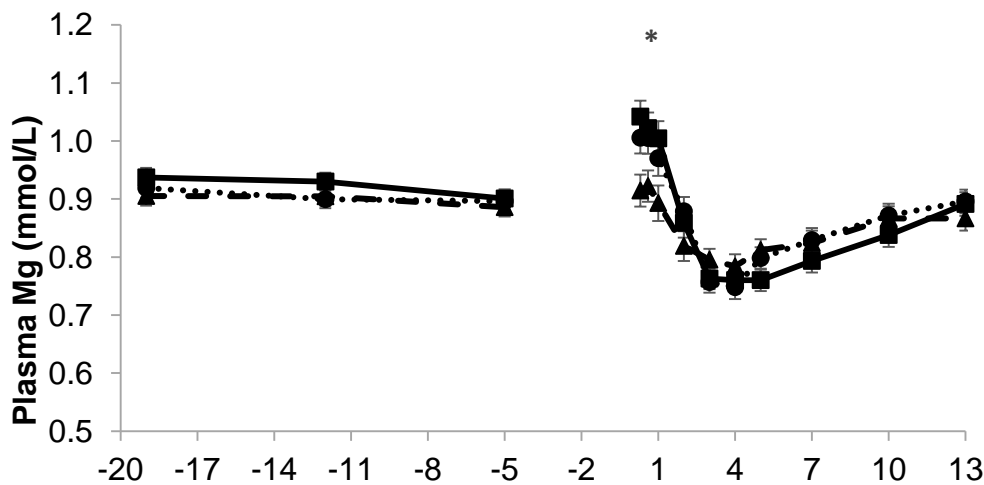
C
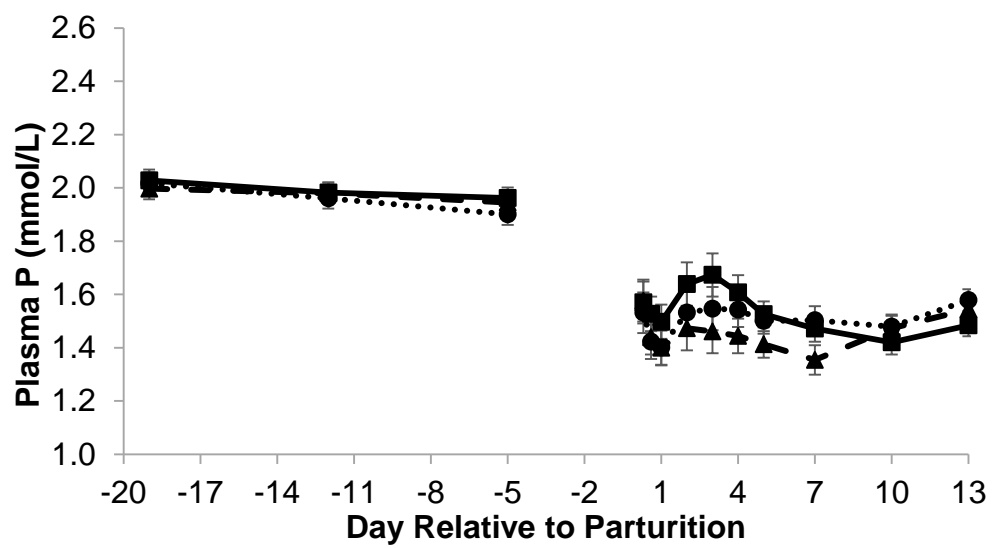
Figure 5- 4. Least squares means and standard errors for plasma Mg (A) and P (B) in the peripartum period (mmol/L) for cows fed decreasing levels of DCAD beginning 24 d prior to expected parturition by parity group.Parity by day interactions were observed for prepartum ($P = 0.02$) and postpartum ($P = 0.0004$) plasma Mg. The interaction of parity and day for prepartum plasma P had $P > 0.10$ and therefore the effect was removed from the model and least squares means were not determined. A parity by day interaction was observed for postpartum plasma P ($P = 0.03$). Asterisks (*) indicate timepoints where means differ by parity ($P \leq 0.05$). Trends ($0.05 < P \leq 0.10$) are indicated by a cross (†).

A
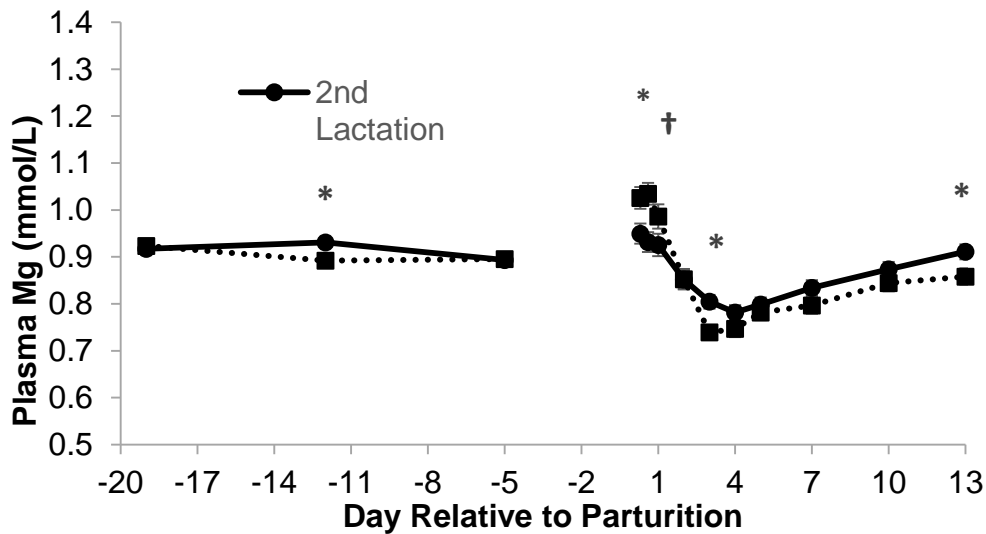
B
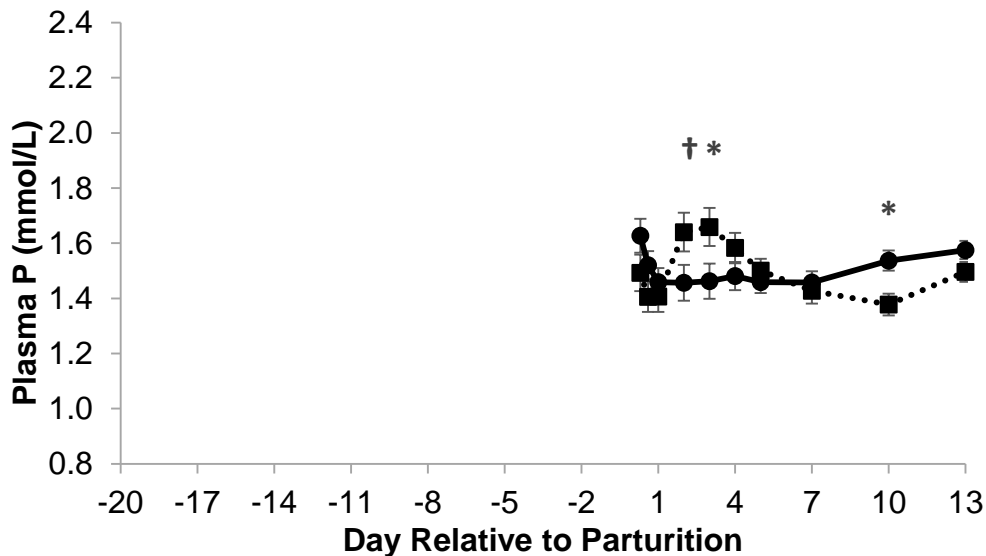
Figure 5- 5. Prevalence of hypocalcemia at sampling timepoints from parturition through 5 DIM for (A) second lactation cows with plasma Ca $\leq$ 2.0 mmol/L, (B) second lactation cows with plasma Ca $\leq$ 2.125 mmol/L, (C) third and greater lactation cows with plasma Ca <2.0 mmol/L and (D) third and greater lactation cows with plasma Ca < 2.125 mmol/L. Cows were fed decreasing levels of DCAD beginning 24 d prior to expected parturition. Treatments: CON = low K ration with no anion supplementation (n = 30, DCAD = +18.3 mEq/100 g DM), MED = partial anion supplementation to a low K ration (n = 30, DCAD = +5.9 mEq/100 g DM) and LOW = anion supplementation to a low K ration to reach a targeted average urine pH between 5.5 and 6.0 (n = 29, DCAD = -7.4 mEq/100 g DM). Asterisks (*) indicate timepoints where prevalence of hypocalcemia differs by treatment ($P \leq 0.05$). Trends ($0.05 < P \leq 0.10$) are indicated by a cross (†).
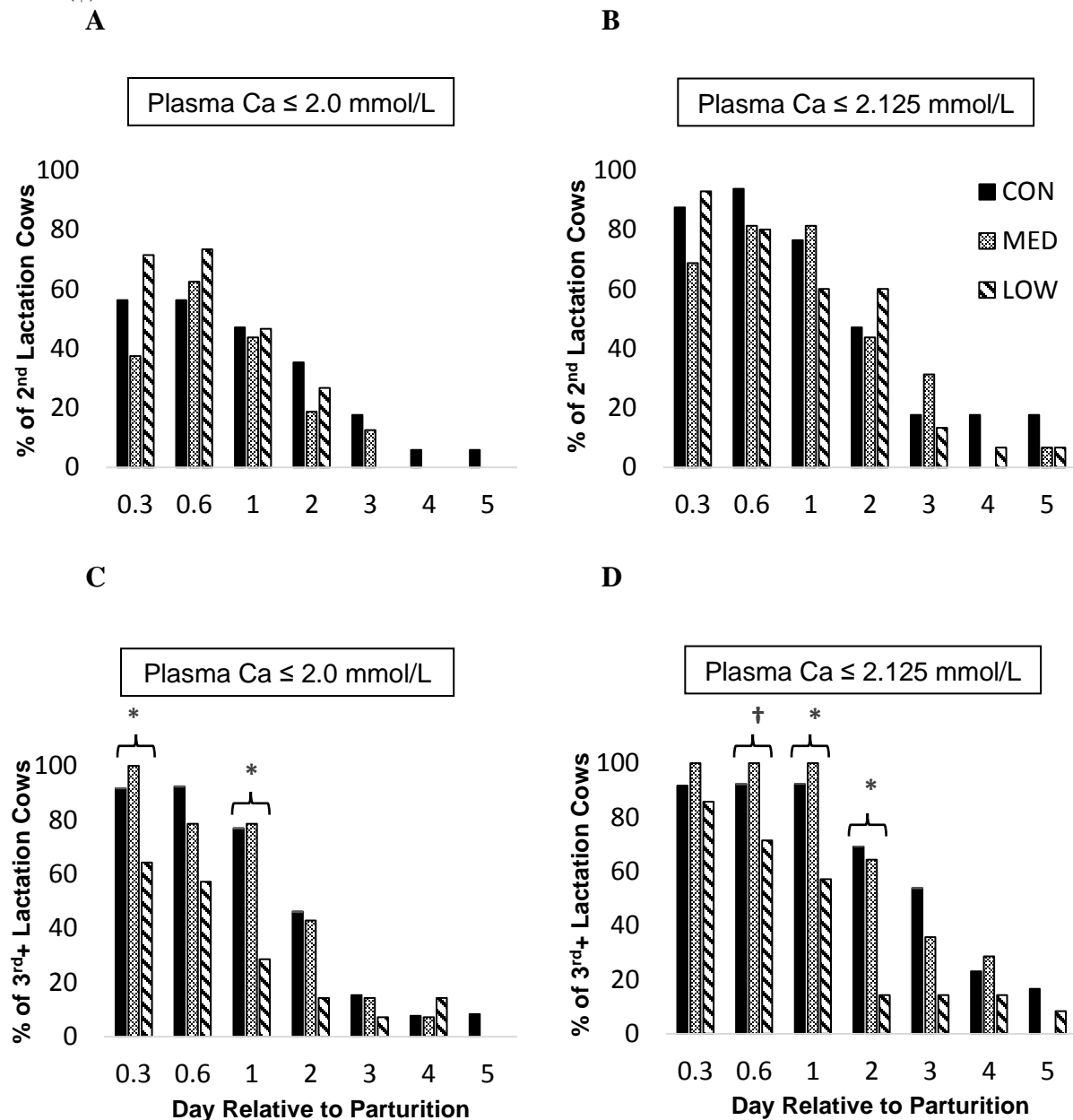
DISCUSSION

The primary hypothesis of this study was that feeding a lower DCAD diet prepartum would result in improved postpartum Ca status and that those benefits would increase as prepartum DCAD decreased. Linear increases in postpartum plasma Ca with decreasing prepartum DCAD supported these hypotheses. Reported success of improving blood Ca status by feeding low K diets with added anionic supplements to reach a low or negative DCAD varies across the literature. Feeding a negative DCAD for 21 d prepartum (-16 mEq/100 g DM; urine pH 7.0) did not result in improvements in blood Ca concentrations in the study by Weich et al. (2013) compared to feeding a positive DCAD, whereas feeding the same negative DCAD for 42 d prepartum (urine pH 6.5) in that study did increase postpartum blood Ca concentrations. DeGroot et al. (2010) observed numerical differences in prepartum blood Ca in cows fed a negative DCAD (-10 to -12 mEq/100 g DM; urine pH 5.93 to 6.66) compared to a positive DCAD but did not observe differences in blood Ca postpartum. Moore et al. (2000) formulated rations with similar relative DCAD to the current trial (+15, 0 and -15 mEq/100 g DM), while also increasing ration Ca concentration in the lower DCAD rations (Ca = 0.44, 0.97 and 1.50% of DM). In that trial, postpartum ionized Ca was improved for cows fed the lower DCAD. The reason for inconsistent effects on blood Ca is potentially due to degree and consistency of the metabolic acidosis experienced by the cow as a consequence of the diet as well as differences in statistical power and sampling frequency. The number of animals in the present study exceeded that of the previously mentioned studies and repeated postpartum blood sampling allowed for detailed assessment of blood Ca dynamics through the highly variable period immediately after parturition.

Although blood pH was not measured in the current trial, the average urine pH during the treatment period for cows fed LOW was consistent with significant alterations in acid-base balance when compared to trials in which more detailed assessments of acid-base status were conducted. Constable et al. (2009) described the relationship between urine pH and net acid excretion, which is a more accurate reflection of the disturbance in systemic acid-base balance, and found that this relationship was strong for urine pH values above 6.3. However, when urine pH fell below this value, the relationship to net acid excretion was poor and improved only when urine ammonium concentration was accounted for and resulted in a nonlinear relationship. This suggests that when urine pH values are below 6.3, the ammonia-ammonium system becomes an important component of removal of excess acids from circulation. In the current trial, average urine pH was below 6.3 in the cows fed LOW for the entire 3 wk prior to parturition. The average urine pH of 5.96 in cows fed LOW in the current trial was similar to urine pH in the negative DCAD (-18 mEq/100 g DM) fed group in the trial by Goff et al. (2014) in which it was demonstrated that pH of the blood was also decreased compared to the positive DCAD fed group. In the trial by Goff et al. (2014), cows fed the negative DCAD diet responded to exogenous PTH administration with more rapid increases in circulating 1,25-dihydroxyvitamin D and blood Ca concentrations. This suggests that one mechanism of action for increased plasma Ca concentrations for cows fed LOW in the current trial may have been increased sensitivity of tissues to PTH stimulation as a result of inducing a metabolic acidosis. Interestingly, urine pH responded in a quadratic manner to decreasing prepartum DCAD. Least squares mean urine pH for cows MED was only slightly lower than for cows fed CON, while mean urine pH of cows fed LOW was drastically decreased. This supports the idea of a quadratic relationship between DCAD and urine pH found in the meta-analysis by Charbonneau

et al. (2006). The impact of the prepartum diet on acid-base balance for cows fed MED was likely less than for cows fed LOW and may not have been sufficient to improve tissue sensitivity to PTH.

Another mechanism of action for low DCAD diets in preventing hypocalcemia in the transition period is through increases in urinary Ca excretion. Decreasing DCAD consistently increases urinary Ca excretion to some degree (Gaynor et al., 1989; Joyce et al., 1997; Goff et al., 2014) and the magnitude of increase is highly correlated with urine pH (Grunberg et al., 2011; Weiss et al., 2015). Similar results were observed in this study, with quadratic effects on urine pH, urine Ca:creatinine ratio, and estimated daily urinary Ca excretion as prepartum DCAD decreased. A potential mechanism for this effect on urine Ca flux is a direct inhibition of Ca reabsorption from the filtrate in the kidney due to the additional acid load (Stacy and Wilson, 1970), which is supported by the strong linear relationship observed between urine pH and urine Ca:creatinine ratio in the current trial as well as in a previous study (Grunberg et al., 2011). The mechanism by which increasing urine Ca excretion in the prepartum period can contribute to improving blood Ca status is likely two fold. Because urine Ca excretion was increased in cows fed LOW by approximately 8 g/d, it could be said that the perceived Ca "requirement" was increased, triggering the homeostatic mechanisms that increase influx, such as increasing bone resorption or intestinal absorption efficiency, or both. Calcium balance studies in goats fed cationic, neutral or anionic diets demonstrated that Ca flux in the urine was increased with anionic diets and this was accompanied by an increase in apparent absorption efficiency of Ca during lactation but not during the prepartum period (Fredeen et al., 1988). Those authors hypothesized that Ca balance was maintained prepartum with increased bone resorption and other studies in which negative DCAD rations were fed to dairy cows have found elevated

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plasma hydroxyproline in the prepartum period which is indicative of bone mobilization (Block, 1984; Goff and Horst, 1997). Other authors have found evidence that decreasing DCAD results in effects on intestinal absorption of Ca. Lomba et al. (1978) investigated the correlation between Ca absorption and ration DCAD in a range of experimental diets and found that digestibility of dietary Ca was increased as ration DCAD decreased in both dry and lactating dairy cows. Schonewille et al. (1994) demonstrated that feeding a negative DCAD diet (-17 mEq/100g DM) to dry, non-pregnant dairy cows increased the apparent absorption efficiency of Ca compared to a positive DCAD control ration.

It is recognized that older cows are more susceptible to clinical and subclinical hypocalcemia (Reinhardt et al., 2011; Caixeta et al., 2015) and may be due to age related declines in the function of calcium homeostatic pathways. Both first lactation cows and older cows have increased deoxypridinoline and decreased osteocalcin, metabolic profiles indicative of net bone resorption, throughout the transition period (Taylor et al., 2009). Blood concentrations of both markers are higher throughout that period for first lactation cows compared to older cows and may indicate that bone metabolism is more active in the younger cows, as would be expected in animals still undergoing skeletal development. Although the trial by Taylor et al. (2009) did not separate second lactation cows from third and greater lactation cows, it is plausible that there is still some degree of increased bone metabolism occurring in second lactation versus older cows. Age-related decreases in Ca metabolism have also been described for vitamin D-mediated alterations in intestinal Ca absorption efficiency. Experiments conducted using radiolabeled Ca have shown that as cows age, absorption efficiency of Ca decreased, while obligatory loss of Ca from endogenous origin increased with advancing age (Hansard et al., 1954). Other studies have shown that older cows are able to increase blood concentrations of PTH and 1,25-

dihydroxyvitamin D similarly to their younger counterparts, but still have exacerbated hypocalcemia (Wilkens et al., 2013). One potential link between these factors is the decrease in vitamin D receptors in intestinal epithelial cells that has been described in cows with advancing age (Horst et al., 1990). Previous trials have not assessed the relative response of second lactation cows fed lower DCAD rations to that of older cows. An interaction of treatment and parity group for postpartum plasma Ca revealed that older cows had the most pronounced increase in plasma Ca when fed a lower DCAD prepartum. Improved sensitivity of the PTH receptor to stimulation by PTH in cows fed a lower DCAD, as demonstrated by (Goff et al., 2014), may have elicited a more robust bone mobilization response as well as more efficient production of 1,25-dihydroxyvitamin D. If older cows in this population had fewer vitamin D receptors present in the gastrointestinal tract, greater circulating concentrations of 1,25-dihydroxyvitamin D may have resulted in more effective saturation of vitamin D receptors that were present on gastrointestinal epithelial cells and bone, contributing to a more rapid recovery of plasma Ca. The difference in hypocalcemia prevalence between cows fed CON and LOW increased from 20.9% at 0.6 DIM to 54.9% at 2 DIM, suggesting that the rate of recovery for older cows fed LOW was more rapid than for older cows fed CON, consistent with the theory of increasing Ca turnover prepartum or greater responsiveness of homeostatic signals postpartum or both.

Relative effects of treatment on prevalence of hypocalcemia in the current study are interpreted similarly using a threshold of either 2.0 mmol/L or 2.125 mmol/L to categorize cows as hypocalcemic. No differences in hypocalcemia prevalence for second lactation cows was found using either threshold and hypocalcemia prevalence was decreased for older cows fed LOW at some timepoints. The comparison of hypocalcemia prevalence at two different

thresholds and several timepoints immediately after parturition provides a reference for those working in the field to measure and interpret hypocalcemia occurrence on farms. In a national study, Reinhardt et al. (2011) found that prevalence of hypocalcemia (both subclinical and clinical cases) in the 48 h after parturition in cows entering their 2nd or greater lactation ranged from 45% in 2nd lactation cows to 62% in 5th lactation cows. Using the same threshold, prevalence of hypocalcemia was similar in the current study. The prevalence of hypocalcemia with a plasma Ca threshold of 2.125 mmol/L resulted in a 15 to 23% higher overall prevalence at the time points within the first two days after calving. Higher blood Ca thresholds have been supported by recently published epidemiological datasets (Chapinal et al., 2012; Martinez et al., 2012) due to associations with negative effects on health, reproduction and productivity. Further epidemiologic studies are necessary to determine the most sensitive and specific thresholds and timepoints relative to parturition for diagnosis of SCH based on subsequent risk of disease and compromised performance. This comparison of two thresholds used in recent literature demonstrate that small differences in the blood Ca threshold can dramatically influence the perceived number of cows that are affected by hypocalcemia.

The number of cows treated with intravenous Ca (n = 6/89, 6.7%) was higher than the prevalence of clinical hypocalcemia that has been published in recent field trials (Chapinal et al., 2011). This may have been due to criteria used to identify animals for treatment. It was agreed that farm personnel could administer intravenous Ca during early signs of milk fever [lethargy and muscle weakness as described by Oetzel (2013)] to avoid cows becoming recumbent. This may have resulted in false identification of clinical hypocalcemia due to lack of specificity of these symptoms or the subjectivity of symptom identification.

Despite discrepancies in thresholds and sample timing, prevalence of hypocalcemia in this trial was higher than expected. Jawor et al. (2012) observed that cows with SCH (serum Ca ≤ 1.8 mmol/L), when paired by health disorders with normocalcemic cows, produced more milk in wk 2 through 4 postpartum. It is possible that higher producing cows are at a greater risk for hypocalcemia and cows in this study produced from 40.8 kg/d (CON) to 43.9 kg/d (LOW) in the 3 wk postpartum. However, other factors could have increased risk for hypocalcemia in this trial. A meta-analysis of DCAD feeding trials identified a quadratic relationship between prepartum diet Ca concentration and milk fever risk, with maximum risk occurring at approximately 1.4-1.5% of DM as Ca (Lean et al., 2006). Diet concentration of Ca in the current trial was slightly above that range in all groups. This was an intentional component of the study design based on experimental evidence that higher feeding rates of Ca in conjunction with low DCAD feeding could reduce parturient paresis compared to low DCAD feeding with lower dietary Ca concentrations (Oetzel et al., 1988). The principle of this theory is that lower DCAD diets increase the Ca flux prepartum through increased urine Ca excretion and therefore additional dietary Ca supports the flux of Ca through the system. To allow for isolation of the effects of decreasing DCAD, diet Ca was held constant in all treatment diets as opposed to increasing diet Ca with decreasing DCAD as was the case in the study by Moore et al. (2000). Future work should be conducted to determine the interaction between low K versus negative DCAD feeding and moderate versus high Ca feeding.

Postpartum plasma concentrations of Mg were lower in the two days after parturition for cows fed lower prepartum DCAD. This result has been observed in other studies in which cows with lower blood Ca concentrations had higher blood Mg concentrations in the immediate postpartum period (Green et al., 1981; Kronqvist et al., 2011). Administration of exogenous

PTH has been shown to decrease urinary Mg excretion and subsequently result in increased blood Mg concentrations (Goff et al., 2014). It is likely that the higher plasma Mg for cows fed CON in this trial is a reflection of increased concentrations of PTH due to a more severe Ca challenge at parturition, increasing the renal threshold for Mg. This difference was not detected in the urine Mg:creatinine excretion ratio. Analysis of urine samples only occurred prepartum and at weekly intervals and this sampling intensity was likely not sufficient to detect any differences occurring in the few days around parturition when the PTH response would be most pronounced. A decrease in Mg excretion was observed in all groups as parturition approached. Excretion of Mg has been shown to decrease as Mg intake decreases (Ammerman et al., 1972), and this effect over time may have reflected the decline in intake that occurred as parturition approached in the current study (Chapter 4).

Plasma concentrations of P were not affected by treatment in this study. The relationship between DCAD feeding and P status is unclear, despite the interrelationships between Ca and P metabolism. Increases in blood P concentrations were found in portions of the transition period for cows fed negative DCAD diets prepartum compared to low K diets prepartum in some trials (Ramos-Nieves et al., 2009; DeGroot et al., 2010; Weiss et al., 2015) while other studies showed no differences in blood P concentrations (Grunberg et al., 2011), consistent with the results of the current study. The inconsistency in P response may be due to variation in pre and postpartum dietary P concentration and DMI in different trials or the severity of the blood Ca disturbance around calving. Increases in blood P concentrations have been demonstrated when dietary concentration of P is increased (Barton et al., 1987; Peterson et al., 2005). Peterson et al. (2005) and (Kichura et al., 1982) both demonstrated that feeding very low dietary P concentrations

prepartum could decrease risk of hypocalcemia postpartum, confirming the interconnected metabolism of Ca and P.

Considering the association between SCH and risk for disease and compromised performance (Chapinal et al., 2011; Chapinal et al., 2012; Martinez et al., 2012), it is plausible that inadequate Mg and P concentrations may have similar associations with risk of subsequent diseases and performance since these minerals also play important roles in many physiological functions (Goff, 2006). Little information is available describing "normal" patterns of blood mineral concentrations, thresholds that identify subclinical disorders of Mg or P based on downstream consequences, or the impact of parity on the dynamics of these minerals in the transition period. Further descriptions of these temporal patterns and the influence of age may aid in interpretation of alterations in these patterns. Plasma concentrations of Mg were higher in older cows immediately after parturition. This may have been due to lower plasma Ca in older cows at parturition, and the subsequent PTH response that may increase the renal threshold for Mg, as discussed previously. Plasma P concentrations were higher in older cows between 2 and 5 DIM. Phosphorus metabolism is affect by hormonal control of Ca status in that increases in bone resorption and intestinal absorption efficiency of Ca will similarly increase resorption of bone P and absorption efficiency of P. However, plasma P is maintained secondary to plasma Ca. This is supported by the study from Kichura et al. (1982) in which prepartum dairy cows responded to low Ca diets with transient reductions in plasma Ca and increases in plasma hydroxyproline (an indicator of bone resorption) and 1,25-dihydroxyvitamin D concentrations, all of which were sustained throughout the prepartum period. Conversely, low P feeding resulted in sustained reductions in plasma P during the prepartum period, but no effects on plasma concentrations of hydroxyproline or 1,25-dihydroxyvitamin D. In the current study, the rebound

of plasma P in older cows as plasma Ca recovers potentially reflects a more prolonged PTH and 1,25-dihydroxyvitamin D response in older cows, and thus greater resorption of P from bone and elevated intestinal P absorption.

CONCLUSIONS

Linearly decreasing the prepartum DCAD in multiparous Holstein cows lowered urine pH and increased urinary Ca excretion prepartum in a quadratic manner. Postpartum plasma Ca concentrations increased linearly with decreasing prepartum DCAD and cows entering their 3rd lactation and greater showed the most pronounced increases in plasma Ca when fed the lowest prepartum DCAD. Older cows fed the lowest prepartum DCAD also had decreased prevalence of SCH in the days immediately after parturition. This work supports the use of lower DCAD diets that reduce urine pH between 5.5 and 6.0 to improve Ca status throughout the transition period.

ACKNOWLEDGEMENTS

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CHAPTER 6

THE EFFECT OF SOURCE OF SUPPLEMENTAL DIETARY CA AND MG IN THE PERIPARTUM PERIOD, AND LEVEL OF DIETARY MG POSTPARTUM, ON MINERAL STATUS, PERFORMANCE AND ENERGY METABOLITES IN MULTIPAROUS HOLSTEIN COWS

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ABSTRACT

The objective of this study was to determine the effects of feeding different supplemental sources of Ca and Mg in the peripartum period, and different dietary levels of Mg postpartum, on plasma mineral status, performance, and aspects of energy metabolism in transition dairy cows. Multiparous Holstein cows (n = 41) were utilized in a completely randomized design with a 2 × 2 factorial arrangement of treatments starting at 28 d prior to expected parturition. At 21 d prior to expected parturition, cows were assigned randomly to source treatments. Main effects were source assignments (**CS** = common sources of supplemental Ca and Mg or **MA** = a blend of common and commercial mineral sources with supplemental minerals primarily from a commercial Ca-Mg dolomite source; MIN-AD, Papillon Agricultural Company, Inc., Easton, MD) beginning at 21 d prior to due date; cows were further randomized within source treatments to one of two levels of Mg supplementation (**LM** = formulated postpartum diet Mg at 0.30% of DM or **HM** = formulated postpartum diet Mg at 0.45% of DM) beginning within 1 d after parturition. Final treatment groups included the following; common source, low Mg (**CS-LM**, n = 11), common source, high Mg (**CS-HM**, n = 11), MIN-AD, low Mg (**MA-LM**, n = 10) and MIN-AD, high Mg (**MA-HM**, n = 9). Treatment diets were fed and data collected through 42 d in milk. Postpartum plasma Mg concentrations tended to be higher for cows fed HM and cows fed CS, but there were no effects on peripartum plasma Ca concentrations. Peripartum plasma P concentrations were higher for cows fed MA. Dry matter intake (**DMI**) in the prepartum period was higher for cows fed MA (CS = 15.9 vs. MA = 16.8 kg/d) and postpartum DMI was higher in some groups depending on week. Plasma non-esterified fatty acid concentrations were lower for cows fed MA during both the prepartum and postpartum periods. A source by level interaction was observed for postpartum plasma β-hydroxybutyrate (BHB) concentrations such that cows

fed CS-LM had numerically higher BHB and cows fed MA-LM had numerically lower BHB (geometric means; CS-LM = 7.9, CS-HM = 6.9, MA-LM = 6.3 and MA-HM = 7.3 mg/dL) than cows fed the other two treatments. Higher milk fat yield, milk fat content and fat- and energy-corrected yield during wk 1 for cows fed MA resulted in source by week interactions for these outcomes. This study demonstrated that varying supplemental Ca and Mg sources and feeding rates had minimal impact on plasma Ca status despite differences in plasma Mg and P concentrations. Effects on DMI and plasma energy metabolites suggest opportunity for strategic use of mineral sources in the transition period to promote metabolic health.

INTRODUCTION

Hypocalcemia is a disorder in which blood Ca concentrations are compromised as the result of inadequate adaptation to the lactational demands for Ca that begin at the onset of colostrum production in dairy cows (Ramberg et al., 1970). Recent research has determined that 11-25% of first lactation animals and 42-60% of multiparous cows can be categorized as having subclinical hypocalcemia (**SCH**; low blood Ca with no clinical signs of hypocalcemia) in the day after parturition (Reinhardt et al., 2011; Caixeta et al., 2015). Martinez et al. (2012) demonstrated an association between SCH and compromised energy metabolism, risk of uterine disease and delayed reproduction. Additional work described similar associations with reproduction (Chapinal et al., 2012) and energy metabolism (Chamberlin et al., 2013) and has further demonstrated increased risk for displaced abomasum and early lactation culling (Chapinal et al., 2011; Roberts et al., 2012) as well as decreased early lactation milk production (Chapinal et al., 2012) in cows with SCH. Taken together, the body of evidence suggests that SCH is a highly prevalent and costly disorder.

Magnesium is known to be an important mineral in the homeostatic pathway for regulating blood Ca based upon work conducted in cows (van Mosel et al., 1990; van Mosel et al., 1991) and humans (Rude et al., 1978; Rude et al., 1985). Feeding higher concentrations of dietary Mg prepartum (0.45-0.50% of DM) has become common practice to aid in prevention of hypocalcemia at parturition and was supported by a meta-analysis conducted by Lean et al. (2006). Decreased blood Mg concentrations during the week after parturition have been reported in several published studies (Green et al., 1981; Shappell et al., 1987; Ramos-Nieves et al., 2009; Kronqvist et al., 2011); however, little work has been done to understand the causes and consequences of this blood Mg pattern. Theoretically, feeding higher dietary rates of Mg postpartum to increase blood Mg may help in the recovery of plasma Ca since plasma Ca concentrations have been shown to take several days to return to prepartum levels (Ramos-Nieves et al., 2009). To the authors' knowledge, feeding varying rates of Mg postpartum to support the recovery of blood Ca has not been investigated.

Dietary mineral source may have impacts on mineral status due to variation in bioavailability, which can be affected by calcination temperature, chemical structure and particle size (Moore et al., 1971; Jesse et al., 1981; Van Ravenswaay et al., 1989; Xin et al., 1989). Further, mineral sources of different chemical structures have been shown to have varying buffering capacities (Schaefer et al., 1982), which may aid in transition period intake and performance where diet transitions have been shown to challenge rumen health (Penner et al., 2007). Investigation of the performance of cows in the transition period fed varying supplemental mineral sources may provide evidence for strategic use of mineral sources to promote successful diet transitions.

The objectives of this experiment were to determine the effects of dietary source of supplemental Ca and Mg, and postpartum dietary level of Mg, on intake, performance and aspects of energy and mineral metabolism in multiparous Holstein cows. We hypothesized that plasma mineral status would be altered by feeding supplemental minerals from a commercial Ca-Mg dolomite and feeding a higher rate of dietary Mg postpartum. If plasma mineral status was improved by either factor, it was hypothesized that intake and performance would also be improved in those cows.

MATERIALS AND METHODS

Study Population, Experimental Design, and Treatments

All animal protocols were approved by the Cornell University Institutional Animal Care and Use Committee. Animals were enrolled in the experiment between May and July of 2015. Multiparous Holstein cows (n = 47) were enrolled in a completely randomized design with a 2×2 factorial arrangement of treatments starting at 28 d prior to expected parturition. Cows were fed a control diet for one week, and at 21 d prior to expected parturition cows were assigned randomly to treatment with randomization restricted to balance for parity group (second vs. third and greater lactation) and previous 305 d mature equivalent milk production. During the prepartum period, cows were randomized to one of two source treatments in which supplemental dietary Ca and Mg were provided primarily from common sources [Mg oxide (BRAZAMAG, Timab Industries, Dinard, France) and limestone; **CS**] or from a blend of common and commercial mineral sources with supplemental minerals primarily from a commercial Ca-Mg dolomite source (MIN-AD, Papillon Agricultural Company, Easton, MD; **MA**). At the next feeding that occurred after parturition, both CS and MA groups were further randomized into two groups within their source treatments in which one received diets formulated to contain Mg

at about NRC (2001) recommendations (**CS-LM** and **MA-LM** = 0.30% of DM) or at a higher

rate (**CS-HM** and **MA-HM** = 0.45% of DM). Cows were fed experimental diets and data were

collected through 42 DIM. The target study population was 40 cows total. Sample size

determination was based on detecting biologically important differences in plasma mineral

concentrations in the week following parturition. The average and standard deviations for these

outcomes were based on previous work conducted in our group for a treatment group fed similar

prepartum rations (Leno et al., In press.). With $\alpha = 0.05$ and $\beta = 0.20$, the detectable difference

in wk 1 for Ca, Mg and P for the main effects of source and level were 0.27 mmol/L, 0.11

mmol/L and 0.30 mmol/L, respectively. Criteria for removal from the trial included twin births

(n= 5) and calving with less than 10 d fed the experimental prepartum diet (n = 1). Cows

excluded before the end of the enrollment period were replaced and, in anticipation of the loss of

cows from the trial, one additional cow was enrolled into each treatment group at the end of the

enrollment period. The final dataset included 41 cows from which 11 were in the common

source, low Mg group (**CS-LM**), 11 were in the common source, high Mg group (**CS-HM**), 10

were in the MIN-AD, low Mg group (**MA-LM**) and 9 were in the MIN-AD, high Mg group

(**MA-HM**).

Feeding Management, Feed Sampling and Analysis

Cows were housed in tiestalls and fed once daily between approximately 0700 h and

0900 h for lactating cows and 0900 h and 1030 h for dry cows. Individual feed intake was

measured on a daily basis throughout the experiment by weighing feed delivered and refused.

Cows were fed for a targeted refusal rate of 10% to allow for ad libitum intake. Rations were

formulated using the Cornell Net Carbohydrate and Protein System (CNCPS v. 6.5; Cornell

University, Ithaca, NY). Ingredient composition and analyzed diet composition of all prepartum

and postpartum treatment diets are presented in Table 6-1 and Table 6-2. All rations were composed of a base TMR containing forages and a base grain mix, which were common to all diets within the prepartum and postpartum periods, as well as a small inclusion rate grain mix. The small inclusion rate grain mixes were unique to each treatment group and contained the majority of supplemental minerals as well as ingredients to offset the higher inclusion rate of MA compared to CS. The base TMR was mixed in one batch for all prepartum cows and in one batch for all postpartum cows. Prior to delivery to the cow, smaller batches were made which included the small inclusion rate grain mixes.

Samples of TMR and all feed ingredients were collected weekly for determination of DM (dried at 40°C for 96 h in a forced-air oven). Weekly DM values were used to adjust as-fed inclusion rates of all forages and grain ingredients. At the end of the experiment, dried samples were ground to 2 mm in a Wiley mill and composited at 4-wk intervals for TMR samples and over the duration of the trial for all forages and grains. Composited samples were sent to a commercial laboratory (Cumberland Valley Analytical Services, Hagerstown, MD) for wet chemistry analysis of DM at 135°C (Method 930.15, AOAC International, 2000), CP (Method 990.03, AOAC International, 2000), ADF (Method 973.18, AOAC International, 2000), NDF (Van Soest et al., 1991), starch (Hall, 2009), sugar (Dubois et al., 1956), ether extract (Method 2003.05, AOAC International, 2006), minerals (Method 985.01, AOAC International, 2000), chloride (silver nitrate titration after extraction with 0.5% nitric acid using a Brinkman Metrohm 848 Titrino Plus, Brinkmann Instruments Inc, Westbury, NY) and sulfur (according to Leco Organic Application Note "Sulfur and Carbon in Plant, Feed, Grain and Flower" Form 203-821-321, 5/08-REV1). Values for NE_L of TMR composite samples were calculated according to

NRC (2001). For calculation of daily DMI, weekly DM determinations from fresh TMR samples were further corrected for residual moisture in composite samples.

Individual Animal Sampling, Analytical Methods and Calculations

Cows were observed daily throughout the experiment for health disorders by farm personnel. Body weights were measured weekly and BCS assigned by two scorers weekly according to Edmonson et al. (1989) beginning during the week prior to assignment to treatment and continuing through 42 d postpartum. Body condition scores were averaged over two scorers before statistical analysis.

After calving, all cows were milked three times daily at 0600 h, 1400 h and 2200 h. Daily milk weights were recorded and daily milk yield calculated as the sum of yields at all three milkings through the 42-d study period. Milk samples were collected at 3 consecutive milkings each week, mixed with a bronopol preservative and stored at 4°C until transportation to a commercial laboratory (DairyOne, Ithaca, NY) within 72 h of collection for analysis of milk fat, protein, lactose, total solids, MUN using mid-infrared techniques (Method 972.16, AOAC International, 2006), and SCC was determined using optical fluorescence (Method 978.26, AOAC International, 2006). Somatic cell scores were calculated from SCC [SCS = $\log_2$(SCC/100,000)+3; (Shook, 1993)]. Milk yield at the corresponding milking was used to weight milk composition and calculate yield of fat, protein, lactose and total solids. Weekly average yield of 3.5% FCM was calculated [3.5% FCM = (0.432 × kg average weekly milk yield) + (16.216 × kg of fat); (Gaines, 1928; Erdman, 2011)] as well as weekly average yield of ECM [ECM = (0.327 × kg average weekly milk yield) + (12.95 × kg of fat) + (7.65 × kg of true protein); (Tyrrell and Reid, 1965)]. Milk production efficiency was calculated from weekly average DMI and ECM (efficiency = kg of ECM/ kg of DMI).

Blood samples were collected via coccygeal venipuncture between 0600 h and 0730 h twice weekly (Monday and Friday) from d -28 relative to expected parturition until parturition, within 2 h of parturition (d 0), daily from d 1 through 7 in milk, and three times per week (Monday, Wednesday, and Friday) thereafter through 21 DIM. Samples were collected using 10 mL sodium heparin evacuated tubes (158 USP, Beckton Dickinson, Franklin Lakes, NJ) and 20 G vacutainer needles (Beckton Dickinson, Franklin Lakes, NJ) and placed on ice immediately after collection. Plasma was harvested after centrifugation at 2,000 x g for 20 min at 4°C, aliquoted into 1.7 mL microfuge tubes, snap frozen in liquid nitrogen and stored at -20°C until analysis.   A subset of samples were analyzed for BHB and non-esterified fatty acids (**NEFA**). A commercial kit (Catachem Inc., Oxford, CT) was adapted for analysis of plasma BHB concentrations in a 96-well plate. Briefly, 5 µL of standards, controls, and unknowns were pipetted in triplicate. Readings (340 nm) were taken after addition of 150 µL of the sample diluent reagent and after 2 min of incubation (37°C) after the addition of 30 µL of the catalyst reagent. The difference between readings was compared to the standard curve to determine BHB concentration. Analysis of plasma NEFA concentrations were conducted in triplicate using a commercial enzymatic kit (HR Series NEFA HR (2), Wako Pure Chemical Industries, Osaka, Japan). Spectrophotometric measurements were conducted using a tunable microplate reader (SpectraMax 190, Molecular Devices, Sunnyvale, CA). Coefficients of variation (both inter- and intra-assay) for all assays were maintained below 10%. A different subset of samples was analyzed for mineral concentrations at the Cornell Animal Health and Diagnostic Center (Ithaca, NY) on an automated analyzer (Hitachi Modular P800, Roche Diagnostics, Indianapolis, IN).

Weekly calculations of prepartum and postpartum energy balance (**EBAL**) were determined according to NRC (2001) equations for energy intake (Mcal/d) = weekly DMI

average (kg/d) $\times$ diet NE_L (Mcal/kg of DM); maintenance requirement (Mcal) = week metabolic

BW (MBW; $kg^{0.75}$) $\times$ 0.08 (Mcal/$kg^{0.75}$ per d); pregnancy requirement (Mcal) = (0.00318 $\times$ d of

gestation $-$ 0.0352) $\times$ (1/0.218); and lactation requirement (Mcal/d) = wk average milk yield

(kg/d) $\times$ [(0.0929 $\times$ fat percentage) + (0.0563 $\times$ true protein percentage) + (0.0395 $\times$ lactose

percentage)]. Weekly values for EBAL were determined as follows:

Prepartum NE_L (Mcal/d) balance = energy intake (Mcal of NE_L/d) $-$ [maintenance

requirement (Mcal of NE_L/d) + pregnancy requirement (Mcal of NE_L/d)]

Postpartum NE_L (Mcal/d) balance = energy intake (Mcal of NE_L/d) $-$ [maintenance

requirement (Mcal of NE_L/d) + lactation requirement (Mcal of NE_L/d)]

Statistical Analysis

Prepartum data were restricted to the 3 wk prior to actual parturition for each cow. Daily

DMI and milk production were reduced to weekly means before statistical analysis. To account

for the prepartum twice weekly and postpartum three times weekly blood sampling schedule,

average day of sampling relative to actual parturition is reported. Prepartum and postpartum data

were analyzed separately and plasma measurements from d 0 were included in prepartum

analysis because samples were taken before commencing postpartum treatment assignments.

Postpartum plasma minerals were analyzed separately for effects between d 1 to 7 and between d

9 to 21 to determine effects that may have been manifested in the days immediately following

parturition and because of differences in sampling intensity. All statistical analyses were

conducted with the statistical software SAS (version 9.4, SAS Institute Inc., Cary, NC). The

distribution of parity group by source, level and overall treatment group was assessed using a

chi-square test using the FREQ procedure. Differences in previous lactation 305-d mature

equivalent milk production and covariate BW and BCS were tested by ANOVA using the

MIXED procedure and the model included effects of source, level and the interaction of source and level. Continuous outcomes were subjected to ANOVA using the MIXED procedure. Continuous measures not repeated over time were analyzed for the fixed effects of source, level (postpartum only), parity group (second vs. third lactation) and the interaction between source and level (postpartum only). All measurements repeated over time were subjected to repeated measures ANOVA using the REPEATED statement in the MIXED procedure of SAS (Littell et al., 1996). The fixed effects of time, source, level (postpartum only), parity group (second vs. third lactation) and the two- and three-way interactions of source, level (postpartum only) and time were included in the model. Cow within source (prepartum) or source and level (postpartum) was the random effect. When available, covariate measurements collected in the week prior to treatment assignment were included in all models. Previous lactation 305-d mature equivalent milk production was included as a covariate for milk yield. The Kenward Rogers method was used for estimation of denominator degrees of freedom. Four covariance structures were tested for each model and the model with the lowest Akaike's Information Criterion was selected (Littell et al., 1996). For data on all plasma measurements the following covariance structures were tested; compound symmetry, heterogeneous compound symmetry, antedependence 1 and unstructured. For all other data the following covariance structures were tested; compound symmetry, heterogeneous compound symmetry, autoregressive order 1 and heterogeneous autoregressive order 1.When $P \leq 0.15$ for interactions with time, the SLICE option was used in the LSMEANS statement to conduct an F-test to determine at which levels of time the treatment groups differed. Plots of studentized residuals were visually inspected for normality and homogeneity of variance. When non-normality of residual variance was evident (NEFA and postpartum BHB), data were log transformed and analysis repeated. Least squares

means and standard errors, or geometric mean and 95% CI (NEFA and postpartum BHB), are reported throughout. Significance was declared at $P \leq 0.05$ and trends are discussed at $0.05 < P \leq 0.15$.

RESULTS

Study Population and Experimental Diets

Data from one cow fed CS-HM were removed from 21 DIM to the end of the observation period due to clinical mastitis that caused a severe drop in milk production. Data from a cow fed MA-HM were removed past 33 DIM due to an injury that required her to be removed from treatment diet and moved to a bedded pack. All data for these two cows prior to illness or injury remained in the dataset. The description of the final study population after exclusion of cows that calved early or calved with twins, including distribution of parity and means of previous lactation 305 d mature equivalent milk production, days on treatment, and BW and BCS at enrollment, are presented in Table 6-3. There were no differences in any of these parameters with the exception of a trend for a source by level interaction in covariate BCS; however, differences were relatively small.

Analyzed dietary Mg concentration of postpartum treatment diets were different from formulated (Table 6-2). Both of the LM diets had analyzed Mg concentrations of 0.35% of DM, above the targeted 0.30% of DM. The concentration of Mg in the HM diets was different from the formulated 0.45% of DM with the CS-HM diet at 0.40% of DM and the MA-HM diet at 0.48% of DM. When analyzed composition of forage composites and formulated grain mix compositions were inputted into CNCPS, calculated Mg composition of the prepartum rations was 0.45 and 0.50% of DM for CS and MA, respectively. For postpartum rations, calculated Mg composition was 0.33, 0.46, 0.33, and 0.47% of DM for CS-LM, CS-HM, MA-LM, and MA-

HM, respectively. Variation in ingredient inclusion rate as well as cumulative inherent error in

TMR sampling, processing, and laboratory analysis could have contributed to those differences

in composite composition. Using the analyzed TMR composite Mg concentrations and

formulated inclusion rates of supplemental mineral sources, percentage of Mg and Ca from

supplemental sources was calculated and the percentage from the basal ingredients was

calculated by difference. These estimations are presented in Table 6-2. Overall, differences in

dietary Mg concentrations were apparent between LM and HM groups, justifying analysis of

data for any effects of level.

Effects of Prepartum Source of Ca and Mg on Prepartum Outcomes

     DMI, EBAL, BW and BCS. Prepartum DMI, EBAL, BW, BW change and BCS are

presented in Table 6-4. Prepartum DMI was higher for cows fed MA (CS = 15.9 vs. MA = 16.8

kg/d, $P = 0.03$; Figure 6-1). Dry matter intake as a percentage of BW was also higher in cows

fed MA (CS = 2.00 vs. MA = 2.10% of BW, $P = 0.02$). Calculated prepartum EBAL was

similarly affected by supplemental mineral source and EBAL tended to be higher for cows fed

MA (CS = 7.2 vs. MA = 8.1 Mcal/d, $P = 0.12$). Prepartum BCS tended to be higher for cows fed

MA (CS = 3.42 vs. MA = 3.47, $P = 0.13$); however, absolute differences were small. No

differences were detected for prepartum BW or BW change.

     Plasma NEFA, BHB and Mineral Concentrations. Prepartum plasma NEFA and BHB

concentrations are presented in Table 6-5 and prepartum plasma mineral concentrations are

presented in Table 6-6. Plasma concentrations of NEFA prepartum were lower in cows fed MA

[geometric mean (back transformed 95% CI); CS = 142 (130-155) vs. MA = 117 (107-129)

µEq/L; $P = 0.005$)]. No differences were detected for prepartum plasma concentrations of BHB,

Ca or Mg. Cows fed MA had higher concentrations of P in plasma prepartum (CS = 1.69 vs.

MA = 1.79 mmol/L; P = 0.02) and there tended to be an interaction of source and day where the difference in plasma P was larger for samples collected closer to calving (P = 0.10).

Effects of Source and Level on Postpartum Outcomes

      DMI, EBAL, BW and BCS. Postpartum DMI, EBAL, BW, BW change and BCS are presented in Table 6-7. An interaction of source, level and time was observed for postpartum DMI (P = 0.03; Figure 6-1) such that DMI was numerically higher for cows fed MA-HM in wk 2 postpartum and for cows fed CS-HM in wk 4. While different patterns were apparent over time for different treatment groups, tests of treatment effects at individual weeks were not significant. There also tended to be a source, level and time interaction for DMI as a percent of BW (P = 0.14). There tended to be an effect of source on BW change such that cows fed MA lost less BW between wk 1 and wk 6 postpartum (CS = -61 vs. MA = -43 kg, P = 0.11). Body condition score tended to be higher for cows fed MA than cows fed CS (CS = 3.14 vs. MA = 3.20, P = 0.14); differences were small but were consistent with effects on BW change and plasma NEFA concentrations. No differences in calculated EBAL nor average BW were detected postpartum.

      Milk Yield and Composition. Results for milk yield, milk composition and milk production efficiency are presented in Table 6-7. Differences were not detected by source or level for milk yield or content and yield of true protein, lactose, or total solids. Similarly no differences in milk production efficiency nor somatic cell score were observed. A source by wk effect was found for fat content (P = 0.07), fat yield (P =0.02), 3.5% fat-corrected yield (P = 0.04), total solids yield (P = 0.05) and energy-corrected yield (P = 0.03). These effects were driven primarily by higher content and yield of fat in wk 1 for cows fed MA (P < 0.05; Figure 6-2). The DIM at which the first milk sampling occurred did tend to differ by source; however, it was closer to parturition by approximately one day for cows fed CS compared to cows fed MA

($P = 0.09$; CS = 3.25 ± 0.42 vs. MA = 4.34 ± 0.46). Controlling for the random effect of DIM at

sampling within sample week did not alter interpretation of the data and therefore the effect was

not included in the final model. There was a level by week effect on milk fat content ($P = 0.01$;

Figure 6-5) such that milk fat content was similar in all weeks except wk 5 when cows fed LM

had lower milk fat content ($P = 0.03$). Although this effect was statistically significant, the effect

in wk 5 likely has minimal biological importance. A trend for a level by week effect was also

found for MUN concentration ($P = 0.01$) and groups differed only in wk 1 ($P = 0.01$) and cows

fed LM had higher MUN.

   Plasma NEFA, BHB, Mineral Concentrations. Results for postpartum plasma NEFA

and BHB are presented in Table 6-5. Cows fed MA tended to have lower NEFA during the

postpartum period [CS = 604 (528-690) vs. MA = 512 (445-590) µEq/L; $P = 0.09$]. There was a

trend for an interaction of source and level on postpartum plasma BHB concentrations [CS-LM =

7.9 (6.7-9.2), CS-HM = 6.9 (5.9-8.1), MA-LM = 6.3 (5.3-7.3) and MA-HM = 7.3 (6.2-8.6)

mg/dL; $P = 0.09$]; BHB concentrations were numerically lowest in cows fed MA-LM, but

multiple comparisons using Tukey's adjustment did not detect differences between specific

groups.

   Results for postpartum plasma mineral concentrations are presented in Table 6-6 and

were analyzed separately as d 1 to 7 and d 9 to 21. Plasma Mg from d 1 to 7 tended to be lower

for cows fed LM postpartum (LM = 0.82 vs. HM = 0.85 mmol/L, $P = 0.11$) and plasma Mg also

tended to be lower for cows fed MA (CS = 0.85 vs. MA = 0.82 mmol/L, $P = 0.10$). From d 9 to

21, similar trends for source and level were found for plasma Mg concentrations and plasma Mg

tended to be lower for cows fed LM (LM = 0.89 vs. HM = 0.93 mmol/L, $P = 0.07$) and for cows

fed MA (CS = 0.93 vs. MA = 0.89 mmol/L, $P = 0.14$). Postpartum plasma P from d 1 to 7

tended to be higher for cows fed MA (CS = 1.40 vs. MA = 1.51 mmol/L, P = 0.09). There was no effect of source on plasma P from d 9 to 21 but cows fed LM tended to have lower plasma P in that period (LM = 1.65 vs. HM = 1.74 mmol/L, P = 0.13). There were no differences detected for plasma Ca concentrations in either period.

Hypocalcemia Prevalence and Health Disorders. Incidence of hypocalcemia was low in this trial. Peak prevalence of hypocalcemia occurred at 1 DIM and 54% of cows had plasma Ca concentrations ≤ 2.125 mmol/L (Martinez et al., 2016) with only 26% of cows having plasma Ca ≤ 2.0 mmol/L (Reinhardt et al., 2011). By 3 DIM prevalence of plasma Ca ≤ 2.125 mmol/L was 7% and prevalence of plasma Ca ≤ 2.0 mmol/L was 5%. Incidence of health disorders were as follows; retained placenta (MA-LM, n = 1), metritis (CS-LM, n = 3; CS-HM, n = 1; MA-LM, n = 2; MA-HM, n = 1), mastitis (CS-HM, n =1; MA-HM, n = 1), displaced abomasum (CS-LM, n = 1), hyperketonemia (defined by urine ketones "moderate" or higher for two consecutive days within 10 DIM, or severe drops in intake and milk production > 10 DIM; CS-LM, n =2; MA-LM, n = 1; MA-HM, n = 3). The sample size was insufficient to test the difference in incidence of health disorders between treatment groups.

Table 6- 1. Ingredient composition of prepartum and postpartum diets

Ingredient, % of DM	Prepartum Diet[1]		Postpartum Diet[1]			
	CS	MA	CS-LM	CS-HM	MA-LM	MA-HM
Brown mid-rib corn silage	37.59	37.59	38.00	38.00	38.00	38.00
Alfalfa hay	-	-	7.60	7.60	7.60	7.60
Wheat straw	23.23	23.23	6.21	6.21	6.21	6.21
Ground shelled corn	2.29	2.29	17.10	17.10	17.10	17.10
Wheat midds	6.54	6.54	4.71	4.71	4.71	4.71
Citrus pulp	4.31	4.31	4.75	4.75	4.75	4.75
Soybean hulls	6.50	6.50	2.13	2.13	2.13	2.13
Canola meal	3.33	3.33	3.80	3.80	3.80	3.80
Corn gluten feed	1.67	1.67	2.37	2.37	2.37	2.37
Distillers grains, ethanol	1.10	0.62	1.29	1.02	1.13	0.58
Amino Plus[2]	2.32	2.32	5.70	5.70	5.70	5.70
Gemini Protein[3]	1.99	1.99	2.28	2.28	2.28	2.28
Energy Booster 100[4]	-	-	1.14	1.14	1.14	1.14
Biochlor[5]	5.56	5.56	-	-	-	-
Alimet[6]	0.07	0.07	0.06	0.06	0.06	0.06
Salt	0.33	0.33	0.57	0.57	0.57	0.57
Sodium bicarbonate	-	-	0.38	0.38	0.38	0.38
Limestone	2.46	1.49	1.35	1.38	1.08	0.47
Ca sulfate	-	-	0.25	0.25	0.25	0.25
Mg oxide	0.41	0.09	0.13	0.38	0.05	0.08
MIN-AD[7]	-	1.78	-	-	0.52	1.66
Mineral oil	0.02	0.02	0.02	0.02	0.02	0.02
Rumensin[8]	0.04	0.04	0.06	0.06	0.06	0.06
Mineral & vitamin mix[9]	0.21	0.21	0.04	0.04	0.04	0.04

[1]Treatments consisted of a 2×2 factorial arrangement of source assignments (CS = common sources of supplemental Ca and Mg or MA = supplemental minerals from a commercial source) beginning at 21 d prior to due date, and level assignments (LM = postpartum formulated diet Mg at 0.30% of DM or HM = postpartum formulated diet Mg at 0.45% of DM) beginning within 1 d after parturition. Both the source and level assignments were continued through the 42 d postpartum study period.
[2]Heat treated soybean meal, Ag Processing, Inc. Omaha, NE
[3]Protein blend, Papillon Agricultural Company, Inc., Easton, MD
[4]Commercial fat source, Milk Specialties Global, Eden Prairie, MN
[5]Anionic supplement, Church & Dwight Co., Inc., Trenton, NJ
[6]2-hydroxy-4-(methylthio)-butanoic acid, Novus International, Saint Charles, MO
[7]Ca-Mg dolomite; Papillon Agricultural Company, Easton, MD
[8]Premix contained 26,400 g/ton monensin, Elanco Animal Health, Greenfield, IN
[9]Prepartum mix contained 21,164 ppm Zn, 5,580 ppm Cu, 16,931 ppm Mn, 147 ppm Se, 144 ppm Co, 611 ppm I, 2,407 KIU/kg Vit D, 87,571 IU/kg Vit E; Postpartum mix contained 103,050 ppm Zn, 22,009 ppm Cu, 82,440 ppm Mn, 693 ppm Se, 712 ppm Co, 3,012 ppm I, 11,869 KIU/kg Vit D, 241,913 IU/kg Vit E

Table 6- 2. Analyzed nutrient composition (mean ± SD) and partitioning of mineral intake by sources

	Prepartum Diet[1]		Postpartum Diet[1]			
	CS	MA	CS-LM	CS-HM	MA-LM	MA-HM
Nutrient, mean ± SD[2]						
DM, %	43.0 ± 1.8	43.8 ± 1.4	43.0 ± 1.0	43.2 ± 1.1	42.8 ± 1.0	43.1 ± 1.0
CP, % of DM	14.3 ± 0.4	14.1 ± 0.6	14.9 ± 0.2	15.0 ± 0.3	15.2 ± 0.4	15.4 ± 0.4
ADF, % of DM	28.1 ± 0.9	29.5 ± 0.6	20.9 ± 0.2	21.5 ± 0.6	21.2 ± 1.0	21.1 ± 0.5
NDF, % of DM	43.4 ± 0.8	45.4 ± 0.9	32.5 ± 0.2	32.9 ± 0.3	33.2 ± 0.9	33.4 ± 0.9
Lignin, % of DM	3.9 ± 0.2	4.1 ± 0.2	3.3 ± 0.1	3.3 ± 0.1	3.2 ± 0.1	3.1 ± 0.1
Starch, % of DM	15.8 ± 0.8	14.5 ± 1.5	25.5 ± 0.9	25.3 ± 0.6	24.6 ± 0.4	25.2 ± 0.4
NFC, % of DM	33.0 ± 1.2	31.2 ± 0.9	45.2 ± 0.7	43.7 ± 0.29	43.5 ± 1.3	43.6 ± 1.2
Fat, % of DM	2.17 ± 0.08	2.22 ± 0.15	3.25 ± 0.26	3.10 ± 0.09	3.04 ± 0.13	2.87 ± 0.24
Ca, % of DM	1.44 ± 0.00	1.40 ± 0.00	1.21 ± 0.08	1.13 ± 0.06	1.17 ± 0.07	1.24 ± 0.03
P, % of DM	0.35 ± 0.00	0.34 ± 0.00	0.36 ± 0.01	0.34 ± 0.01	0.37 ± 0.01	0.36 ± 0.00
Mg, % of DM	0.49 ± 0.02	0.52 ± 0.01	0.35 ± 0.02	0.40 ± 0.01	0.35 ± 0.01	0.48 ± 0.00
K, % of DM	1.08 ± 0.02	1.08 ± 0.03	1.00 ± 0.03	0.98 ± 0.03	1.02 ± 0.03	1.01 ± 0.04
S, % of DM	0.45 ± 0.01	0.44 ± 0.01	0.32 ± 0.01	0.33 ± 0.02	0.33 ± 0.02	0.33 ± 0.02
Na, % of DM	0.26 ± 0.01	0.25 ± 0.02	0.42 ± 0.01	0.42 ± 0.00	0.43 ± 0.01	0.43 ± 0.01
Cl, % of DM	0.79 ± 0.04	0.80 ± 0.05	0.53 ± 0.01	0.53 ± 0.02	0.54 ± 0.01	0.53 ± 0.02
DCAD, mEq/100 g DM	-11.2 ± 1.0	-11.1 ± 1.4	8.7 ± 1.1	7.7 ± 1.2	8.9 ± 1.7	9.2 ± 1.9
NE_L, Mcal/kg	1.46 ± 0.02	1.42 ± 0.01	1.64 ± 0.01	1.61 ± 0.01	1.62 ± 0.01	1.60 ± 0.01
MP, g/kg DM[3]	90.5	90.2	113.0	113.0	112.7	112.5
MP Intake, g/d[4]	1439	1515	2158	2237	2141	2284
Mineral Intake Sources[4]						
Mg from MIN-AD, % of Mg	-	39.3	-	-	17.1	39.8
Mg from MgO, % of Mg	45.4	9.2	20.6	51.9	7.1	9.3
Mg from Other, % of Mg	54.7	51.5	79.4	48.1	75.8	51.0
Ca from MIN-AD, % of Ca	-	27.3	-	-	9.6	28.8
Ca from Limestone, % of Ca	65.0	40.4	42.4	46.6	35.1	14.5
Ca from Other, % of Ca	35.0	32.4	57.6	53.4	55.4	56.7

[1]Treatments consisted of a 2 × 2 factorial arrangement of source assignments (CS = common sources of supplemental Ca and Mg or MA = supplemental minerals from a commercial source) beginning at 21 d prior to due date, and level assignments (LM = postpartum formulated diet Mg at 0.30% of DM or HM = postpartum formulated diet Mg at 0.45% of DM) beginning within 1 d after parturition. Both the source and level assignments were continued through the 42 d postpartum study period

[2]Weekly samples were composited over 4 week intervals, chemical analysis conducted on 2 composites for each prepartum diet and 4 composites for each postpartum diet

[3]Metabolizable protein (**MP)** intake as predicted by CNCPS (v. 6.5) based on analyzed composition of forages

[4]Based on actual 21 d intake prepartum and postpartum, predicted diet MP concentration, formulated supplemental mineral source inclusion rates and analyzed ration composite mineral concentrations, Mg and Ca from "other" represents basal ingredients and was determined by difference

Table 6- 3. Distribution of lactation number and means ± S.D. for previous lactation 305 d mature equivalent milk production, days on prepartum treatment diets, BW and BCS at enrollment for treatment groups

Variable	Treatment[1]				P-values[2]		
	CS-LM	CS-HM	MA-LM	MA-HM	S	L	S × L
Entering parity							
2nd	7	7	6	4	0.54	0.76	0.84
3rd+	4	4	4	5			
Previous 305 ME, kg	15,753 ± 1,819	15,959 ± 2,042	15,650 ± 2,109	15,327 ± 1,208	0.53	0.92	0.65
Days on treatment	22 ± 6	21 ± 4	21 ± 6	21 ± 6	0.81	0.94	0.59
BW, kg	775 ± 90	767 ± 58	793 ± 42	777 ± 88	0.54	0.61	0.87
BCS	3.42 ± 0.20	3.33 ± 0.25	3.25 ± 0.12	3.39 ± 0.25	0.40	0.71	0.09

[1]Treatments consisted of a 2 × 2 factorial arrangement of source assignments (CS = common sources of supplemental Ca and Mg or MA = supplemental minerals from a commercial source) beginning at 21 d prior to due date, and level assignments (LM = postpartum formulated diet Mg at 0.30% of DM or HM = postpartum formulated diet Mg at 0.45% of DM) beginning within 1 d after parturition. Both the source and level assignments were continued through the 42 d postpartum study period
[2]S = source; L = level

Table 6- 4. Least squares means and standard errors for prepartum DMI, DMI as a percentage of BW, energy balance (EBAL), BW, BW change, and BCS for cows fed one of two sources of supplemental dietary Ca and Mg

Variable[3]	Treatments[1]		SEM	P-values[2]	
	CS	MA		S	S×W
DMI, kg/d	15.9	16.8	0.3	0.03	0.52
DMI, % of BW	2.00	2.10	0.03	0.02	0.88
EBAL, Mcal/d	7.2	8.1	0.4	0.12	0.71
BW, kg	800	801	3	0.70	0.19
BW change, kg[4]	20	22	3	0.58	-
BCS	3.42	3.47	0.02	0.13	0.56

[1]Treatments consisted of a 2×2 factorial arrangement of source assignments (CS = common sources of supplemental Ca and Mg or MA = supplemental minerals from a commercial source) beginning at 21 d prior to due date, and level assignments (LM = postpartum formulated diet Mg at 0.30% of DM or HM = postpartum formulated diet Mg at 0.45% of DM) beginning within 1 d after parturition. Both the source and level assignments were continued through the 42 d postpartum study period
[2]S = source; W = week
[3]Data collected in the 3 wk prior to parturition
[4]Difference between BW measurement at week -3 and week -1

Table 6- 5. Least squares means and standard errors, or geometric means and back transformed 95% CI, for prepartum and postpartum non-esterified fatty acids (NEFA) and BHB for cows fed one of two sources of supplemental dietary Ca and Mg in the peripartum rations and one of two dietary Mg levels postpartum

	Treatments[1]				P-values[2]					S×L×
Variable	CS-LM	CS-HM	MA-LM	MA-HM	S	L	S×D	L×D	S×L	D
Prepartum[3]										
NEFA, µEq/L	142 (130-155)		117 (107-129)		0.005	-	0.17	-	-	-
BHB, mg/dL	5.0 ± 0.2		5.0 ± 0.2		0.86	-	0.90	-	-	-
Postpartum[4]										
NEFA, µEq/L	616 (511-743)	591 (491-713)	519 (427-631)	505 (411-620)	0.09	0.72	0.22	0.64	0.95	0.39
BHB, mg/dL	7.9 (6.7-9.2)	6.9 (5.9-8.1)	6.3 (5.3-7.3)	7.3 (6.2-8.6)	0.27	0.84	0.76	0.90	0.09	0.51

[1]Treatments consisted of a 2 × 2 factorial arrangement of source assignments (CS = common sources of supplemental Ca and Mg or MA = supplemental minerals from a commercial source) beginning at 21 d prior to due date, and level assignments (LM = postpartum formulated diet Mg at 0.30% of DM or HM = postpartum formulated diet Mg at 0.45% of DM) beginning within 1 d after parturition. Both the source and level assignments were continued through the 42 d postpartum study period
[2]S = source; L = level; D = day
[3]Samples collected twice weekly in the 3 wk before parturition
[4]Samples collected on days 1, 3, 5, 7, and three times per week through 21 DIM

Table 6- 6. Least squares means and standard errors for prepartum and postpartum plasma mineral concentrations for cows fed one of two sources of supplemental dietary Ca and Mg in the peripartum rations and one of two dietary Mg levels postpartum

	Treatments[1]					P-values[2]					
Variable	CS-LM	CS-HM	MA-LM	MA-HM	SEM	S	L	S×D	L×D	S×L	S×L×D
Prepartum[3]											
Ca, mmol/L	2.44		2.44		0.02	0.85	-	0.93	-	-	-
Mg, mmol/L	0.96		0.96		0.01	0.59	-	0.33	-	-	-
P, mmol/L	1.69		1.79		0.03	0.02	-	0.10	-	-	-
Postpartum (d 1-7)[4]											
Ca, mmol/L	2.35	2.37	2.41	2.38	0.04	0.38	0.85	0.49	0.57	0.54	1.00
Mg, mmol/L	0.84	0.87	0.81	0.84	0.02	0.10	0.11	0.99	0.59	0.92	0.78
P, mmol/L	1.43	1.38	1.47	1.55	0.07	0.09	0.86	0.82	0.63	0.32	0.49
Postpartum (d 9-21)[5]											
Ca, mmol/L	2.56	2.63	2.56	2.58	0.03	0.44	0.17	0.19	0.38	0.40	0.82
Mg, mmol/L	0.91	0.94	0.87	0.92	0.02	0.14	0.07	0.83	0.34	0.64	0.18
P, mmol/L	1.67	1.70	1.63	1.79	0.06	0.68	0.13	0.84	0.91	0.30	0.69

[1]Treatments consisted of a 2 × 2 factorial arrangement of source assignments (CS = common sources of supplemental Ca and Mg or MA = supplemental minerals from a commercial source) beginning at 21 d prior to due date, and level assignments (LM = postpartum formulated diet Mg at 0.30% of DM or HM = postpartum formulated diet Mg at 0.45% of DM) beginning within 1 d after parturition. Both the source and level assignments were continued through the 42 d postpartum study period
[2]S = source; L = level; D = day
[3]Samples collected twice per week in the 3 wk before parturition and within 2 h after parturition
[4]Samples collected daily from 1 through 7 DIM
[5]Samples collected three times per week from 8 through 21 DIM

Table 6- 7. Least squares means and standard errors for DMI, EBAL, BW and BCS parameters as well as milk yield and composition over the first 6 wk postpartum for cows fed one of two sources of supplemental dietary Ca and Mg in the peripartum rations and one of two dietary Mg levels postpartum

| Variable[3] | Treatments[1] | | | | | P-values[2] | | | | | |
	CS-LM	CS-HM	MA-LM	MA-HM	SEM	S	L	S×W	L×W	S×L	S×L×W
DMI, kg/d	20.7	21.3	20.7	21.4	0.7	0.98	0.32	0.57	0.35	0.88	0.03
DMI, % BW	2.97	3.07	2.92	3.03	0.09	0.57	0.21	0.37	0.40	0.95	0.14
EBAL, Mcal/d	-8.0	-8.5	-9.1	-7.9	1.0	0.79	0.73	0.67	0.50	0.36	0.90
BW, kg	699	698	699	713	7	0.25	0.32	0.34	0.93	0.26	0.24
BW change, kg[4]	-59	-63	-42	-42	13	0.11	0.87	-	-	0.87	-
BCS	3.14	3.14	3.20	3.19	0.04	0.14	0.95	0.78	0.17	0.78	0.97
Milk yield, kg/d	45.0	46.5	45.9	44.0	1.8	0.64	0.89	0.19	0.47	0.30	0.54
Fat, %	3.85	3.72	3.84	3.94	0.11	0.35	0.90	0.07	0.01	0.34	0.84
Fat, kg/d	1.68	1.68	1.75	1.72	0.09	0.51	0.81	0.02	0.44	0.88	0.82
3.5% FCM, kg/d[5]	46.9	47.5	48.4	46.7	2.0	0.85	0.77	0.04	0.27	0.54	0.73
Protein, %	2.86	2.81	2.83	2.87	0.07	0.78	0.90	0.64	0.50	0.49	0.55
Protein, kg/d	1.24	1.24	1.29	1.23	0.05	0.67	0.49	0.18	0.41	0.55	0.24
Lactose, %	4.86	4.84	4.81	4.87	0.05	0.85	0.72	0.29	0.97	0.50	0.97
Lactose, kg/d	2.18	2.23	2.24	2.14	0.10	0.86	0.76	0.26	0.90	0.44	0.66
Total solids, %	12.5	12.3	12.4	12.6	0.2	0.51	0.94	0.38	0.32	0.36	0.77
Total solids, kg/d	5.53	5.56	5.72	5.50	0.25	0.78	0.69	0.05	0.68	0.61	0.51
ECM, kg/d [6]	46.1	46.5	47.6	45.9	1.9	0.80	0.70	0.03	0.50	0.54	0.58
ECM/DMI	2.26	2.22	2.30	2.15	0.07	0.80	0.19	0.27	0.83	0.40	0.46
MUN, mg/dL	6.74	6.58	7.30	6.61	0.35	0.37	0.20	0.74	0.06	0.43	0.47
SCS	1.90	2.09	2.46	1.81	0.47	0.76	0.60	0.83	0.40	0.35	0.57

[1]Treatments consisted of a 2×2 factorial arrangement of source assignments (CS = common sources of supplemental Ca and Mg or MA = supplemental minerals from a commercial source) beginning at 21 d prior to due date, and level assignments (LM = postpartum formulated diet Mg at 0.30% of DM or HM = postpartum formulated diet Mg at 0.45% of DM) beginning within 1 d after parturition. Both the source and level assignments were continued through the 42 d postpartum study period
[2]S = source; L = level; W = week
[3]Data collected weekly, or daily and reduced to weekly means (DMI and milk yield), from parturition through 6 wk postpartum
[4]Difference between BW measurement at week 1 and week 6
[5]3.5% FCM = $(0.432 \times$ kg of wk average milk yield) + $(16.216 \times$ kg of fat)
[6]ECM = $(0.327 \times$ kg of wk average milk yield) + $(12.95 \times$ kg of fat) + $(7.65 \times$ kg of true protein)

Figure 6- 1. Least squares means and standard errors for DMI (kg/d) for 3 wk prepartum and 6 wk postpartum. Treatments consisted of a 2×2 factorial arrangement of source assignments (CS = common sources of supplemental Ca and Mg or MA = supplemental minerals from a commercial source) beginning at 21 d prior to due date, and level assignments (LM = postpartum formulated diet Mg at 0.30% of DM or HM = postpartum formulated diet Mg at 0.45% of DM) beginning within 1 d after parturition. Prepartum and postpartum data were analyzed separately. An effect of source was detected for prepartum DMI (P = 0.03) with no source by week effect (P = 0.52). A three way interaction of source, level and week was detected for postpartum DMI (P = 0.03).
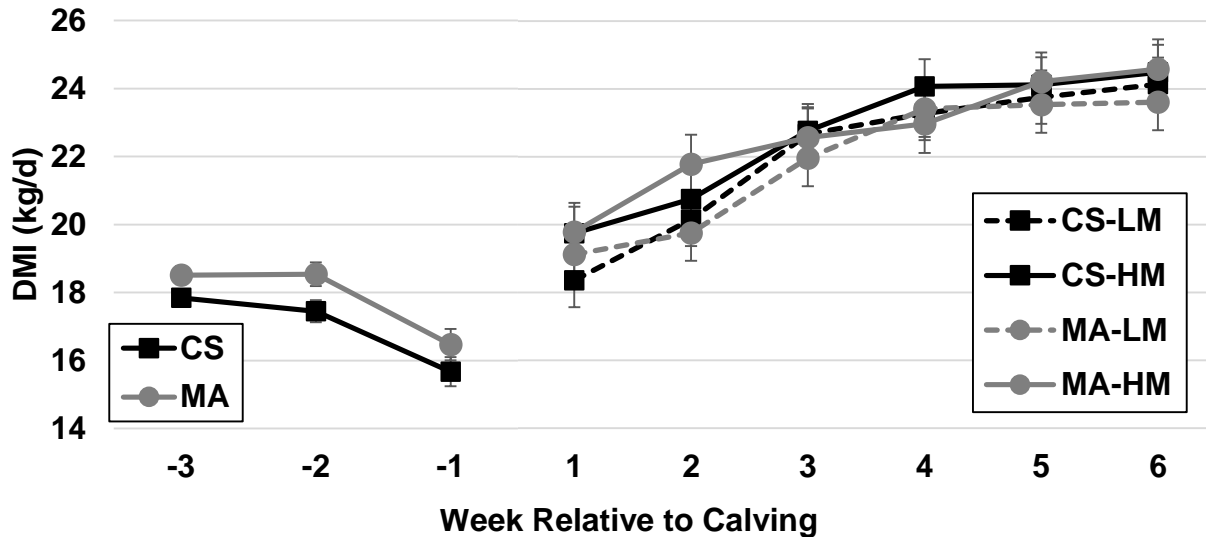
Figure 6- 2. Least squares means and standard errors for milk fat content by supplemental mineral source (A), milk fat content by postpartum dietary Mg level (B), milk fat yield by supplemental mineral source (C), and milk fat yield by postpartum dietary Mg level. Treatments consisted of a 2×2 factorial arrangement of source assignments (CS = common sources of supplemental Ca and Mg or MA = supplemental minerals from a commercial source) beginning at 21 d prior to due date, and level assignments (LM = postpartum formulated diet Mg at 0.30% of DM or HM = postpartum formulated diet Mg at 0.45% of DM) beginning within 1 d after parturition. There were no interactions of source and level therefore results are presented as main effects. A source by week interaction was observed milk fat content ($P = 0.07$) and milk fat yield ($P = 0.02$) and a level by week interaction was observed for milk fat content ($P = 0.01$) but there was no effect of level or interaction of level and time on milk fat yield ($P > 0.44$). For outcomes with treatment by time interactions, asterisks (*) indicate timepoints at which treatment means significantly differ ($P \leq 0.05$).
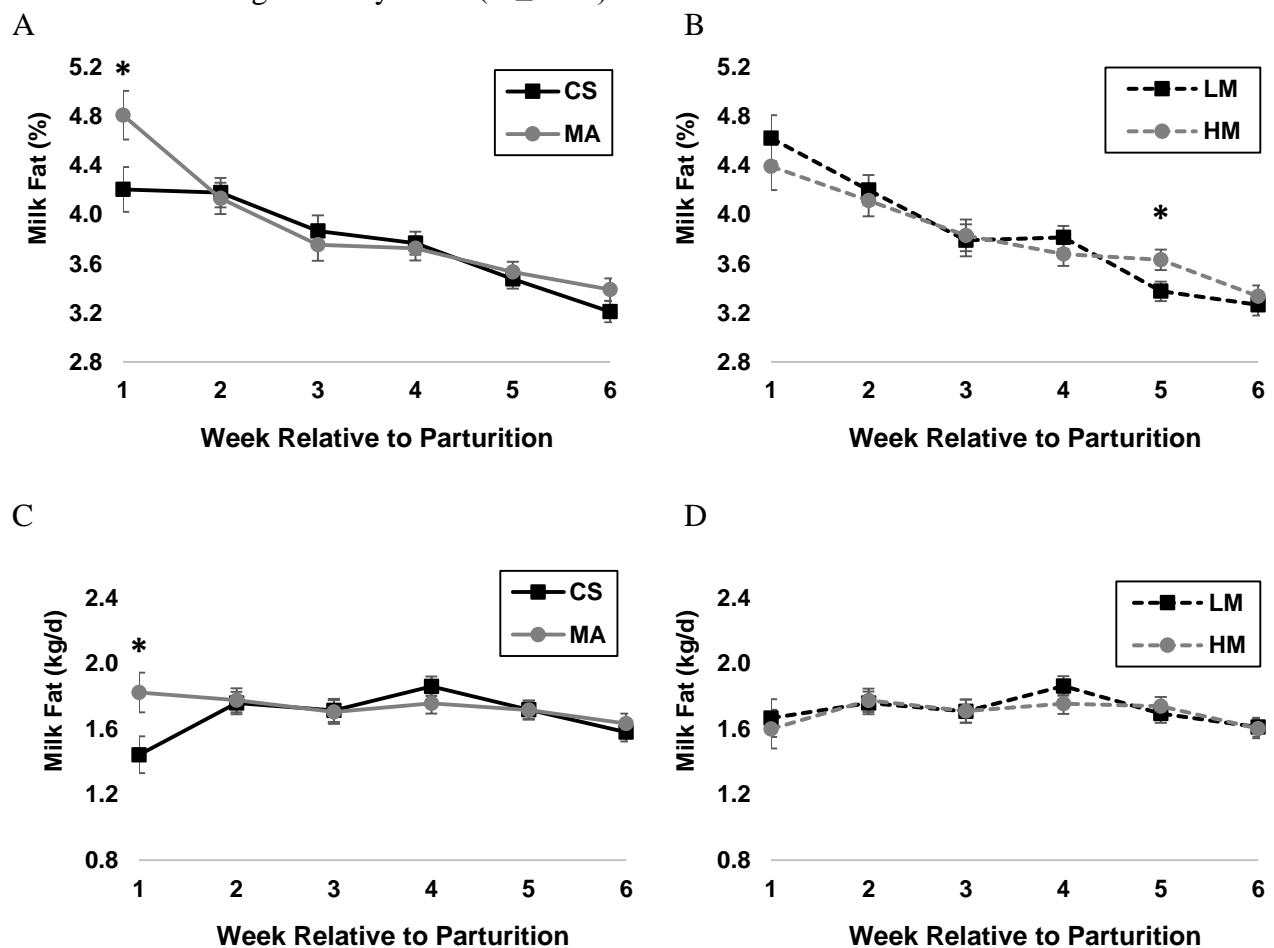
DISCUSSION

Mineral Status

The primary objective of this experiment was to determine the effect of varying the primary supplemental source of Ca and Mg in the peripartum rations, and postpartum feeding rate of Mg, on plasma mineral status during the transition period. Based on the small numerical differences observed for several outcomes in the study, the statistical power to detect these differences is low. Especially for the interaction between source and level, the probability of type II error is high and the results should be interpreted in that context. The trend for a difference in postpartum plasma Mg concentration indicated an effect of both dietary concentration of Mg and source of supplemental minerals. Increasing dietary Mg concentration increased blood concentrations of Mg for ruminants in some studies (Chicco et al., 1972; Greene et al., 1983a) but not others (Van Ravenswaay et al., 1989; Jittakhot et al., 2004). Urine Mg excretion more consistently responds to the increased Mg supply (Chicco et al., 1972; Van Ravenswaay et al., 1989; Jittakhot et al., 2004). This response indicates more total absorbed Mg and is consistent with an increase in absorption of Mg as ruminal supply of Mg increase (Care et al., 1984; Weiss, 2004). Contrary to our hypotheses, minimal effects of dietary Mg level on other aspects of plasma mineral status were found, indicating that the small differences in plasma Mg did not affect Ca homeostasis. Marginal effects of dietary Mg level on other outcomes measured in this experiment indicated that the differences in plasma Mg concentrations observed in cows fed LM did not limit intake or performance compared to cows fed HM. Formulated Mg concentrations of all rations were above NRC (2001) requirements, and the analysis of TMR composite samples showed that the LM rations had higher Mg concentrations than formulated. Increasing ration K concentration has been shown to inhibit Mg absorption in ruminants (Care et

al., 1984; Jittakhot et al., 2004; Weiss, 2004) and dietary K concentration in this study was quite low. Taken together, it is plausible that all diets allowed for total absorbed Mg above requirements in both the LM rations as well as the HM rations, and therefore physiological functions requiring Mg were supplied adequately despite differences in plasma Mg concentrations.

A trend for a difference in postpartum plasma Mg concentrations was also observed for cows fed MA compared to cows fed CS where cows fed MA had lower plasma Mg concentrations. One potential mechanism for this difference in blood Mg is greater bioavailability of Mg from Mg oxide as opposed to Mg from the commercial Ca-Mg dolomite. Early work in steers comparing Mg oxide to dolomitic limestone suggested that the latter had reduced bioavailability of Mg (Moore et al., 1971) or Mg and Ca (Gerken and Fontenot, 1967). Similar results have been observed in sheep fed Mg oxide or dolomitic limestone (Rahnema and Fontenot, 1983). In contrast, Crawford et al. (2008) observed no difference in apparent absorption of Ca, Mg, or P for cows fed a Ca-Mg dolomite in partial replacement of Mg oxide and limestone. The discrepancy between studies may be a result of the solubility of the Ca and Mg in the particular mineral sources used. A large amount of variation in apparent absorption of Mg from different Mg oxide sources has been identified in the literature (Ammerman et al., 1972; Van Ravenswaay et al., 1989; Xin et al., 1989). These differences are potentially due to the calcination temperature used in manufacturing and the resulting solubility of Mg (Van Ravenswaay et al., 1989) as well as physical processing of these mineral sources and the resulting particle size (Jesse et al., 1981). It is plausible that the same variability exists for Ca-Mg dolomites, although to the author's knowledge this variation has not been described in ruminant feed sources.

No differences were detected in prepartum plasma Mg in the current study. This may have been due to the high mineral feeding rates and high DMI observed in that period, offsetting potential differences in bioavailability with higher total supply. Because demand for Mg is relatively low in the dry period for fetal development (House and Bell, 1993) compared to during lactation when milk production causes a drain on circulating Mg (Shappell et al., 1987; Tsioulpas et al., 2007) concentrations of Mg in blood are likely more easily maintained in the prepartum period. Differences in bioavailability of Mg in this period would likely have been more sensitively reflected in the urine (Jesse et al., 1981; Van Ravenswaay et al., 1989). Plasma Mg concentrations do not always increase in response to feeding mineral sources with apparently higher Mg bioavailability (Xin et al., 1989), however, strong linear correlations between apparently absorbed Mg and urinary Mg as well as urinary Mg and plasma Mg in sheep (Chicco et al., 1972) indicate that these measures provide insight into the Mg absorbed from the diet in some situations. Since bioavailability was not measured in this trial, this cannot be definitively identified as the cause of differences in plasma Mg concentrations.

Interactions between ruminal concentrations of Ca and Mg and subsequent absorption have been investigated in sheep, demonstrating decreased absorption of Mg as ruminal Ca concentrations increase (Behar, 1975; Care et al., 1984). Whereas the site of Ca absorption in ruminants is primarily thought to be post-ruminal (Yano et al., 1979; Greene et al., 1983c; Rahnema and Fontenot, 1983) evidence suggests that significant ruminal absorption of Ca can occur in some situations (Greene et al., 1983b; Khorasani et al., 1997) and this could contribute to the antagonistic relationship between absorption of these two divalent cations. In a trial conducted in transition dairy cows, Kronqvist et al. (2011) fed one of three levels of Ca prepartum and found that as prepartum dietary Ca was increased, apparent Mg digestibility and

postpartum plasma Mg concentrations were decreased. In that study, dietary Ca concentration was only varied prepartum and did not affect blood Ca concentrations but differences in plasma Mg concentrations were evident in the postpartum period, suggesting a carryover effect of prepartum dietary Ca level. In the current study, despite the fact that prepartum dietary concentrations of Ca were similar, ruminal supply of Ca may have been higher in cows fed MA due to higher intakes or greater solubility of Ca from the Ca-Mg dolomite. This may have impaired Mg absorption resulting in compromised postpartum plasma Mg concentrations in those cows. The lack of a detected difference in plasma Ca concentrations due to supplemental mineral source may indicate that the difference in postpartum plasma Mg concentrations did not impair Ca homeostasis.

Higher plasma P concentrations for cows fed MA was observed both prepartum and in the week after parturition. Blood P concentrations have been demonstrated to increase when higher dietary P concentrations are fed (Barton et al., 1987; Peterson et al., 2005) and if the same response can be expected when total intake of P is increased, the higher intake observed in cows fed MA prepartum may have contributed to this difference. In a study conducted in steers in which feeding a dolomitic form of Mg was compared to feeding Mg oxide and Mg carbonate, apparent P absorption tended to increase for steers fed the dolomite (Moore et al., 1971). Baseline blood P concentrations was lower in steers fed the dolomite in that study so it is unclear if this is an effect of source. A possible antagonistic effect of Ca or Mg, depending on the amount and solubility of these minerals in different parts of the gastrointestinal tract, may have been responsible for altering solubility of P. This has been demonstrated in ruminant diets with increasing Ca concentrations (Yano et al., 1979) and potentially could lead to decreased absorption of P due to the formation of chelates.

DMI and Performance

Despite detecting minimal effects on plasma mineral concentrations in this study, meaningful differences in intake and plasma energy metabolites were found for cows fed supplemental minerals from the commercial Ca-Mg dolomite source, as compared to commonly used Mg oxide and limestone. This suggests that differences in performance were at least partly the result of some mechanism other than differences in mineral status in the transition period. Different Mg oxide and Mg carbonate sources have been demonstrated *in vitro*, using rumen fluid from dairy cows (Jesse et al., 1981; Schaefer et al., 1982), and *in vivo*, in dairy heifers, to have differences in buffering capacity (Schaefer et al., 1982). In addition to the effects observed on DMI, the differences in milk fat percentage and yield in wk 1 for cows fed MA suggest that the differences in ration mineral sources may have altered ruminal fermentation. Although buffering capacity was not measured in the current trial with these particular mineral sources, it is plausible that differences in buffering capacity between sources could influence rumen health during the transition period. The transition onto diets immediately after parturition with higher concentrations of rapidly fermentable carbohydrates has been demonstrated to result in subacute ruminal acidosis (Penner et al., 2007). Although variation in buffering capacity of the mineral sources and feeding rates may contribute to the differences observed in postpartum DMI, it is unlikely that this accounts for the increase in DMI observed prepartum.

A difference in the palatability of mineral sources in this study is a potential contributing factor to the effects observed on DMI. To the author's knowledge, direct comparisons of Mg oxide, limestone, and Ca-Mg dolomites fed to transition dairy cows with similar diets to those in the current study are not available for comparison. A similar approach in steers in which Mg oxide and limestone were partially replaced with Ca-Mg dolomites determined that DMI of

steers fed a high concentrate diet were not affected by these mineral source differences (Crawford et al., 2008). In a review conducted by Erdman et al. (1980), several studies investigating the inclusion of Mg oxide as a buffer were compiled and feeding Mg oxide resulted in decreased DMI compared to controls (no buffer included in ration) when fed in conjunction with low (<30%) forage diets, whereas no effect was observed in high forage diets. The rations in the current study all had > 50% forage (DM basis); however, different diet characteristics, such as the inclusion rate of the mineral sources, may influence effects on DMI. Feeding buffers in ruminant rations has been demonstrated to increase the liquid passage rate of rumen contents (Haaland and Tyrrell, 1982) and in steers fed Ca-Mg dolomites compared to Mg oxide and limestone, a decrease in the flow of fluid out of the rumen has been demonstrated (Crawford et al., 2008). Differences in liquid passage rate between source treatments in the current study may have resulted in altered ruminal fermentation. In addition, ruminal Mg supply has been identified as an important component of voluntary feed intake (Ammerman et al., 1971; Ammerman et al., 1972; Chicco et al., 1973) as well as cellulose digestion (Ammerman et al., 1971) in sheep, and decreased Mg supply resulted in decreased DMI as well as decreased *in vivo* and *in vitro* cellulose digestion. If the Mg oxide fed in the current study were more ruminally soluble, this may have resulted in unintended consequences for rumen fermentation, such as increased liquid passage rate and fluctuations in rumen Mg concentrations. In the high fiber prepartum rations fed in the current trial, differences in rumen fermentation efficiency due to lower liquid passage rates or a more consistent release of Mg in the rumen could have resulted in increased fiber digestion rates for cows fed MA and subsequently higher DMI. The speculated alterations in palatability, passage rate and ruminal digestion cannot be confirmed from the current trial, but

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future work assessing mineral sources in transition cow rations should investigate these interactions.

Although effects of treatment on DMI in the postpartum period were not as consistent as in the prepartum period, other factors may have altered the response to these sources postpartum. First, prepartum ration composition and expected digestion and passage rates vary from the postpartum rations and probably influence the effect of the mineral sources on rumen functions. Second, inclusion rate of the Ca-Mg dolomite in the postpartum ration as a percent of DM was similar in the MA-HM group compared to the prepartum MA ration, but was much lower in the MA-LM group. Higher inclusion rates of the mineral source may be necessary to observe the effects seen in the prepartum period. Third, other diet factors aside from alterations in passage rate and Mg supply may have limited ruminal fermentation postpartum. Milk urea N concentrations are related to measures of ration crude protein, intake and productivity (Broderick and Clayton, 1997) and previous work has observed MUN concentrations similar to that observed in the current study in groups with limitations in performance (Olmos Colmenero and Broderick, 2006). Decreased MUN concentrations can be a reflection of feeding diets in which rumen degradable protein and rumen ammonia concentrations are limiting which can result in decreased microbial growth and ultimately reduce milk and milk protein yield (Olmos Colmenero and Broderick, 2006). Low MUN concentrations in the current study suggest that there may have been limitations in microbial growth; however, diets were formulated for adequate metabolizable protein supply and it is possible that performance was not compromised because protein requirements not met with microbial supply were met with rumen undegradable sources.

Energy Metabolites, BW and BCS

Plasma concentrations of NEFA were lower for cows fed MA during the prepartum period in this study, consistent with the higher DMI observed in those cows. Postpartum plasma NEFA concentrations tended to remain lower over the first 3 wk after parturition for cows fed MA despite the time and Mg feeding rate dependent effects on intake. Consistent with lower plasma NEFA concentrations, cows fed MA tended to maintain higher BCS and lose less BW in the postpartum period. When effects on energy metabolites are considered with the increase in milk fat percentage and yield in wk 1 for cows fed MA, the data suggest that the source of additional milk fat was not mobilized adipose tissue and further supports altered ruminal fermentation in cows fed MA.

CONCLUSIONS

Varying postpartum feeding rate of Mg, as well as the primary source of supplemental Ca and Mg, for periparturient multiparous cows resulted in altered plasma Mg and P concentrations. The consequences of these changes are unclear because performance was similar for all groups. Ultimately, these differences in plasma Mg concentrations did not impact plasma Ca status and suggests that any of the Mg level or mineral source approaches tested in this study could be implemented to maintain similar Ca status postpartum. The population of cows in this study had low incidence of hypocalcemia overall. Increases in DMI prepartum period, and in parts of the postpartum period, due to supplemental source of Ca and Mg, as well as decreases in plasma NEFA concentrations, suggest that there may be opportunity for strategic use of mineral sources in the transition period to promote intake and metabolic health.

ACKNOWLEDGEMENTS

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CHAPTER 7

DIFFERENTIAL EFFECT OF A SINGLE DOSE OF ORAL CA BASED ON

POSTPARTUM PLASMA CA CONCENTRATION IN HOLSTEIN COWS

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ABSTRACT

Our objectives were to determine: 1) the effect of a single dose of an oral Ca bolus within 24 h after parturition on plasma Ca concentration, 2) the response of primiparous (**PP**) and multiparous (**MP**) cows to this supplementation strategy, and 3) differential responses based on plasma Ca at enrollment. For objective 1, cows from 1 commercial dairy in New York State were enrolled within 19 h after parturition (mean $\pm$ SD = 8.3 $\pm$ 5.3 h) and randomized within parity group (1st, 2nd, and $\geq$ 3rd) to control [**CON** (n = 25); no placebo] or a single dose bolus treatment [**BOL** (n = 25); 3 oral Ca boluses supplying 53 to 63 g of Ca]. Plasma Ca was measured repeatedly between 1 and 24 h following treatment. For objectives 2 and 3, cows on 6 commercial farms in New York State were assigned to treatment as for objective 1 (CON n = 1,973; BOL n = 1,976). Herd records for health, reproduction and DHIA test day milk production were collected. Mixed effect multivariable models were developed using repeated measures ANOVA, Poisson regression or proportional hazard models. Objective 2 analyses considered treatment with periparturient risk factors while objective 3 analyses also considered Ca status. No difference was observed for plasma Ca between 1 and 24 h after treatment. Primiparous cows assigned to BOL calving at > 712 d old had decreased risk of one or more health disorders ($\leq$ 30 DIM; risk ratio (**RR**) = 0.65, 95% confidence interval (**CI**) = 0.51 to 0.84) and those with BCS > 3.5 responded to BOL with increased milk production (CON = 31.7 $\pm$ 1.1, BOL = 35.1 $\pm$ 1.1 kg/d), as did those with days carried calf >277 (CON = 31.9 $\pm$ 1.0, BOL = 34.7 $\pm$ 1.0 kg/d). Reduced risk of one or more health disorders was observed in parity $\geq$ 3 (RR= 0.85, 95% CI = 0.81 to 0.89) and MP cows with BCS >3.5 (retained placenta; RR = 0.70, 95% CI = 0.58 to 0.84) or that were lame (displaced abomasum; RR = 0.49, 95% CI = 0.32 to 0.75). Differential responses for PP cows by Ca status were minimal. For MP cows with low plasma

Ca, BOL decreased risk of additional Ca treatment (≤ 1.8 mmol/L; RR = 0.57, 95% CI = 0.40 to 0.80) as well as risk of one or more health disorders (≤ 2.15 mmol/L; RR = 0.90, 95% CI = 0.85 to 0.95). Supplementation with a single oral dose of Ca could be targeted to periparturient risk groups for improved health. Calcium status did not differentiate responses of PP cows, but MP cows with low Ca at parturition had improved health status when supplemented.

INTRODUCTION

Dairy cows experience myriad metabolic adaptations around the time of parturition (Bauman and Currie, 1980), one of which is meeting the increased demand for Ca that occurs at the onset of colostrum and milk production (Ramberg et al., 1970) compared to the relatively low Ca demand for fetal development in late gestation (House and Bell, 1993). Even with multiple physiological adaptations in place to maintain normocalcemia, many cows fail to meet this demand without compromising systemic Ca status. As a result, these cows can experience clinical hypocalcemia or, in the absence of clinical symptoms, be categorized as having subclinical hypocalcemia (**SCH**) based on blood Ca concentration (Reinhardt et al., 2011). Recent research has demonstrated that cows with SCH suffer exacerbated negative energy balance and metabolic disease (Chapinal et al., 2011; Chamberlin et al., 2013) as well as increased susceptibility to infectious diseases in the early postpartum period (Martinez et al., 2012). Subclinical hypocalcemia has been associated with greater risk of early lactation culling (Roberts et al., 2012) and compromised reproductive performance (Chapinal et al., 2012; Martinez et al., 2012).

In an effort to mitigate some of the downstream consequences for cows that experience SCH, supplemental Ca can be provided in the form of injections of readily available Ca or oral administration of Ca salts in the form of pastes or boluses. While injection of readily available

Ca is necessary for cows displaying clinical signs of hypocalcemia to prevent or treat

recumbency (Oetzel, 2013), oral Ca supplements in the form of boluses may be more appropriate

for suspected cases of SCH and have been demonstrated to provide a more moderate but

sustained increase in blood Ca concentration (Blanc et al., 2014). Due to the lack of cow side

tests validated for measurement of blood Ca, selection of cows to receive supplemental Ca is

often based primarily on parity, recognizing that older cows are more susceptible to SCH, with at

least 50% of older cows affected (Reinhardt et al., 2011; Caixeta et al., 2015). Recent research

conducted with oral Ca boluses indicates that group level responses are minimal (Martinez et al.,

2016b) or not evident (Oetzel and Miller, 2012). When specific risk groups are assessed, effects

of Ca supplementation in the immediate postpartum period can be beneficial, such as in cows

with high production potential and cows that are lame near parturition (Oetzel and Miller, 2012;

Martinez et al., 2016b). However, detrimental responses have been observed in primiparous

cows and multiparous cows with low productive potential or at lower risk of uterine disease

(Martinez et al., 2016a; Martinez et al., 2016b). Accounting for blood Ca status of cows at

parturition in conjunction with presence or absence of certain risk factors may further identify

subgroups for more targeted supplementation.

The efficacy of a single dose of oral Ca at parturition on health and performance

outcomes has not been previously addressed. The primary objective of our study was to

determine the effect of administering a single dose of an oral Ca bolus within 24 h after

parturition in an all-inclusive approach on blood Ca concentration, health outcomes and

productive and reproductive performance with consideration of additional periparturient risk

factors. Our secondary objective was to determine the differential response to bolus

administration based on blood Ca status prior to treatment allocation. We hypothesized that a

single dose of oral Ca would increase blood Ca concentration and that differential responses to oral Ca supplementation would be observed in health and performance outcomes due to periparturient risk factors and Ca status.

MATERIALS AND METHODS

Farms and Study Population

All animal use and procedures were approved by the Cornell University Institutional Animal Care and Use Committee (protocol 2014-0017). This study was conducted at 6 commercial dairy farms in New York State between February and December 2015. Criteria for farm enrollment included at least 500 milking cows, use of herd management software (DairyComp 305; Valley Agricultural Software, Tulare, CA), enrollment in monthly DHIA testing, no prophylactic use of supplemental Ca after calving or willingness to discontinue prophylactic use, and willingness of farm personnel to allocate animals to treatments and adhere to study protocols. These farms represent a convenience sample of eligible farms in close proximity to Lowville or Ithaca, NY. General herd information and details on transition cow management for each farm are outlined in Appendix 1.

Treatments, Animal Sampling, and Sample Analysis

Experiment 1. A subset of cows at one location (Farm A) were enrolled to determine the effect of a single oral dose of Ca on blood Ca concentrations over the first 24 h after bolus administration. A total of 50 cows [10 primiparous (**PP**) and 40 multiparous (**MP**)] were enrolled within 24 h after parturition (mean $\pm$ SD = 8.3 $\pm$ 5.3 h) between June and July of 2015. A sample size of 50 cows was determined in order to detect a difference in plasma Ca concentration of 0.12 mmol/L with 95% confidence and 80% power based on the variation in plasma Ca observed in the day following parturition in previous work conducted in our group

(Leno et al., In press.). All cows that had been moved to the close up dry cow pen prior to parturition that were not displaying clinical signs of hypocalcemia at the time of enrollment were eligible. Cows were enrolled sequentially by calving date and time and were randomly allocated to treatment group based on enrollment sheets that were generated separately for parity groups (1st, 2nd, and 3rd and greater). The random number function of Microsoft Excel (Microsoft Corporation, Redmond, WA) was used to allocate sequential pairs in enrollment sheets to one of two treatments. Cows assigned to control (**CON**; n = 25) received no intervention after parturition, and cows assigned to the bolus treatment (**BOL**; n = 25) received a single dose of an oral Ca bolus (Quadrical, Bio-vet, Inc., Barneveld, WI) which delivered between 53 and 63 g of Ca per dose (3 boluses) in the form of Ca chloride, Ca sulfate, Ca propionate, and Ca lactate. Boluses also contained niacin and vitamin D_3. Treatments were administered by trained farm personnel.

Blood samples were collected just prior to treatment allocation (time 0) and at 1, 2, 4, 8, 12 and 24 h after treatment administration. Samples were collected using 20 G vacutainer needles and 10 mL lithium heparin vacutainer tubes (Greiner Bio-One, Monroe, NC). Plasma was separated by centrifugation at 2,000 x g for 10 min in a portable centrifuge, harvested, snap frozen in liquid nitrogen, and stored at -20°C. Within 14 h after sample collection, samples were transported to the laboratory for storage at -80°C. Plasma samples were analyzed for total Ca concentration at the University of Illinois Veterinary Diagnostic Laboratory (Urbana, IL) using an Olympus AU680 automated chemistry analyzer (Beckman Coulter, Inc., Brea, CA) with reagents from Beckman Coulter.

Experiment 2. This experiment was conducted at all 6 farms to determine: 1) the effect of a single oral dose of Ca on health and performance outcomes and potential differential responses

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to treatment based on periparturient risk factors and 2) responses to this supplementation strategy based on blood Ca status prior to bolus administration. Eligibility criteria and enrollment were as described for Experiment 1. A sample size of 500 per group for PP cows was planned in order to detect a difference between 8 and 3.5% for retained placenta, 12 and 6.5% for metritis, 15% and 9% for mastitis, 38 and 47% for pregnancy to first service and a 1.5 kg/d response in milk yield with 95% confidence and power ≥80%. For MP cows, a sample size of 1,500 per group was planned to detect a difference between 8 and 5% for RP, 12 and 8% for metritis, 5 and 2.5% for DA, 15 and 11% for mastitis, 35 and 41% for pregnancy to first service and a 1 kg/d response in milk yield with 95% confidence and power ≥90%. The variation in early lactation milk yield (SD = 6 kg/d) was determined from previous work in our group (Leno et al., In press.)

Cows enrolled in Experiment 2 had a blood sample collected prior to treatment administration using a 20 G needle and 6 mL polypropylene syringe containing no anticoagulant which was then dispensed into a 4 mL vacutainer tube containing lithium heparin (Greiner Bio-One, Monroe, NC). Sample collection was conducted by trained farm personnel. Blood samples were stored at 5°C and shipped to a central location twice per week at which point they were centrifuged at 1,000 x g for 10 min at 22°C and plasma stored at -20°C until analysis.

Samples of the TMR fed to the close up dry cow groups and groups housing cows in early lactation were collected once per week at each farm for the duration of cow enrollment. Wet TMR samples were frozen at -20°C, dried at 55°C for 48 h in a forced air oven, and ground to 2 mm in a Wiley mill. A composite sample of each TMR was sent to a commercial laboratory (Cumberland Valley Analytical Services, Hagerstown, MD) for analysis of chemical composition according to wet chemistry methods as described by McCarthy et al. (2015). Average ingredient composition and chemical analysis of diets are presented in Appendix II.

Health, Milk Production, and Reproduction Data Collection

Cows were assigned BCS and locomotion scores (**LS**) between 0 and 10 DIM. Body condition scores were assigned within three categories (≤ 2.5, 2.75 to 3.5, ≥ 3.75) based on a five point scale in quarter point increments (Edmonson et al., 1989). Locomotion scores were assigned into two categories (not lame, lame). Cows with normal gait or an abnormal gate without a favored limb were categorized as not lame, while cows favoring a limb, as evidenced by limping or a hunched back, were categorized as lame. At farms B, C, D and F, BCS and LS were assigned by trained farm personnel. At farms A and E, scores were assigned by one trained researcher.

Farms were provided case definitions for identification of clinical health disorders that were identified and recorded by farm personnel in the herd management software DairyComp 305. Clinical hypocalcemia was defined as signs of weakness or recumbency with cold extremities causing the inability to rise within 72 h after parturition and no evidence of physical injury. Retained placenta (**RP**) was diagnosed if the placenta was not dispelled within 24 h after parturition. Metritis was defined as foul-smelling, watery uterine discharge that is red to brown in color within 14 d of parturition. Mastitis was defined as abnormal color (red-tinged or yellow) or texture (watery, flakes, or clumps) of milk. Displaced abomasum (**DA**) was defined as a high-pitched ping upon simultaneous percussion and auscultation on the right or left side of the cow along the line from the elbow to the hip which extends forward of the 10th rib if on the right side. Farm recorded occurrence of health disorders and culling (sold or died) within 30 DIM were collected from DairyComp 305 along with monthly test day milk production for the first 4 tests, success or failure of first service, and d to pregnancy by 150 DIM. For test day milk production, tests occurring less than 7 DIM were deleted due to variability in reporting, and the following

test was considered the first test day. For cows that aborted and were then rebred, pregnancy

date was considered to be the date of the breeding for which the first pregnancy was diagnosed.

Statistical Analysis

     Experiment 1. Statistical analysis was conducted using SAS (version 9.4, SAS Institute,

Cary, NC). A mixed effect repeated measures ANOVA model was conducted using the MIXED

procedure. The repeated effect was h with the effect of cow within treatment included as the

subject of repeated measures. There was no difference between CON and BOL for plasma Ca

concentration prior to treatment assignment when analyzed by univariate ANOVA in the GLM

procedure, and therefore plasma Ca concentration prior to treatment assignment was included as

a covariate in the model. Other fixed effects included treatment, h, parity group (1st, 2nd or 3rd

and greater) and the interaction between treatment and h. The Kenward Rogers method was used

for estimating denominator degrees of freedom. Five variance-covariance structures were tested

(compound symmetry, heterogeneous compound symmetry, toeplitz, antedependence 1,

unstructured) and the option resulting in the lowest Akaike's Information Criterion was selected.

Model residuals were inspected for normality and homogeneity of variance.

     Experiment 2. All milk production, health disorders, culling and reproductive outcomes

were analyzed separately for PP and MP cows. Descriptive data were generated using the FREQ

and UNIVARIATE procedures. The difference between treatment groups for PP and MP cows

for the occurrence of twinning, stillbirth, lameness and the distribution of BCS category were

subject to a chi-square test with the FREQ procedure, and the difference in DIM at first test day

was tested using univariate ANOVA with the GLM procedure. Two separate multivariable

models were developed for each outcome to address the 2 objectives of Experiment 2. First, to

address the efficacy of an all-inclusive approach to treatment with consideration of periparturient

risk factors, multivariable models were developed without considering blood Ca measured in samples collected prior to treatment assignment. In the second model for each outcome, Ca status prior to treatment assignment was also considered and therefore only the cows with Ca concentration determined on a blood sample collected prior to enrollment were included in the analysis. Since BCS and LS were not available for all cows, multivariate modelling within each objective was initially conducted for just the subset of cows with complete data. If BCS or LS did not remain in the final model, the process was repeated with the complete study population and BCS and LS were no longer considered as predictors.

Potential predictor variables considered in all models included calving season (winter = December, January, February; spring = March, April, May; summer = June, July, August; fall = September, October, November), days carried calf (**DCC**), LS, and BCS (recategorized as ≤3.5 and >3.5 because of few observations ≤2.75). Models developed for analysis of PP cow outcomes also included age at first calving (**AFC**). Models developed for analysis of MP cow outcomes also included parity group (2nd or 3rd and greater) and dry period length. For all models, the effect of farm was included as a random effect (repeated measures and proportional hazards models) or cows were clustered within farm and an exchangeable correlation matrix was used (Poisson regression models). The multivariable modelling approach involved initially offering all potential variables, as well as the treatment variable, to the model. Manual backward stepwise elimination was conducted to reach the base model by removing main effects with $P > 0.10$, however, the effect of treatment and parity group (for MP cows) were forced into all models regardless of P-value. Subsequently, interactions between treatment and additional variables that had biologically plausible relationships were offered to the base model one at a time. All possible interactions were considered, regardless of the significance of the main effect,

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considering that differential responses to treatment within stratified groups could mask the effects observed when the variable is only offered to the model as a main effect. All interactions resulting in $P < 0.10$ when considered individually where then added to the final multivariable model. At this point, any variables that were no longer important ($P > 0.10$) were removed. Type III *P*-values for model effects were used throughout the modelling process so that the order of variable inclusion did not affect interpretation. If interactions between treatment and continuous predictors were retained, the continuous predictor was categorized into quartiles and the categorized variable was forced into the model in place of the continuous variable for ease of interpretation. Estimates of treatment effects at each quartile were observed to determine any groups that responded similarly to simplify categories. The categorized variables were then considered in the model for the same outcome in objective 2. For interaction terms in the final model, the LSMEANS statement with the PDIFF option was used to generate pairwise comparisons between CON and BOL within each level of the confounding variable. Importance of these multiple contrasts was adjusted using a Bonferroni correction for the number of levels of the interacting variable.

Milk production across the first 4 test days after parturition was analyzed by repeated measures ANOVA using the MIXED procedure. Based on previous work demonstrating that production responses to oral Ca bolus supplementation were dependent on previous lactation 305-d mature equivalent milk production (Oetzel and Miller, 2012; Martinez et al., 2016b) this variable was dichotomized based on the DairyComp 305 reported relative value of the P305ME and considered in multivariable models for test day milk production of MP cows. Cows with relative value > 105% were categorized as high RANK305ME and cows with relative value ≤ 105% were categorized as low RANK305ME. The random effect of farm and the repeated

measure of test number with cow within treatment as the subject of repeated measures were included. In addition to treatment and parity group (for MP cows), the effect of test number and the interaction between treatment and test number were forced into the model. Three variance-covariance structures were considered: heterogeneous autoregressive 1, Toeplitz, and unstructured. The initial model containing all potential main effects was analyzed with each structure and the option with the lowest Akaike's Information Criterion was selected for further modelling. After interaction consideration and variable selection, the final model was analyzed with each of the 3 structures again to ensure that the structure chosen remained the best option.

Health event outcomes that were analyzed included additional supplemental Ca (administration of oral or injectable Ca ≤ 3 DIM), RP, metritis, DA, mastitis and culling within 30 DIM as well as the occurrence of one or more health outcome (RP, metritis, DA, or mastitis). To be included in the analysis as eligible to have the above health disorders, cows had to either be diagnosed with the disorder or have survived within the herd until at least 2 DIM for RP, 10 DIM for metritis, and 30 DIM for DA, mastitis, and one or more health disorders. The distribution between treatments of cows excluded from each health event category due to DIM at culling was tested using the chi-square test of the FREQ procedure to ensure that results were not biased by this screening. Analysis of these outcomes was conducted using a mixed effect Poisson regression model with a log link function with the GENMOD procedure to enable reporting of risk ratios.

Reproductive outcomes assessed included pregnancy risk to first service and days to conception within 150 DIM. Pregnancy risk to first service was analyzed using the same methods as described for health events. Additional variables considered included breeding code at first service (timed A.I. or heat breeding) and previous lactation days open for MP cows. Days

to pregnancy from the end of the voluntary waiting period (**VWP**) to 150 DIM was analyzed

with a Cox proportional hazard regression model using the PHREG procedure of SAS.

Voluntary waiting period was accounted for by specifying different entry times into the analysis

and therefore results represent the hazard for pregnancy between the end of the voluntary waiting

period and 150 DIM. Cows that were removed from the herd, classified as "do not breed" or bred

before the end of the VWP were removed from the analysis. Censoring was used to account for

cows that were removed from the herd or classified as "do not breed" between the end of the

VWP and before conceiving, or were not pregnant by 150 DIM. Variables offered to the model

were the same as for the analysis of pregnancy to first service with the exception of breeding

code at first service. Sensitivity analysis was conducted with final models to ensure that

censoring was noninformative. Kaplan-Meier analysis was used to generate survival curves by

treatment (stratified by the level of interacting variables if applicable based on the final

multivariable model) to determine median days from the end of the VWP to conception.

To address objective 2 of Experiment 2, Ca status prior to treatment administration was

dichotomized according to thresholds in 0.05 mmol/L intervals ranging from 1.6 to 2.3 mmol/L.

To ensure enough statistical power to assess the interaction between treatment and Ca status,

only the thresholds resulting in at least 10% of cows within each level of the contingency table

between treatment and the dichotomized Ca variables were considered. This resulted in a range

of thresholds for PP cows of 2.15 to 2.3 mmol/L and for MP cows 1.8 to 2.15 mmol/L. Models

were developed according to the method described previously with the addition of one of the

dichotomized Ca status variables as an additional predictor. This Ca status variable and its

interaction with treatment were forced into all models. For MP models, parity was included as a

main effect but due to the strong association between Ca status and parity ($P < 0.0001$ for all

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thresholds) the interaction between treatment and parity was not considered to allow for interpretation of the treatment by Ca status interaction, the effect of interest. A separate model was developed with each Ca status threshold. The final reported model was decided by the treatment by Ca status interaction with lowest probability of type I error.

RESULTS

Experiment 1

The results for plasma Ca concentration over the 24 h following treatment administration are presented in Figure 7-1. The median plasma Ca concentration prior to treatment administration was 2.00 mmol/L (range = 1.23 mmol/L to 2.48 mmol/L). There was no effect of treatment on average plasma Ca concentration over the observation period (CON = 2.00 and BOL = 1.96 mmol/L, $P = 0.36$), and there was no interaction between treatment and time ($P = 0.14$). There tended to be an effect of h, and plasma Ca concentration decreased over time.

Experiment 2

Off the 4,438 cows that calved during the enrollment period, 3,949 were enrolled in Experiment 2 and remained in the data set. Reasons for exclusion from the study population are outlined in Table 7-1. The final number of cows in each group were 1,973 for CON and 1,976 for BOL. Descriptive data for the final study population by farm is presented in Table 7-2. The balance of treatments for parity, twin calving, stillbirth, BCS, LS, and DIM at first DHIA test day are presented separately for PP and MP cows in Table 7-3. Prevalence of lameness tended to differ by treatment in the PP cow population ($P = 0.06$); however, lameness was offered to all statistical models. The balance of treatments was similarly analyzed for the subset of cows with BCS and LS, as well as the subset with plasma Ca concentration measured prior to treatment

administration, and the treatment balance of subpopulations was not markedly different for those subpopulations.

   Objective 1. Incidence of health disorders, culling and reproductive outcomes by treatment for PP cows are presented in Table 7-4 along with the associated contrasts comparing BOL to CON (by level of periparturient risk factors when applicable). Incidence of additional Ca supplementation (0.3% overall) and DA (0.6% overall) in PP cows were too low for statistical analysis. There were no effects of treatment on risk of metritis in PP cows ($P = 0.68$) while controlling for BCS category ($P = 0.09$) and no effect of treatment, pregnancy to first service ($P = 0.72$) and hazard of pregnancy between the VWP and 150 DIM ($P = 0.63$). No other variables were retained in the models for mastitis, pregnancy to first service and days to pregnancy. An interaction between treatment and AFC group was observed for risk of RP ($P = 0.04$) while controlling for calving season ($P = 0.03$) and DCC ($P = 0.06$). An interaction between treatment and AFC group was also observed for risk of mastitis ($P = 0.04$). Overall, there tended to be an interaction between treatment and AFC group for risk of having one or more health disorder ($P = 0.08$) while controlling for DCC ($P = 0.10$). There tended to be increased risk for early removal from the herd for PP cows receiving BOL while controlling for DCC ($P = 0.04$). An interaction between treatment and BCS category ($P = 0.008$) and an interaction between treatment and DCC group ($P = 0.05$) were observed for milk yield over the first 4 DHIA test days; the treatment contrasts for milk yield by level of BCS and DCC are presented in Table 7-5. Other variables retained in the model for test day milk production included AFC ($P = 0.02$) and treatment by DHIA test ($P = 0.68$).

   Incidence of health disorders, culling and reproductive outcomes by treatment for MP cows are presented in Table 7-4 along with the associated contrasts comparing BOL to CON (by

level of periparturient risk factors when applicable). No association between treatment and risk of additional Ca supplementation ($P = 0.16$) was detected while controlling for parity ($P = 0.07$) and dry period length ($P = 0.09$). Mastitis was unaffected by treatment ($P = 0.49$) in a model containing parity ($P = 0.06$). There was no association between treatment and risk of early removal from the herd ($P = 0.28$) while controlling for parity ($P = 0.05$), locomotion score ($P = 0.08$), and dry period length ($P = 0.04$). Similarly, there was no association between treatment and risk of pregnancy at 1st service ($P = 0.76$) controlling for parity ($P = 0.11$) and previous lactation days open ($P = 0.04$). An interaction between treatment and BCS for risk of RP ($P = 0.04$) was detected and there tended to be an interaction between treatment and parity ($P = 0.10$) while controlling for DCC ($P = 0.09$) and locomotion score ($P = 0.10$). There tended to be an interaction between treatment and locomotion score ($P = 0.06$) for risk of metritis while controlling for parity ($P = 0.04$) and BCS ($P = 0.05$). There tended to be an interaction between treatment and locomotion score for risk of DA ($P = 0.09$) while controlling for parity ($P = 0.07$). An interaction between treatment and parity for risk of one or more health disorder ($P = 0.05$) was detected while controlling for DCC ($P = 0.10$), locomotion score ($P = 0.04$) and BCS ($P = 0.09$). An interaction between treatment and dry period length group was observed for days to pregnancy from the end of the VWP ($P = 0.0006$) while controlling for parity ($P < 0.0001$), calving season ($P = 0.02$), and previous lactation days open ($P < 0.0001$). For milk yield across the first 4 DHIA test days, there was an interaction between treatment and DCC group ($P = 0.06$) while controlling for parity ($P < 0.0001$), locomotion score ($P = 0.0007$), BCS ($P = 0.0002$), RANK305ME ($P < 0.0001$), calving season ($P = 0.05$) and treatment by DHIA test number ($P = 0.59$). Results of the treatment contrasts for milk yield by DCC group are presented in Table 7-5.

Objective 2. The differential responses of PP cows to treatment based on Ca status for

health disorders, culling and reproductive outcomes are presented in Table 7-6 with the contrasts

comparing BOL to CON within each level of Ca status. This analysis was not conducted for risk

of early removal from the herd since there were too few observations to estimate the treatment by

Ca status interaction. The Ca status threshold resulting in the smallest probability of type I error

for the interaction between Ca status and treatment in all models was 2.20 mmol/L, with the

exception of metritis and days to pregnancy for which a threshold of 2.15 mmol/L was used.

There were no effects of treatment for PP cows in either Ca status group for risk of metritis ($P \geq$

0.12), conception to first service ($P \geq 0.30$) or days to pregnancy from the end of the VWP ($P \geq$

0.43). Risk of RP was decreased for PP cows assigned to BOL with high plasma Ca ($P = 0.01$)

while controlling for DCC ($P = 0.10$) and treatment by AFC group ($P = 0.04$). Primiparous cows

in the higher Ca status group had increased risk of mastitis when administered a bolus ($P <$

0.0001) and other predictors in the model included an interaction between treatment and AFC as

a continuous variable ($P = 0.04$). Risk of one or more health disorder tended to increase for PP

cows administered boluses in the higher Ca status group ($P = 0.03$) no other predictors were

included in the model. Differential responses to treatment based on Ca status for test day milk

production of PP cows are presented in Table 7-5. Milk production tended to be higher for PP

cows with high plasma Ca that received BOL ($P = 0.03$). Other variables retained in the model

for test day milk production included AFC ($P = 0.09$), treatment by DHIA test number ($P =$

0.69), and treatment by DCC group ($P = 0.03$).

The differential responses to treatment of MP cows based on Ca status for health

disorders, culling, and reproductive outcomes are presented in Table 7-7 with the contrasts

comparing BOL to CON for each level of Ca status. For MP cows, the plasma Ca threshold

utilized in the final model varied by outcome and ranged from 1.80 to 2.15 mmol/L. Risk of additional Ca treatment was reduced for cows with low plasma Ca that received BOL ($P = 0.001$) while controlling for parity ($P = 0.09$) and dry period length ($P = 0.05$). Risk of RP was reduced for cows in both high ($P = 0.001$) and low ($P = 0.0007$) Ca status groups while controlling for parity ($P = 0.08$), DCC ($P = 0.08$), and treatment by BCS ($P = 0.04$). Bolus administration reduced metritis risk for cows with low plasma Ca ($P = 0.0001$) and increased risk for cows with high plasma Ca ($P = 0.02$) and other predictors retained in the model included parity ($P = 0.04$), locomotion score ($P = 0.05$) and BCS ($P = 0.07$). Risk of DA was reduced for cows with low plasma Ca that received BOL ($P = 0.01$) in a model containing parity ($P = 0.05$) and dry period length ($P = 0.06$) also retained as predictors. Mastitis risk did not differ by treatment for cows in either Ca status group ($P > 0.11$) while controlling for parity ($P = 0.08$). Risk of one or more health disorder was decreased for cows with low plasma Ca assigned to BOL ($P < 0.0001$) while controlling for parity ($P = 0.02$) and locomotion score ($P = 0.04$). Risk of culling was not affected by treatment in either Ca status group ($P \geq 0.34$) while controlling for parity ($P = 0.04$), locomotion score ($P = 0.04$), dry period length ($P = 0.03$), and treatment by BCS ($P = 0.08$). Risk of pregnancy to first service was increased for cows receiving BOL with higher plasma Ca ($P = 0.001$), other variables retained in the model included parity ($P = 0.23$) and previous lactation days open ($P = 0.08$). Days to pregnancy from the end of the VWP was not affected by treatment in either Ca status group ($P \geq 0.06$) while controlling for parity ($P = 0.002$), previous lactation days open ($P < 0.0001$), calving season ($P = 0.01$), and treatment by dry period length ($P = 0.13$). Treatment by dry period length was significant when dichotomized (as in the model for objective 1, $P = 0.003$) but was retained as a continuous variable in this model to allow for interpretation of the interaction of interest at an average dry period length.

Differential responses to treatment based on Ca status for test day milk production of MP cows are presented in Table 7-5. Milk production over the first 4 DHIA test days did not differ for MP cows at either level of Ca status ($P \geq 0.06$). Other variables retained in the model include parity ($P < 0.0001$), locomotion score ($P = 0.003$), BCS ($P = 0.002$), RANK305ME ($P < 0.0001$), calving season ($P = 0.08$), treatment by DHIA test number ($P = 0.70$) and treatment by DCC group ($P = 0.06$).

Table 7- 1. Total number of eligible cows calving during the enrollment period, reasons for exclusion from the study, and the final study population

Exclusion reason	N
Calving not recorded by farm personnel	172
Farm personnel identified abortion or DCC <260	118
Enrolled prior to calving or >24 h after calving	83
Missing enrollment information	41
Clinical hypocalcemia at parturition	20
Dry period <30 d	18
Extreme calving difficulty (C-section/prolapse)	7
Inappropriate treatment allocation or administration	7
Sold as replacements	7
Died the day of calving	5
Other[1]	11
Total eligible	4,438
Final study population for Objective 1	3,949
Final study population for Objective 2[2]	3,341

[1]Includes conformational or behavioral issues prohibiting treatment administration, mastitis in the dry period, and calving off of the farm premises
[2]Cows with blood Ca concentration determined prior to treatment administration

Table 7- 2. Descriptive statistics of the study population and health outcomes by farm

	Farm					
Variable	A	B	C	D	E	F
Total (n)	595	478	897	513	543	923
Parity (n)						
1st	170	133	256	130	146	152
2nd	172	144	264	176	156	285
$\geq$3rd	253	201	377	207	241	486
Twin calving (%)	6.1	3.1	5.9	3.5	2.4	5.7
Stillbirth (%)	6.2	3.6	3.9	4.3	5.0	5.9
BCS (%)[1]						
≤ 2.5	3.4	6.8	3.7	5.6	1.6	2.5
2.75 to 3.5	81.8	82.6	78.7	83.8	68.2	73.2
≥ 3.75	14.8	10.7	17.6	10.6	30.1	24.3
Lame (%)[1]	28.7	22.4	42.1	22.3	37.2	10.3
Ca treatment risk (%)[2]	2.5	1.3	2.7	1.4	8.3	3.9
RP risk (%)[3]	3.5	3.8	10.5	0.2	8.5	10.7
DA risk (%)[4]	3.0	0.9	2.9	0.6	2.8	2.8
Metritis risk (%)[5]	15.7	4.8	9.8	1.2	8.5	14.7
Mastitis risk (%)[6]	2.1	7.4	5.8	6.1	1.9	4.5
Health disorder risk (%)[7]	21.2	16.7	23.2	7.9	17.9	23.3
Early removal risk (%)[8]	3.5	5.9	5.4	4.7	3.3	4.0
Pregnancy risk at 1st service (%)[9]	39.6	34.1	42.4	34.7	-	40.1

[1]BCS and locomotion scoring conducted by farm personnel (farms B, C, D and F) or researchers (farms A and E) between 0 and 10 DIM.
[2]Ca treatment risk = number of cows treated with supplemental Ca (injection or oral) $\leq$ 3 DIM / number of fresh cows.
[3]RP risk = number of cows diagnosed with retained placenta (RP) / number of fresh cows.
[4]DA risk = number of cows diagnosed with displaced abomasum (DA) $\leq$ 30 DIM / number of fresh cows (excluding those that were not diagnosed with DA and were culled $\leq$ 30 DIM).
[5]Metritis risk = number of cows diagnosed with metritis / number of fresh cows (excluding those that were not diagnosed with metritis and were culled $\leq$ 10 DIM).
[6]Mastitis risk = number of cows diagnosed with mastitis / number of fresh cows (excluding those that were not diagnosed with mastitis and were culled $\leq$ 30 DIM).
[7]Health disorder risk = number of cows diagnosed with RP, metritis, DA, or mastitis / number of fresh cows (excluding those that were not diagnosed with disorders and were culled $\leq$ 30 DIM).
[8]Early removal risk = number of cows that died or were sold $\leq$ 30 DIM / number of fresh cows.
[9]Pregnancy risk at 1st service = number of cows that conceived to the first breeding after parturition / number of cows that were bred at least once after parturition. Reproductive data for farm E was removed from analysis due to an extensive superovulation program

Table 7- 3. The distribution of parity, twin calving, stillbirth, BCS category and lameness (LS) and mean ± SD for DIM at first DHIA test day for treatment groups by parity

Variable	Primiparous			Multiparous		
	CON[1]	BOL[1]	*P*-value	CON[1]	BOL[1]	*P*-value
Cows, n	492	495		1481	1481	
Cows with LS & BCS (%)	76.6	78.0		72.5	73.5	
Cows with blood Ca (%)	74.6	73.5		88.0	87.0	
Parity (%)						0.91
2nd	-	-		40.3	40.5	
≥ 3rd	-	-		59.7	59.5	
Twin calving (%)	1.4	1.0	0.55	5.8	6.1	0.76
Stillbirth (%)	9.6	7.9	0.35	3.9	3.2	0.32
BCS (%)[2]			0.89			0.33
≤ 2.5	3.9	4.5		2.9	4.0	
2.75 to 3.5	78.5	78.6		77.2	76.9	
≥ 3.75	17.6	16.9		20.0	19.2	
Lame (%)[2]	7.2	11.1	0.06	36.4	36.2	0.91
1st DHIA test day (DIM)	22.5 ± 9.1	22.7 ± 9.0	0.68	22.2 ± 9.1	22.3 ± 9.2	0.72

[1]Treatments: CON = no intervention, BOL = a single dose of an oral Ca bolus providing 53 to 63 g of Ca in the form of 3 boluses within 24 h after parturition.
[2]BCS and locomotion scoring (LS) conducted by farm personnel (farms B, C, D and F) or researchers (farms A and E) between 0 and 10 DIM.

Table 7- 4. Contrasts generated from multivariable Poisson regression models comparing risk of health disorders and pregnancy to 1st AI service postpartum, as well as the contrasts generated from proportional hazards models for hazard of conception between the end of the VWP and 150 DIM, for cows receiving a single oral dose of Ca within 24 h following parturition (BOL) to those receiving no intervention (CON)

Outcome	Level of Interaction[1]	Treatment		RR[2]	95% CI	P-value[3]
		CON	BOL			
Primiparous						
RP risk (%)[4]	AFC Q1 to Q3[5]	5.2% (19/364)	4.8% (18/374)	0.98	0.70 to 1.37	0.91
	AFC Q4[5]	5.6% (7/126)	2.5% (3/119)	0.42	0.18 to 0.98	0.04
Metritis risk (%)[6]	-	11.0% (41/373)	11.0% (42/383)	1.03	0.89 to 1.19	0.68
Mastitis risk (%)[7]	AFC Q1 & Q2	1.6% (4/248)	4.6% (11/238)	3.10	1.47 to 6.55	0.003
	AFC Q3 & Q4	3.8% (9/238)	1.6% (4/245)	0.45	0.24 to 0.86	0.02
Health disorder risk (%)[8]	AFC Q1[5]	21.6% (27/125)	28.5% (35/123)	1.46	0.81 to 2.64	0.20
	AFC Q2 & Q3[5]	14.8% (35/237)	16.3% (40/246)	1.09	0.61 to 1.96	0.77
	AFC Q4[5]	16.0% (20/125)	10.3% (12/117)	0.62	0.43 to 0.88	0.008
Early removal risk (%)[9]	-	1.4% (7/492)	2.6% (13/495)	1.91	1.12 to 3.25	0.09
Pregnancy risk at 1st service (%)[10]	-	43.7% (171/391)	44.6% (176/395)	1.02	0.92 to 1.14	0.72
Median d to conception, d from VWP[11]	-	29 (26-35)	29 (26-37)	1.04[12]	0.89 to 1.22	0.63
Multiparous						
Ca treatment risk (%)[13]	-	4.9% (73/1481)	3.8% (57/1481)	0.78	0.53 to 1.15	0.16
RP risk (%)[4]	Parity = 2	5.0% (21/423)	6.7% (29/431)	1.11	0.72 to 1.73	0.64
	Parity ≥ 3	9.9% (64/647)	7.4% (48/651)	0.66	0.52 to 0.83	0.0003
	BCS ≤ 3.5	7.5% (65/865)	7.6% (67/885)	1.05	0.76 to 1.46	0.17
	BCS > 3.5	9.8% (20/205)	5.1% (10/197)	0.70	0.58 to 0.84	0.0002
Metritis risk (%)[6]	Not lame	6.4% (43/673)	8.4% (57/679)	1.31	0.99 to 1.74	0.06
	Lame	11.1% (42/380)	7.4% (28/381)	0.66	0.39 to 1.11	0.12
DA risk (%)[14]	Not lame	2.0% (13/665)	2.3% (15/668)	1.15	0.56 to 2.35	0.70
	Lame	6.3% (23/366)	3.3% (12/368)	0.52	0.32 to 0.83	0.01
Mastitis risk (%)[7]	-	5.3% (76/1422)	5.1% (72/1410)	0.95	0.84 to 1.08	0.49
Health disorder risk (%)[8]	Parity = 2	12.2% (51/419)	15.9% (68/427)	1.29	1.04 to 1.60	0.02
	Parity ≥ 3	25.8% (161/623)	20.6% (129/625)	0.80	0.70 to 0.90	0.0005
Early removal risk (%)[9]	-	4.4% (47/1073)	5.2% (57/1089)	1.18	0.89 to 1.57	0.28
Pregnancy risk at 1st service (%)[10]	-	37.5% (432/1151)	37.0% (431/1166)	0.99	0.91 to 1.07	0.76
Median d to conception, d from VWP[11]	Dry ≤ 52 d	41 (30-46)	50 (46-57)	0.81[12]	0.71 to 0.93	0.002
	Dry > 52 d	48 (42-54)	39 (32-46)	1.14[12]	0.99 to 1.32	0.07

[1]Periparturient risk factors resulting in interactions with treatment with $P \leq 0.10$.

[2]Control was the reference group for all contrasts.

[3]The threshold for significance is adjusted using a Bonferroni correction (PP health disorder risk $P \leq 0.016$, all other comparisons $P \leq 0.025$).

[4]RP risk = number of cows diagnosed with retained placenta (RP) / number of fresh cows.

[5]Age at first calving (AFC) quartiles were as follows: Q1 $\leq$ 649 d, Q2 = 650 to 673 d, Q3 = 674 to 712 d, Q4 > 712 d

[6]Metritis risk = number of cows diagnosed with metritis / number of fresh cows (excluding those that were not diagnosed with metritis and were culled $\leq$ 10 DIM).

[7]Mastitis risk = number of cows diagnosed with mastitis / number of fresh cows (excluding those that were not diagnosed with mastitis and were culled $\leq$ 30 DIM).

[8]Health disorder risk= number of cows diagnosed with RP, metritis, DA, or mastitis / number of fresh cows (excluding those that were not diagnosed with disorders and were culled $\leq$ 30 DIM).

[9]Early removal risk = number of cows that died or were sold $\leq$ 30 DIM / number of fresh cows.

[10]Pregnancy risk at 1st service = number of cows that conceived to the first breeding after parturition / number of cows that were bred at least once after parturition.

[11]Median days to conception from the end of the farm's voluntary waiting period (VWP), expressed as median (95% CI).

[12]Hazard ratio.

[13]Ca treatment risk = number of cows treated with supplemental Ca (injection or oral) $\leq$ 3 DIM / number of fresh cows.

[14]DA risk = number of cows diagnosed with displaced abomasum (DA) $\leq$ 30 DIM / number of fresh cows (excluding those that were not diagnosed with DA and were culled $\leq$ 30 DIM).

Table 7- 5. Least squares means for DHIA test day milk production over the first 4 tests following parturition for primiparous (PP) and multiparous (MP) cows receiving a single oral dose of Ca within 24 h following parturition (BOL) and those receiving no intervention (CON)

Outcome	Level of Interaction[1]	Treatment		P-value[2]
		CON	BOL	
Objective 1[1]				
PP milk yield, kg/d	BCS ≤ 3.5	33.0 ± 0.8	33.0 ± 0.8	0.96
	BCS > 3.5	31.7 ± 1.1	35.1 ± 1.1	0.003
	DCC ≤ 277[3]	32.7 ± 0.9	33.7 ± 0.9	0.12
	DCC > 277[3]	31.9 ± 1.0	34.7 ± 1.0	0.0009
MP milk yield, kg/d	DCC ≤ 277[3]	47.0 ± 1.2	46.8 ± 1.2	0.64
	DCC > 277[3]	46.8 ± 1.2	47.7 ± 1.2	0.03
Objective 2[1]				
PP milk yield, kg/d	> 2.2 mmol/L	32.1 ± 1.0	33.3 ± 1.0	0.03
	≤ 2.2 mmol/L	34.2 ± 1.1	33.4 ± 1.1	0.32
MP milk yield, kg/d	> 2.15 mmol/L	46.2 ± 1.2	45.8 ± 1.2	0.55
	≤ 2.15 mmol/L	47.1 ± 1.1	47.8 ± 1.1	0.06

[1]For objective 1, periparturient risk factors resulting in interactions with treatment with $P \leq 0.10$ were retained in the model and resulting contrasts are presented, for objective 2, the interaction between treatment and Ca status was forced into the model using several thresholds. The threshold resulting in the smallest probability of type I error for the interaction between treatment and Ca status is presented with the resulting contrasts.

[2]P-values are the result of contrasts between CON and BOL at each level of the interacting variable. The threshold for significance was adjusted using a Bonferroni correction ($P \leq 0.025$).

[3]DCC = Days carried calf.

Table 7- 6. Contrasts generated from multivariable Poisson regression models comparing risk of health disorders and pregnancy to 1st AI service postpartum, as well as the contrasts generated from proportional hazards models for hazard of conception between the end of the VWP and 150 DIM, for cows receiving a single oral dose of Ca within 24 h following parturition (BOL) to those receiving no intervention (CON) by blood Ca status prior to treatment

Outcome	SCH Group[1]	Treatment CON	BOL	RR[2]	95% CI	P-value[3]
RP risk (%)[4]	> 2.2 mmol/L	5.9% (14/236)	3.8% (9/236)	0.52	0.31 to 0.87	0.01
	≤ 2.2 mmol/L	4.6% (6/130)	7.1% (9/127)	1.36	0.75 to 2.46	0.32
Metritis risk (%)[5]	> 2.15 mmol/L	10.4% (29/278)	11.1% (32/289)	1.03	0.92 to 1.16	0.56
	≤ 2.15 mmol/L	9.3% (8/86)	2.7% (2/74)	0.45	0.17 to 1.24	0.12
Mastitis risk (%)[6]	> 2.2 mmol/L	0.9% (2/234)	4.3% (10/232)	4.20	2.27 to 7.74	<0.0001
	≤ 2.2 mmol/L	4.7% (6/128)	3.2% (4/124)	0.80	0.38 to 1.69	0.56
Health disorder risk (%)[7]	> 2.15 mmol/L	13.7% (38/277)	17.8% (51/286)	1.29	1.02 to 1.62	0.03
	≤ 2.15 mmol/L	14.1% (12/85)	9.7% (7/72)	0.83	0.49 to 1.41	0.49
Pregnancy risk at 1st service (%)[8]	> 2.2 mmol/L	41.4% (72/174)	46.3% (81/175)	1.12	0.90 to 1.39	0.30
	≤ 2.2 mmol/L	44.6% (45/101)	41.9% (44/105)	0.95	0.74 to 1.21	0.66
Median d to conception, d from VWP[9]	> 2.15 mmol/L	28 (24-35)	26 (24-31)	1.00[10]	0.81 to 1.24	1.00
	≤ 2.15 mmol/L	31 (21-49)	47 (24-66)	0.85[10]	0.55 to 1.29	0.43

[1]The blood Ca threshold used to categorize subclinical hypocalcemia (SCH) for each outcome was determined by the treatment by SCH interaction with the lowest probability of type I error for models derived along a range of Ca thresholds.

[2]Control was the reference group for all contrasts

[3]The threshold for significance is adjusted using a Bonferroni correction and is $P \leq 0.025$ for all comparisons.

[4]RP risk = number of cows diagnosed with retained placenta (RP) / number of fresh cows.

[5]Metritis risk = number of cows diagnosed with metritis / number of fresh cows (excluding those that were not diagnosed with metritis and were culled ≤ 10 DIM).

[6]Mastitis risk = number of cows diagnosed with mastitis / number of fresh cows (excluding those that were not diagnosed with mastitis and were culled ≤ 30 DIM).

[7]Health disorder risk = number of cows diagnosed with RP, metritis, DA, or mastitis / number of fresh cows (excluding those that were not diagnosed with disorders and were culled ≤ 30 DIM).

[8]Pregnancy risk at 1st service = number of cows that conceived to the first breeding after parturition / number of cows that were bred at least once after parturition.

[9]Median d to conception from the end of the farm's voluntary waiting period (VWP), expressed as median (95% CI).

[10]Hazard ratio.

Table 7- 7. Contrasts generated from multivariable Poisson regression models comparing risk of health disorders and pregnancy to 1st AI service postpartum, as well as the contrasts generated from proportional hazards models for hazard of conception between the end of the VWP and 150 DIM, for cows receiving a single oral dose of Ca within 24 h following parturition (BOL) to those receiving no intervention (CON) by blood Ca status prior to treatment

Outcome	SCH Group[1]	Treatment		RR[2]	95% CI	P-value[3]
		CON	BOL			
Ca treatment risk (%)[4]	> 1.8 mmol/L	2.5% (25/1005)	3.0% (30/990)	1.26	0.72 to 2.19	0.42
	≤ 1.8 mmol/L	13.4% (40/299)	8.0% (24/299)	0.57	0.40 to 0.80	0.001
RP risk (%)[5]	> 1.9 mmol/L	8.4% (50/599)	8.2% (50/609)	0.81	0.71 to 0.92	0.001
	≤ 1.9 mmol/L	7.6% (27/357)	5.2% (18/345)	0.57	0.41 to 0.79	0.0007
Metritis risk (%)[6]	> 2.15 mmol/L	8.1% (18/222)	11.8% (28/237)	1.56	1.07 to 2.26	0.02
	≤ 2.15 mmol/L	7.8% (56/720)	6.3% (44/697)	0.80	0.71 to 0.90	0.0001
DA risk (%)[7]	> 2.15 mmol/L	1.4% (4/297)	1.9% (6/317)	1.46	0.56 to 3.81	0.44
	≤ 2.15 mmol/L	4.3% (41/951)	2.4% (22/904)	0.53	0.33 to 0.86	0.01
Mastitis risk (%)[8]	> 1.95 mmol/L	5.2% (38/731)	3.7% (27/726)	0.71	0.45 to 1.10	0.13
	≤ 1.95 mmol/L	5.5% (29/523)	7.0% (35/500)	1.25	0.95 to 1.66	0.11
Health disorder risk (%)[9]	> 2.15 mmol/L	19.7% (43/218)	23.4% (55/235)	1.24	0.91 to 1.68	0.17
	≤ 2.15 mmol/L	21.0% (150/714)	17.0% (118/693)	0.80	0.72 to 0.89	<0.0001
Early removal risk (%)[10]	> 1.8 mmol/L	4.0% (29/724)	5.7% (41/718)	1.14	0.71 to 1.81	0.59
	≤ 1.8 mmol/L	5.6% (13/234)	4.9% (12/243)	0.71	0.36 to 1.43	0.34
Pregnancy risk at first service (%)[11]	> 2.1 mmol/L	37.5% (126/336)	43.6% (154/353)	1.16	1.06 to 1.28	0.001
	≤ 2.1 mmol/L	37.1% (247/666)	34.6% (226/653)	0.94	0.87 to 1.01	0.11
Median d to conception, d from VWP[12]	> 1.95 mmol/L	43 (33-47)	34 (29-34)	1.02[13]	0.90 to 1.16	0.18
	≤ 1.95 mmol/L	48 (42-54)	58 (49-65)	0.90[13]	0.75 to 1.07	0.06

[1]The blood Ca threshold used to categorize subclinical hypocalcemia (SCH) for each outcome was determined by the treatment by SCH interaction with the lowest probability of type I error for models derived along a range of Ca thresholds.

[2]Control was the reference group for all contrasts

[3]The threshold for significance is adjusted by a Bonferroni correction and is $P \leq 0.025$ for all comparisons.

[4]Ca treatment risk = number of cows treated with supplemental Ca (injection or oral) $\leq$ 3 DIM / number of fresh cows.

[5]RP risk = number of cows diagnosed with retained placenta (RP) / number of fresh cows.

[6]Metritis risk = number of cows diagnosed with metritis / number of fresh cows (excluding those that were not diagnosed with metritis and were culled $\leq$ 10 DIM).

[7]DA risk = number of cows diagnosed with displaced abomasum (DA) ≤ 30 DIM / number of fresh cows (excluding those that were not diagnosed with DA and were culled ≤ 30 DIM).

[8]Mastitis risk = number of cows diagnosed with mastitis / number of fresh cows (excluding those that were not diagnosed with mastitis and were culled ≤ 30 DIM).

[9]Health disorder risk = number of cows diagnosed with RP, metritis, DA, or mastitis / number of fresh cows (excluding those that were not diagnosed with disorders and were culled ≤ 30 DIM).

[10]Early removal risk = number of cows that died or were sold ≤ 30 DIM / number of fresh cows.

[11]Pregnancy risk at 1st service = number of cows that conceived to the first breeding after parturition / number of cows that were bred at least once after parturition.

[12]Median days to conception from the end of the farm's voluntary waiting period (VWP), expressed as median (95% CI).

[13]Hazard ratio.

Figure 7- 1. Blood Ca concentration (mmol/L) by h after treatment administration for a subset of cows (n = 25 per treatment) enrolled on farm A. Cows were randomly assigned to treatments within parity groups (1st, 2nd and 3rd and greater lactation) within 19 h after parturition [CON = no intervention, BOL = a single dose of an oral Ca bolus providing 53 to 63 g of Ca in the form of 3 boluses].There tended to be an effect of h ($P = 0.07$) but there was no overall effect of treatment ($P = 0.36$) or interaction between treatment and h ($P = 0.14$).
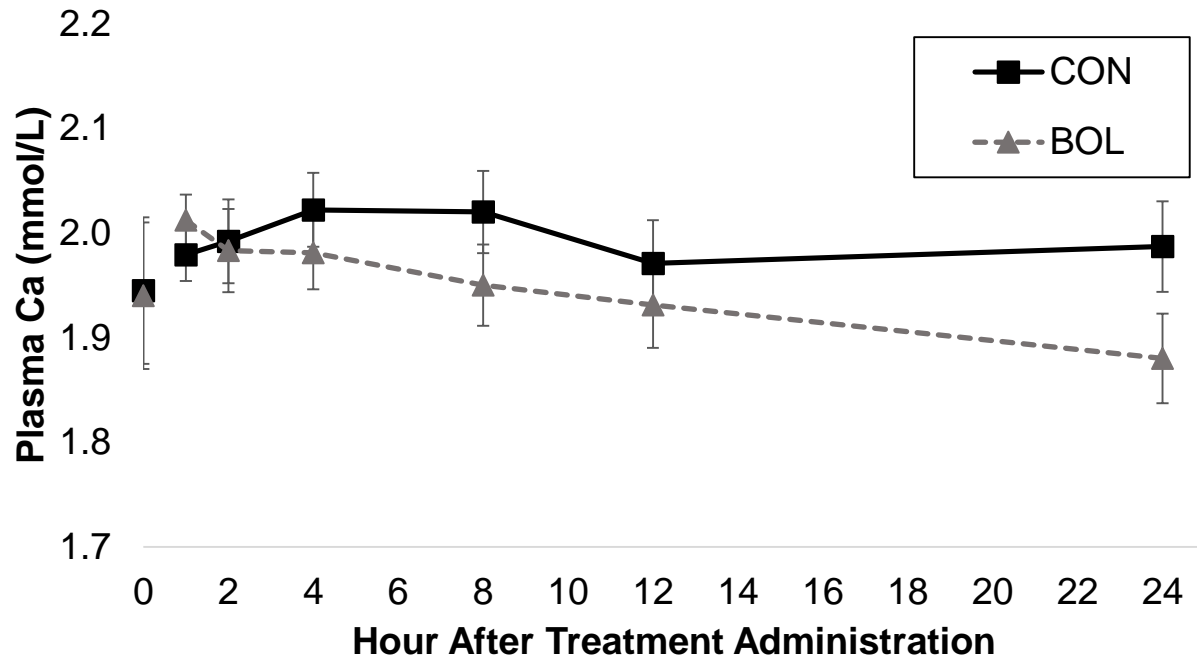
DISCUSSION

Experiment 1

Our primary hypothesis of Experiment 1 was that a single dose of an oral Ca bolus within 24 h after parturition would result in increased plasma Ca concentration. Recent studies that have assessed oral Ca bolus supplementation have implemented multiple dosing strategies. While this strategy has proven effective for increasing blood Ca concentration (Blanc et al., 2014), the additional doses require either more time away from the pen or more time restrained in headlocks within the days immediately following parturition. The additional handling time required to administer doses that occur after cows are handled at parturition are an added management complication and may interfere with cow behavior. Results of Experiment 1 demonstrated that administering a single dose of an oral Ca bolus did not increased blood Ca concentration between 1 and 24 h following administration. The amount of Ca supplied by this single dose of oral Ca may have been insufficient to result in increased systemic blood Ca concentration. Alternatively, the resulting increase in blood Ca from this supplementation strategy may have been too transient to be detected with the sampling scheme used due to the composition and form of the product. Martinez et al. (2016a) demonstrated that supplying either 43 or 86 g of Ca in the form of an oral bolus resulted in transiently increased blood Ca concentration with increases lasting for 2 to 4 h. Although the amount of Ca supplied in our study was intermediate to those two treatments, the product used in the trial by Martinez et al. (2016a) was different from our study in both the Ca salts incorporated in the product as well as the presence of a fat coating on the bolus, which may alter the dissolution time of the bolus in the rumen. Previous work using oral Ca drenches have demonstrated that different Ca salts (ie. Ca chloride vs. Ca propionate) result in a different magnitude and duration of increased blood Ca

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(Goff and Horst, 1994). Martinez et al. (2016a) demonstrated that the most pronounced response in blood Ca was observed at the first blood sample collected after bolus administration at 0.5 h, indicating that release and absorption of Ca occurs rapidly after bolus administration. With the first sample collected 1 h after bolus administration in the current study, a possible short spike in plasma Ca would not be detected. Additional limitations to Experiment 1 include the timing of enrollment of cows relative to parturition (mean $\pm$ SD = 8.3 $\pm$ 5.3 h) which may have increased the variation in blood Ca changes over the 24 h observation period as well as the low power to detect true differences due to the number of animals that were enrolled. A further limitation is that cows enrolled were not identified as hypocalcemic prior to enrollment, and responses could differ for cows with compromised Ca status.

Experiment 2

Objective 1. Previous work conducted on the use of oral Ca boluses for the mitigation of downstream consequences associated with SCH has demonstrated that responses at the group level are minimal, but specific risk groups can be identified that have positive responses to bolus administration (Oetzel and Miller, 2012; Martinez et al., 2016a; Martinez et al., 2016b). Further, the negative responses to bolus administration observed in some groups of cows (Martinez et al., 2016a; Martinez et al., 2016b) necessitate the determination of specific target groups to optimize the profitability of supplementation. Our primary hypothesis of this portion of the study was that responses to a single dose of oral Ca at parturition would similarly be dependent on periparturient risk factors. Indeed, the majority of responses that were observed were the result of treatment interactions.

Positive responses to bolus administration for PP cows were limited to cows calving with higher AFC and to those that were overconditioned at parturition. Positive responses to

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supplementation based on these risk factors for PP cows have not been previously identified. In

this study population, increasing AFC was positively associated with milk yield over the first 4

test days. Two previous studies have identified that MP cows with greater production potential

have positive responses to Ca supplementation (Oetzel and Miller, 2012; Martinez et al., 2016b).

Primiparous cows calving with higher AFC in our study might have had increased demand for

Ca, precipitating greater responses to supplementation. Increasing age has also been associated

with decreased intestinal absorption efficiency of Ca (Hansard et al., 1954) and lower indication

of bone metabolism at parturition (Taylor et al., 2008) which might contribute to a greater

challenge for older PP cows at parturition. The additional Ca supplied to those animals might

have been utilized to support metabolic and immune health, contributing to decreased risk of

disease in those cows. Primiparous cows that were overconditioned in the current study also

responded positively to supplemental Ca. Higher BCS around parturition has been associated

with greater risk of metabolic disease and compromised performance (Ospina et al., 2010; McArt

et al., 2012). Supplementing Ca to overconditioned cows could alleviate some of the underlying

causes of increased risk for metabolic disease in these animals such as declines in gut motility

that occur when blood Ca declines (Daniel, 1983; Martinez et al., 2014), supporting improved

intake postpartum and mitigating metabolic disease. Some reduction in the incidence or severity

of metabolic disease and higher dry matter intake would be supportive of greater milk yield in

those cows.

Previous work conducted in PP cows has demonstrated marked detrimental effects of

bolus administration including increased risk of metritis and compromised reproductive

performance (Martinez et al., 2016a; Martinez et al., 2016b). In that study, the amount of Ca

supplemented was much greater than in the current study and in one group, the cessation of bolus

administration in PP cows was followed by an increase in SCH prevalence compared to PP cows that were not supplemented. This suggests that the administration of this quantity of oral Ca may have impaired Ca homeostatic mechanisms, exacerbating the challenge of adapting to the increased demands of early lactation. The dose of Ca administered in the current study might have been sufficient to aid in the recovery of blood Ca in certain risk groups without impairing homeostatic mechanisms in those cows that were not at increased risk of hypocalcemia. Overall, PP cows that received supplemental Ca in the current study did have increased risk of early removal from the herd and PP cows with lower AFC had greater risk of mastitis when administered boluses. Taken together, the data suggest that a blanket treatment approach for PP cows would not be beneficial but that targeting treatment to the risk groups identified could result in positive responses.

Positive responses to Ca supplementation in MP cows were observed in cows entering parity 3 or greater, overconditioned cows, and lame cows. As discussed previously, age is a risk factor for hypocalcemia and compromised Ca metabolism in the peripartum period. Decreased risk of RP and risk of one or more health disorder in older cows supplemented with oral Ca may be a reflection of supplying additional Ca to support immune function in those cows. Functional capacity of innate immune cells is an important component of expelling the placenta after parturition (Kimura et al., 2002) and clearing the uterus of bacterial contamination introduced during parturition. Previous work has demonstrated that cows with hypocalcemia have compromised immune cell function (Kimura et al., 2006; Martinez et al., 2014) and greater risk of uterine disease (Martinez et al., 2012; Wilhelm et al., 2017), and this may have been alleviated to some extent in cows that received boluses in the current study. Cows that are lame have previously been identified to respond to oral Ca supplementation with decreased occurrence of

health disorders (Oetzel and Miller, 2012). Dry matter intake is likely further compromised in lame cows in the periparturient period which will impair the effectiveness of preventative strategies implemented in prepartum rations and result in reduced Ca intake around parturition.

Milk production tended to be higher for cows receiving boluses with DCC greater than 277. Interestingly, a similar response was observed in PP cows with DCC greater than 277. For MP cows, this difference was small (0.9 kg/d more for cows assigned to BOL) whereas the response was larger for PP cows (2.8 kg/d for cows assigned to BOL). The reason for this response is unclear, however, DCC may be an indirect measure of the time spent in the close up pen consuming a preventative negative DCAD ration. Those cows may have been more prepared to respond to supplemental Ca postpartum. Multiparous cows that received BOL with shorter dry periods had decreased hazard of pregnancy from the end of the VWP to 150 DIM. Cows with a shorter dry period may represent a group of cows at lower risk of health and reproductive issues. Taken with the increased risk of health disorders in MP cows entering their 2nd parity, our data suggests that a blanket approach to treatment may not be appropriate even for MP cows. Other researchers have also identified that subsets of MP cows thought to be at lower risk of periparturient health problems responded negatively to bolus administration (Martinez, 2016a).

Objective 2. Within the industry there is increased interest and application of blood Ca monitoring of cows in the immediate postpartum period. To date, this is only practically useful for herd-level monitoring because of the lack of cowside tests available for blood Ca determination which would be necessary for making individual cow treatment decisions. As these technologies advance, the opportunity to use this information for identification of cows to supplement should be investigated to aid in targeting our treatment strategies. In the current

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study, we aimed to determine thresholds for blood Ca measured within the day after parturition that differentiated responses to supplemental Ca. Other studies have investigated thresholds for blood Ca that were predictive of subsequent disease (Chapinal et al., 2011; Chapinal et al., 2012; Martinez et al., 2012); however, these thresholds was developed based on blood Ca measurements determined within the first 3 DIM or over the first week of lactation. The relationship between blood Ca concentration measured in the day following parturition and subsequent health and performance is complicated by the Ca utilization that is required to mount an immune response (Waldron et al., 2003; Martinez et al., 2014) as well as the Ca that is required to be excreted in milk (Shin-ichi and Shinobu, 1993). Consequently, low blood Ca in the day after parturition, within a reasonable range, could be reflective of a strong immune response at parturition or be necessary for appropriate adaptation to the lactational Ca demand. For these reasons, the interaction between Ca status and oral Ca supplementation was not limited to recent thresholds utilized for identification of SCH.

There were minimal differential responses of PP cows to Ca supplementation based on Ca status, indicating that utilizing periparturient risk factors for targeting treatment is more valuable than measurement and utilization of blood Ca concentration within the day following parturition. For MP cows, those with low plasma Ca (≤ 1.8 mmol/L) that received boluses had decreased risk of receiving additional supplemental Ca, suggesting that those cows were less likely to display signs of clinical hypocalcemia. While the results of Experiment 1 did not show differences in plasma Ca concentration, this finding suggests that for cows with low plasma Ca at enrollment, there was likely enough of an increase in blood Ca concentration to prevent muscular and nervous signs that would be detected by farm personnel to identify clinical hypocalcemia (Oetzel, 2013). Further, MP cows with low plasma Ca (≤ 2.15 mmol/L) were less likely to have

a DA when given boluses. Subclinical reductions in blood Ca concentration have been demonstrated to reduce gut motility (Daniel, 1983; Martinez et al., 2014) and supplementation of Ca could have alleviated this to some degree which would support greater intake in the immediate postpartum period and potentially aid in prevention of DA. Risk of RP was reduced for MP cows receiving a bolus that were above and below the blood Ca threshold and risk of metritis was reduced for MP cows with low plasma Ca. The associations between hypocalcemia and immune function discussed previously may have been alleviated by bolus supplementation in those cows. Overall, risk of having one or more health disorder in early lactation was reduced for cows that received boluses that also had low plasma Ca. The thresholds that differentiated responses to Ca supplementation ranged from 1.8 to 2.15 mmol/L suggesting that the thresholds used in recent literature to classify cows as SCH may not be directly applicable to identification of cows in need of supplementation within 24 h of parturition.

Responses of both PP and MP cows that were in the higher Ca status groups were inconsistent. In comes cases, cows receiving boluses in this group responded positively with decreased risk of RP in all parties, increased milk yield in PP cows and decreased risk of mastitis and increased pregnancy risk at first service in MP cows. In other cases, cows in the higher Ca status group responded negatively to bolus administration, such as with increased risk of mastitis in PP cows and increased risk of metritis in MP. Martinez et al. (2016a) also observed that administering Ca boluses increased risk of metritis in MP cows that had normal calving (no dystocia, twins, stillbirth, laceration or RP). While it is possible with the large sample size that some of these findings are the result of type I error, these results do suggest that bolus supplementation in cows with higher blood Ca is inconsistent. This is in contrast with responses observed in cows with low blood Ca which were positive when identified.

A limitation of our study was the variability in herd reporting of disease incidence. While all herds were provided standardized case definitions, very low disease occurrence in one herd suggested some inconsistency in reporting (Table 7-2). Randomization within farm ensured that risk of disease diagnosis was equal across treatments and inclusion of farm as a random effect accounted for the differences in likelihood of disease diagnoses between farms. Another limitation of the study is the lack of blinding of farm personnel to treatments. However, treatments were administered sequentially by calving time and therefore randomization eliminated treatment administration bias and cows were not differentiated by treatment beyond the time of handling at parturition.

CONCLUSIONS

Administration of a single dose of oral Ca at parturition supplying 53 to 63 g of Ca did not result in increased blood Ca concentration between 1 and 24 h postpartum; however, responses observed for health and performance outcomes suggest that cows in certain risk categories and MP cows with low plasma Ca at enrollment responded positively to bolus administration. Supplementation of PP cows with higher AFC or higher BCS at parturition positively affected health status and early lactation performance, respectively. In MP cows, supplementation of cows with higher parity, higher BCS, and lame cows also resulted in improved health status. Differential responses to treatment based on blood Ca concentration demonstrated that blood Ca was less reliable than other periparturient risk factors for identification of PP cows with potential to respond positively to Ca supplementation. For MP cows, those with low plasma Ca responded with decreased health disorders but cows with higher plasma Ca had varied responses.

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Table 7-Appendix I- 1. General herd management information by farm as reported in a herd management survey

Characteristic	Farm					
	A	B	C	D	E	F
Milking cows[1], n ± SD	1,474 ± 15	567 ± 69	1,282 ± 38	899 ± 19	1,677 ± 27	1,222 ± 21
Herd milk production[1], kg ± SD	38.4 ± 1.0	38.9 ± 1.9	37.0 ± 1.3	41.2 ± 2.9	37.3 ± 1.9	36.8 ± 1.1
Milking frequency, times/d	3	3	3	3	3/2[2]	3
Use of bST	Yes	Yes	No	Yes	No	No
Reproductive Management[3]						
Voluntary waiting period, DIM	55	60	63 parity =1 49 parity > 1	70	-	59
1st service management	Presynch-Ovsynch	Ovsynch	Presynch - Ovsynch	Double Ovsynch	-	Presynch-Ovsynch
2nd + service management	Resynch	Resynch	Ovsynch	CIDR synch & Ovsynch	-	Resynch & heat detection

[1]Obtained from weekly DairyComp 305 (Valley Agricultural Software, Tulare, CA) records for the duration of cow enrollment
[2]Cows were milked 3 times per day through 105 DIM and subsequently milked 2 times per day for the remainder of lactation
[3]Farm E used an extensive superovulation program and therefore reproductive data from this farm were not used

Table 7-Appendix I- 2. Management of the prefresh pen and maternity pen by farm as reported in a herd management survey

Characteristic	Farm					
	A	B	C	D	E	F
Prefresh pen						
Comingled[1]	No	Yes	Yes	No	Yes	No
Freestall layout	3-row	3-row	3-row	2-row	3-row	2-row
Feed bunk type	Post & rail	Post & rail	Headlocks	Post & rail	Headlocks	Post & rail/ headlocks
Movement into pen, days carried calf (DCC) or wk relative to calving	255 DCC	-3	-3	-3.5	255 DCC	-3
Frequency of cow movement, times/wk	1	1	1	1	2	2
Stall stocking density[2], %						
Primiparous	70 ± 10	95 ± 13	99 ± 9	67 ± 27	107 ± 30	79 ± 12
Multiparous	79 ± 13			76 ± 16		74 ± 7
Bunk stocking density[2], cm/cow						
Primiparous	62 ± 8	53 ± 8	52 ± 5	113 ± 64	33 ± 6	88 ± 15
Multiparous	56 ± 10			106 ± 30		95 ± 9
Feeding frequency, times/d	1	1	1	1	1	1
Feed push up frequency, times/d	8	10	10	6	8	3
Maternity pen						
Layout of maternity pen	Pack	Pack	Pack	Pack	Pack	Pack
Timing of movement relative to calving	Signs of labor	Signs of labor	6 d prior to calving	Signs of labor	Signs of Labor	Signs of labor
Maximum time in maternity pen, h	2	8	1	8	1	24
Access to feed	Yes	No	Yes	Yes	Yes	Yes
Time of colostrum harvest	Next time fresh pen milked	Immediately post-calving	1 h post-calving	Next time fresh pen milked	Next time fresh pen milked	Immediately post-calving

[1]Primiparous and multiparous cows housed in the same pen
[2]Mean $\pm$ SD, obtained from weekly DairyComp 305 (Valley Agricultural Software, Tulare, CA) records for the duration of cow enrollment

Table 7-Appendix I- 3. Management of the fresh pen by farm as reported in a herd management survey

Characteristic	Farm					
	A	B	C	D	E	F
Comingled[1]	No	No	Yes	No	Yes	Yes
Freestall layout	3-row	3-row	3-row	2-row	3-row	2-row
Feed bunk type						
Primiparous	Headlocks	Headlocks	Headlocks	Headlocks	Headlocks	Headlocks
Multiparous	Headlocks	Post and rail		Headlocks		
Movement out of pen, DIM						
Primiparous	20	21	18	21	13-30	21-28
Multiparous	10-12	10		14		
Stall stocking density[2], %						
Primiparous	90 ± 15	89 ± 14	102 ± 10	99 ± 4	94 ± 16	76 ± 10
Multiparous	94 ± 14	74 ± 10		97 ± 4		
Bunk stocking density[2], cm/cow						
Primiparous	49 ± 10	53 ± 14	50 ± 5	62 ± 3	54 ± 10	82 ± 11
Multiparous	46 ± 7	64 ± 10		63 ± 3		
Feeding frequency, times/d	1	1	1	1	1	1
Feed push up frequency, times/d	8	10	10	6	8	10

[1]Primiparous and multiparous cows housed in the same pen

[2]Mean ± SD, obtained from weekly DairyComp 305 (Valley Agricultural Software, Tulare, CA) records for the duration of cow enrollment

Table 7-Appendix II- 1. Average ingredient composition of the close up dry cow ration and chemical composition of one composite TMR sample for the duration of cow enrollment by farm

Item	Prepartum Diet by Farm							Postpartum Diet by Farm					
	A	B	C	D	E Period 1	E Period 2	F	A	B	C	D	E	F
Ingredient (% of DM)													
Corn silage	51.40	56.87	48.33	42.97	39.81	37.18	60.05	38.08	35.34	30.42	30.37	40.96	44.45
Dry hay or straw	22.35	20.38	18.41	20.76	9.23	8.50	9.10	0.53	3.34	1.28	2.21	2.37	3.94
Haylage	-	-	-	-	22.48	20.37	-	10.50	11.88	18.15	14.71	11.50	3.36
Concentrate mix	18.38	18.74	28.40	31.46	21.48	32.48	26.45	48.90	47.53	48.82	50.57	43.97	46.25
Di-/mono- Ca phosphate	0.41	0.18	-	0.15	0.07	-	-	-	0.25	-	0.26	-	0.19
Limestone	2.63	1.80	2.02	2.08	1.75	0.52	2.02	1.44	1.38	1.15	1.61	1.08	1.53
MIN-AD[2]	0.80	-	-	-	-	-	-	0.45	-	-	-	-	-
Mg oxide	-	0.37	0.21	0.20	0.12	0.32	-	0.11	0.18	0.18	0.24	0.12	0.22
Mg sulfate	0.40	0.15	0.68	0.14	-	-	0.44	-	-	-	-	-	-
Ca sulfate	0.40	-	0.68	-	0.98	0.64	-	-	0.09	-	0.03	-	0.05
Ca chloride	-	0.77	-	-	-	-	-	-	-	-	-	-	-
Animate[3]	2.02	0.74	1.28	2.24	-	-	1.94	-	-	-	-	-	-
Biochlor[4]	1.21	-	-	-	-	-	-	-	-	-	-	-	-
Soychlor[5]	-	-	-	-	4.07	-	-	-	-	-	-	-	-
Nutrient (% of DM)													
CP	14.3	14.8	13.1	15.4	15.2	14.2	14.9	17.4	15.8	16.1	16.2	16.3	15.6
ADF	26.8	26.8	30.8	26.6	27.9	28.5	25.5	22.1	18.9	20.6	19.9	22.6	18.2
NDF	40.4	43.6	45.4	41.9	40.8	43.2	40.3	34.1	31.9	29.8	29.3	34.4	28.5
Lignin	4.2	3.5	4.5	3.8	4.6	4.4	3.9	3.7	2.2	3.5	3.0	3.9	2.4
Sugar	1.9	4.6	6.3	3.8	2.3	3.2	4.3	5.0	3.6	5.4	6.8	5.3	4.8
Starch	22.1	16.1	16.9	19.9	15.7	17.5	22.0	25.2	27.0	26.0	24.5	20.5	28.8
Fat	3.2	3.2	3.1	2.9	4.7	4.5	3.3	5.7	4.2	5.2	4.6	4.6	5.4
Ca	1.78	1.03	1.08	1.11	1.48	0.86	1.12	1.09	0.93	0.98	1.12	1.03	0.97
P	0.4	0.34	0.31	0.37	0.39	0.38	0.37	0.44	0.41	0.38	0.40	0.40	0.41
Mg	0.45	0.40	0.45	0.43	0.39	0.36	0.37	0.37	0.32	0.32	0.32	0.28	0.30
K	1.12	1.15	1.15	1.19	1.81	1.49	1.20	1.37	1.16	1.38	1.37	1.36	1.31
S	0.41	0.27	0.43	0.32	0.36	0.29	0.35	0.29	0.26	0.23	0.24	0.25	0.22
Na	0.18	0.12	0.07	0.05	0.12	0.12	0.09	0.45	0.44	0.63	0.48	0.46	0.40
Cl	0.63	0.73	0.39	0.47	0.77	0.39	0.55	0.57	0.47	0.60	0.56	0.54	0.45
NE_L (Mcal/kg)[6]	1.50	1.52	1.48	1.52	1.52	1.59	1.57	1.70	1.74	1.70	1.70	1.65	1.79
DCAD (mEq/100 g DM)[7]	-6.9	-2.8	-5.5	-0.4	7.3	14.1	-2.8	20.9	19.3	32.0	25.4	23.7	24.4

[1]Period 1 represented the portion of the study during which Farm E was supplementing anions in the ration (start of study until September 21st, 2015) and Period 2 represents the portion of the study during which there were no supplemental anions in the ration (the remainder of the study).
[2]Dolomitic limestone; Papillon Agricultural Company, Inc., Easton, MD
[3]Anionic supplement; Phibro Animal Health, Corp., Quincy, IL
[4]Anionic supplement; Church & Dwight Co., Inc., Trenton, NJ
[5]Anionic supplement; West Central, Ralston, IA
[6]Calculated from chemical composition (NRC, 2001)
[7]DCAD = [(Na % of DM/0.023) + (K % of DM/0.039)] – [(S % of DM/0.016) + (Cl % of DM/0.0355)]
Table 2. Average ingredient composition of the fresh group ration and chemical composition of one composite TMR sample for the duration of cow enrollment by farm

CHAPTER 8

CONCLUSIONS AND FUTURE DIRECTIONS

The transition period presents an opportunity to prepare the dairy cow to be metabolically healthy in order to produce large amounts of milk in the coming lactation while also being prepared to have optimal fertility and become pregnant at the appropriate time. Management of transition cows to achieve this goal requires multifaceted approaches to dealing with the metabolic adaptations to lactation. As with all disorders of transition cows, hypocalcemia requires significant efforts for prevention of the disorder. Nutrition and feeding management are important components of prevention. Additionally, supplementation with Ca after parturition can play a role in handling the variation in response to prepartum management. Specifically for subclinical hypocalcemia (**SCH**), monitoring of blood Ca in the immediate postpartum period will likely become an important component of both monitoring the efficacy of prevention and management at the herd level, as well as identification of cows in need of supplementation.

CONCLUSIONS

The first objective of this research was to investigate strategies and tools for SCH monitoring. The IDEXX VetTest was identified as a reliable tool for blood total Ca monitoring with high sensitivity and specificity for identifying SCH in comparison to a veterinary diagnostic lab measurement. To account for the mean bias of the tool, the threshold for SCH diagnosis using VetTest measurements had to be adjusted. The relationship between ionized and total Ca measured over the 5 d following parturition was demonstrated to vary by day. These results suggest that epidemiological studies associating ionized Ca in the immediate postpartum period with downstream outcomes should be conducted and thresholds for ionized Ca should not be based solely on the assumed relationship with total Ca. Further, the more consistent associations between ionized Ca and neutrophil activity outcomes, as opposed to total Ca, suggest that

ionized Ca might be a more sensitive predictor of functional outcomes associated with blood Ca status in transition cows.

The second objective of this research was to optimize application of negative DCAD feeding for the prevention of hypocalcemia. Feeding a negative DCAD with a targeted average urine pH between 5.5 and 6.0 resulted in increased blood Ca concentration postpartum compared to cows fed a low K ration without supplemental anions or a partially anion supplemented ration that only minimally decreased urine pH. Further, decreasing DCAD resulted in linear increases in milk production and intake in the early postpartum period, suggesting that improved blood Ca status in those cows enabled them to meet their productive potential without negative effects on energy status. Increased Ca excretion for cows fed the lowest DCAD suggests that stimulation of greater Ca turnover prepartum was an important component of this strategy which likely supported more rapid recovery of blood Ca after the onset of lactation. An interesting finding was the decrease in hypocalcemia prevalence seen in cows entering parity 3 or greater, suggesting that this group of cows responds most profoundly to the preventative strategy.

The third objective of this research was to evaluate new approaches to macromineral nutrition in the transition period to support the recovery of blood Ca postpartum. Although feeding higher dietary Mg postpartum did increase plasma Mg concentration in this study, this did not influence the recovery of plasma Ca concentration postpartum, contrary to our hypotheses. Similarly, dietary source of Mg and Ca had minimal influence on plasma mineral status in the transition period. The dietary supply of Mg in the control group in this study was higher than formulated and this may be part of the reason that minimal effects on mineral status were observed. An interesting finding was the increase in prepartum intake and the decrease in apparent adipose tissue mobilization in cows fed a Ca-Mg dolomite as the primary supplemental

source of minerals. Varying the supplemental mineral source in peripartum rations may be an opportunity to aid cows in the energetic adaptations to lactation.

The final objective of this research was to identify opportunities for strategic use of supplemental Ca in the early postpartum period. Supplementing cows with a single dose of oral Ca within the day following parturition did not increase plasma Ca concentration between 1 and 24 h following supplementation in a subset of cows. The lack of response in plasma Ca may have been a result of the small subset of cows used which had a large range of blood Ca status at enrollment, and likely had variation in the Ca dynamics throughout the sampling period. Despite this observation, the supplementation strategy was effective for improving health and early lactation performance when supplementation was targeted to cows with greater risk of experiencing transition disorders. These risk factors included increasing age, high body condition score and lameness. Responses to supplemental Ca were not dependent on Ca status for primiparous cows. Multiparous cows with low plasma Ca that received supplemental Ca had improved health status in early lactation. These results suggest that a single dose of oral Ca sufficiently alleviated hypocalcemia or supported higher demand for Ca or both in certain groups of cows.

FUTURE DIRECTIONS

Throughout the process of conducting the research that has been presented and discussed throughout this dissertation, I have come across several interesting observations that warrant further investigation. I have been fortunate to have a variety of interactions with industry professionals and producers throughout my time at Cornell and the interest in monitoring and managing hypocalcemia is prominent in the industry. To a small degree, monitoring of blood Ca has begun to be implemented in the field. Unfortunately, interpretation of this information is

very challenging. In Chapter 5, I presented a comparison of SCH categorized using two different blood Ca thresholds (2.0 and 2.125 mmol/L) which resulted in large differences in the perceived prevalence of hypocalcemia. Recommendations for blood Ca monitoring should include thresholds determined by day relative to parturition, as well as by parity, since both of these factors can dramatically affect the expected blood Ca concentration. Further, in the study presented in Chapter 6, prevalence of hypocalcemia was very low regardless of treatment group in comparison to the study presented in Chapter 5. Reference values for an achievable goal for SCH prevalence should be investigated, and these findings suggest that there is considerable variation in the achievable prevalence even within a similar population of cows fed similar rations. The data in Chapter 5 also demonstrated that the improvements in blood Ca status when feeding a negative DCAD diet were most pronounced at 2 DIM. Evaluation of prevention and management strategies might be most reliability conducted using samples collected after the day or two following parturition as this likely reflects the rate of recovery of blood Ca. Large scale epidemiological studies that categorize SCH by day relative to parturition are needed to better understand not only the relative impacts of SCH on individual cow outcomes by day, but also the utility of different sampling timepoints for herd level monitoring.

Prevention of hypocalcemia through feeding a negative DCAD ration prepartum was demonstrated to be a very effective approach for improving outcomes for cows at the herd level in my research. Within the industry, many are applying more "aggressive" approaches to negative DCAD feeding in which urine pH values at or below 6.0 are targeted. As demonstrated in my research, blood Ca status, intake and production were all improved with this approach compared to more moderate approaches. Interestingly, there appeared to be alterations in protein status for cows fed the lowest DCAD ration prepartum with decreased milk protein and milk

urea nitrogen concentrations. The interaction between nutritionally induced metabolic acidosis and macronutrient metabolism has been investigated minimally in periparturient dairy cows. As the approach to DCAD feeding becomes more aggressive, attention should be given to effects on protein metabolism during this time of negative protein balance. Because of the importance of acid-base balance in maintaining many physiological functions within the cow, the allocation of resources to this function might take precedent and compromise other functions that require these protein such as immune function and milk protein production. Future work investigating alterations in amino acid metabolism with negative DCAD rations is important to determine any unintended consequences of the approach and perhaps the potential for supplementation of cows with additional protein or specific amino acids in this period to mitigate these effects.

Despite the lack of influence on mineral status of the dietary Mg source and level observed in Chapter 6, it appears that opportunity still exists to further investigate mineral sources in transition diets. The unexpected findings of increased intake, decreased apparent adipose tissue mobilization, and increased milk fat production in the week following parturition suggest that there might be ruminal effects of feeding Ca-Mg dolomite sources in comparison to Mg oxide and limestone. Improved characterization of the bioavailability of these sources as well as the effects on rumen function in the unique situation of the transition period, including the ration and cow characteristics, may bring to light the underlying cause of the observations of that trial. If there are benefits for rumen function for varied sources of supplemental minerals, or even simply a benefit due to palatability, this can have real impacts for transition cow health, since any strategy that improves intake is valuable at this time. Two unique characteristics of the dataset from that trial were the high intake of cows in the prepartum period and the very low prevalence of hypocalcemia in comparison to the trial presented in Chapter 5. Further

investigation of the effects of level of intake on mineral status should be investigated in the future.

Through the process of investigating the response to Ca bolus administration in the immediate postpartum period, I became interested in the underlying reasons behind the positive responses to Ca supplementation. In our study, the response of multiparous cows with low blood Ca consistently suggested improvements in health status. This is logical based on what we know about the role that Ca plays in immune function and metabolic health. What was interesting to me was the responses observed in cows that might not necessarily be considered to be at high risk of health issues in my work and that of others. For example, primiparous cows with higher age at first calving, primiparous and multiparous cows with higher days carried calf, and multiparous cows with high previous lactation 305-d mature equivalent milk production. These subcategories of cows may represent animals that have a predisposition to respond to Ca supplementation that may or may not manifest as SCH. This suggests that Ca supplementation may not simply be a strategy to alleviate low blood Ca, but may also be needed to support cows with a greater demand for Ca. In the future, investigation of dietary Ca supply postpartum may also be a strategy for meeting these needs. This area has been minimally investigated.

As I continue my career in the dairy industry, I look forward to sharing the knowledge that I have gained throughout the process of my PhD. I hope that opportunity exists for me to act as a liaison between academia and producers, in order to bring new research to the field for application, as well as to bring questions from the field back to researchers. While hypocalcemia will continue to be of importance to the industry, the multifaceted approach for management of the disorder has broad application that I will continue to apply throughout the field. Future research that incorporates all of these approaches and investigates nutrition and physiology as an

integration between mineral and macronutrient metabolism will allow for us to achieve greater

levels of cow health and performance.