Calcium and Phosphorus Metabolism in Jersey and Holstein Cows During Early Lactation

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ABSTRACT

The objective of this dissertation was to assess the dynamics of calcium (Ca) and phosphorus (P) metabolism in dairy cattle. Hypocalcemia, or a drop in blood Ca, is a common condition near parturition. All cows experience some degree of hypocalcemia. Maintenance of blood Ca within the acceptable range of 8 to 10 mg/dl is a balancing act between the demand for Ca for milk production and the cow's homeostatic mechanisms to maintain blood Ca. These homeostatic mechanisms include bone resorption that is driven by Ca demand however both Ca and P are released when bone is resorbed. These times of bone resorption and bone mineral replenishment have not been accounted for in current mineral recommendations.

For the first study, it was postulated that dairy producers could administer 25-hydroxyvitamin D_3 (25-OH) in the prepartum period to prevent hypocalcemia. Twenty-seven multiparous Jersey cows were randomly assigned to receive an oral bolus containing corn starch (control, CON) or corn starch plus 15 mg of 25-hydroxyvitamin D_3 (25-OH) or 15 mg of vitamin D_3 (D_3) at 6 d prior to expected parturition. Jugular blood samples were collected at -14, -13, -5, -4, -3, -2, -1 d prior to expected calving, on the day of calving, and 1, 3, 5, 7, 9, 11, 13, 28, 56, and 84 d with respect to calving. Samples were analyzed for 25-OH, Ca, P, magnesium, osteocalcin (OC), and parathyroid hormone (PTH). Blood Ca, P, and Mg decreased near the time of calving and then increased over time. Serum 25-hydroxyvitamin D_3 was higher for cows dosed with 25-OH (119.0 pg/ml) compared with those dosed with D_3 (77.5 pg/ml) or CON (69.3 pg/ml). Cows dosed with 25-OH tended to have lower serum PTH concentration, but treatments did not affect serum Ca, P, or Mg. Serum OC was higher in second lactation cows compared with cows entering their third or fourth lactation but OC was unaffected by treatment. Although results indicated a 60% increase in serum 25-OH due to a single oral dose of 25-OH prior to calving, the amount administered in this study apparently was not sufficient for initiation of any improvement in Ca homeostasis at parturition.

Due to the intimate relationship of Ca and P in bone, it was postulated for the second study that dietary Ca would affect bone mobilization and Ca and P balance in the lactating dairy cow. Eighteen Holstein cows were blocked by parity and calving date and

randomly assigned to one of three dietary treatments: high (1.03%, HI), medium (0.78%, MED), or low (0.52%, LOW) dietary Ca. Dietary P was 0.34% in all diets. Total collection of milk, urine, and feces was conducted 2 wk prior to calving and in wk 2, 5, 8, 11, and 20 of lactation. Blood samples were collected at -14 and -10 d prior to calving and 0, 1, 3, 5, 10, 14, 21, 28, 35, 56, 70, 84, 98, and 140 d after calving. Blood samples were analyzed for Ca, P, PTH, OC, and deoxypyridinoline (DPD). Rib bone biopsies were conducted within 10 d of calving and during wk 11 and 20 of lactation. Dietary Ca concentration affected Ca balance, with cows consuming the HI Ca diet in positive Ca balance for all weeks with the exception of wk 11. Interestingly, all cows across all treatments had a negative Ca balance at wk 11, possibly the result of timed estrous synchronization that occurred during wk 11. At wk 20, Ca balances were 61.2, 29.9, and 8.1 g/d for the HI, MED, and LOW diets, respectively. Phosphorus balances across all treatments and weeks were negative. Dietary Ca concentration did not affect P balance in the weeks examined for this study but there was a clear effect of parity on balance, markers of bone metabolism, and bone P. Regardless of dietary treatment, serum OC concentration peaked around d 35 of lactation. Simultaneously, DPD concentration began to decrease, which may indicate a switch from net bone resorption to net bone formation after day 35. This was not reflected in balance measures however, this information may help refine dietary mineral recommendations for lactating dairy cows and ultimately reduce P excretion into the environment.

Ultimately from the first study it is clear that oral dosing with 25-OH at 6 d prior to expected calving is not justified. However, we learned that parity has an effect on bone formation with younger animals resorbing and forming more bone and that net formation appears to occur after 30 days in milk. Both of these points were corroborated in the second study. Additionally, the second study demonstrated that dietary Ca content has no effect on P balance from 2 to 20 wk of lactation. Finally, the rib bone does not appear to be a sensitive indicator of bone metabolism or at least not at the time points we measured.

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Chapter 1: Introduction

Hypocalcemia is a common periparturient condition that affects 6% of US dairy cows (USDA, 1996). Maintenance of blood calcium (Ca) within the acceptable range of 8 to 10 mg/dl is a balancing act between the demand for Ca for milk production and the cow's homeostatic mechanisms to maintain blood Ca. Blood Ca concentration below 5 mg/dl typically results in parturient paresis, the condition more commonly known as milk fever. As cows age, Ca homeostatic mechanisms are slower to react to the Ca demands of lactation (Horst et al., 1994). Several studies have focused on the relationship of vitamin D and hypocalcemia. Specifically, a commercial product of 25-OH was developed but its efficacy has not been evaluated. It was postulated that dairy producers could administer 25-hydroxyvitaminD$_3$ (25-OH) in the prepartum period to prevent hypocalcemia. This compound is different from 1,25-dihydroxyvitaminD$_3$ (DHVD; the form that has biological activity) by the absence of a hydroxyl group at carbon one. After administration, 25-OH is converted to the active form in the kidney and can then increase blood Ca by activating the Ca homeostatic mechanisms in the animal. Normally there is a 24 to 48 h delay in increasing serum Ca concentration once hypocalcemia has occurred. Prophylactic administration of 25-OH at 6 d prior to expected calving should allow for the homeostatic mechanisms to be primed to maintain Ca balance under lactation demands, this is what we sought to verify.

One of these homeostatic mechanisms to increase blood Ca is mobilization of bone mineral. In bone Ca and phosphorus (P) are intimately associated in hydroxyapatite bone mineral. When hypocalcemia occurs the need for Ca drives the animal to resorb mineral stores from bone. However, at this time P is also freed as a byproduct of bone Ca resorption. Current NRC recommendations for P do not account for useable endogenous bone P that is released in response to Ca demand. Phosphorus is a leading environmental concern for dairy producers and environmentalists. Phosphorus excretion is directly related to P intake; therefore the P that is released from bone can contribute to excess P in excrement from dairy cows.

Due to this relationship of Ca and P in the animal it was postulated that dietary Ca would affect bone mobilization and Ca and P balance in the lactating dairy cow. Our primary objective for the second study was to evaluate the effect of dietary Ca on the timing and extent of bone mobilization, Ca and P balance, and mineral status of the animal. A secondary objective was to validate the markers of bone metabolism with bone biopsy samples and balance data. The following is a brief review of Ca and P metabolism in the lactating cow to set the stage for this research that was conducted.

Chapter 2: Review of Literature

In 1901, the average annual milk yield per cow was below 2,000 kg. The same animal, the dairy cow, in today's times produces over 14,000 kg of milk per year (VandeHarr and St-Pierre, 2006). Many advances in our understanding of dairy cow nutritional and physiological needs have facilitated this rise in milk production. For example, there have been 2,567 articles published in the Journal of Dairy Science pertaining to ruminant nutrition in the last 25 years. The research conducted in the mineral area has formed our current understanding of mineral requirements but there are still knowledge gaps because less than 200 of the nutrition articles mentioned above have examined mineral requirements.

Mineral research is expensive, time consuming, and often specific to the conditions the research was conducted under. The majority of mineral research has been conducted with calcium (Ca) the primary goal being to reduce hypocalcemia, and more recently with phosphorus (P); due to the increase in environmental regulations.

CALCIUM FUNCTION AND METABOLISM

Hypocalcemia in the Dairy Cow

A cow is considered to be hypocalcemic when a significant decline in plasma Ca concentration occurs (Horst et al., 1994). Producing 1 kg of Holstein milk requires that 1.2 g of Ca (NRC, 2001) be delivered to the mammary gland. A complex endocrine regulating system is in place to ensure that extracellular Ca concentration remains within a narrow range (8 – 10 mg /dL, Goff et al., 1991). There are varying degrees of hypocalcemia, but most cows experience some level of hypocalcemia (Goff, 2000) around parturition. The average treatment cost of one case of clinical hypocalcemia is ~ $334 (Hutjens, 2003). However, significant additional costs are usually endured because a hypocalcemic cow has an increased risk of developing other problems such as ketosis and mastitis (Overton and Waldon, 2004). Alleviating the occurrence of hypocalcemia has potential to increase profitability on dairy farms.

Calcium Homeostatic Mechanisms

When the blood Ca is disturbed, Ca-maintaining homeostatic mechanisms are activated, but with increased age these mechanisms have reduced activity. Calcium homeostatic mechanisms include increased intestinal Ca absorption, resorption of bone Ca, and decreased renal Ca excretion. Two hormones, 1,25-dihydroxyvitamin D (DHVD) and parathyroid hormone (PTH) are involved in each of these processes (Goff et al., 1991).

There are two types of vitamin D available to ruminants. Vitamin D_3 is formed in the skin as a result of photochemical conversion of 7-dehydrocholesterol, and vitamin D_2 is found in forages. Both forms of vitamin D can be utilized by the ruminant, but both must be converted to the biologically active DHVD to have activity (Wasserman, 2004). After absorption of intestinal vitamin D_2 and synthesis of D_3, these compounds are transported to the liver and converted to 25-hydroxyvitamin D_3 (25-OH). Parathyroid hormone, low blood Ca, and low blood P can each activate mitochondrial 1α-hydroxylase in the kidney to convert 25-OH into DHVD when the body is deficient of Ca (Goff et al., 1991).

The small intestine accounts for approximately 90% of dietary Ca absorption (Wasserman, 2004). Calcium is absorbed in two ways: via non-saturable diffusion related to Ca concentration in the intestine or via a saturable active mechanism driven by the demand of the animal and independent of intestinal Ca concentration (Wasserman and Taylor, 1977). Calcium freely enters the enterocyte due to the high concentration gradient between the lumen of the gut and the cell.

Active intestinal Ca absorption is controlled by DHVD. In the cell, 1,25-dihydroxyvitamin D-dependant Ca binding protein binds and transports Ca to the basolateral side of the cell. This is the rate limiting step in cellular intestinal Ca absorption, as demonstrated using *in vitro* transport chambers (Nellans and Kimberg, 1978). In that study, rats were fed a diet that stimulated DHVD formation, and an increase in the ileal mucosal-to-serosal flux of Ca was measured. The DHVD dependency of Ca absorption was further documented in rat ileum by Armbrecht et al. (1999).

Calcium resorption from bone is stimulated by DHVD (Figure 2.1). When bone is resorbed and the matrix is demineralized by H+ and Cl- from osteoclasts and the bone minerals Ca and P are released. Calcium and P are intimately related in bone mineral in the form of calcium phosphate $[Ca_2(PO_4)_2]$, which is amorphoric, or hydroxyapatite $[Ca_{10}(PO_4)_6(OH)_2]$, a crystalline structure which provides binding locations for Ca and P. Bone contains two primary types of cells: osteoblasts that synthesize and deposit bone, and osteoclasts that resorb bone. Osteoblasts control osteoclast differentiation and mediate their activity. Mobilization of bone results in ions of Ca and P being released into circulation (Goff, 2000). But mobilization is driven primarily by the concentration of blood Ca rather than blood P. Low blood Ca promotes synthesis of DHVD, which prompts osteoblasts to increase the number of osteoclasts, which will increase bone resorption and ultimately, increase blood Ca and P. The receptor protein for DHVD is expressed in bone but only on the osteoblast cells; osteoclast action is stimulated indirectly (Horst et al., 1994). Receptor activator of NF-κB ligand (RANKL) expression is promoted when DHVD binds to the receptor on osteoblasts (Teitelbaum, 2000). The presence of RANKL promotes the differentiation of macrophages into osteoclasts via osteclastogenesis, thus increasing the number of osteoclasts and ultimately increasing bone resorption.

Parathyroid hormone is also an osteoclastogenic agent that promotes bone resorption by binding to osteoblasts (Figure 2.1). Both hormones, PTH and DHVD, promote RANKL expression which increases osteoclast numbers as described above. However, osteoprotegerin (OPG) produced from osteoblasts binds to RANKL and inhibits RANKL from binding to its receptor RANK thereby preventing eventual osteoclast formation. Mice without the OPG gene become severely osteoporotic because of lack of regulation of formation of osteoclasts (Teitelbaum, 2000).

In rats a synergism occurs when PTH and DHVD are both present (Weisbrode et al., 1974). Thyroparathyroidectomized rats administered pharmacological doses of vitamin D failed to increase the number of osteoclasts. However, when PTH and vitamin D were administered concomitantly osteoclasts proliferated. This synergism is not fully understood but may be attributed to PTH's control of vitamin D conversion to DHVD.

Release of PTH quickly promotes reabsorption of Ca in the kidney, prior to promoting the conversion of 25-OH to DHVD. Parathyroid hormone promotes active absorption of Ca in the ascending limb of Henle's loop and the distal convoluted tubules in the kidney by Ca channels. The insertion of Ca channels into the luminal membrane and opening of the channels is controlled by PTH. The channels mediate Ca entry, or reabsorption of Ca, into the epithelial cells (Hoenderop et al., 2005).

Diffusional Ca entry from the lumen into the cell is also increased by PTH. Parathyroid hormone initiates a higher intracellular negative charge by increasing the basolateral membrane chloride ion conductance which promotes diffusion of positively charged Ca ions across the cell membrane. If the concentration of Ca in the blood is marginally low then these actions in the kidney can bring the concentration of Ca back to an acceptable range in the animal. This mechanism cannot resolve significant hypocalcemia though, because the total amount of Ca that can be recovered from urine is relatively small in the dairy cow (Goff, 2000). Calcitonin has activity inverse of PTH and is released in a state of hypercalcemia. Intestinal Ca absorption and osteoclast resorption decrease in the presence of calcitonin thereby an increase in bone formation occurs.

Outlined above are the mechanisms that increase blood Ca in the dairy cow in response to a lactation demand. However, it is important to note that sudden, severe hypocalcemia will likely require intravenous Ca treatment until the intestinal and bone Ca homeostatic mechanisms can adapt.

Vitamin D and Hypocalcemia

Many vitamin D compounds and combinations of the different compounds have been investigated for their use as prophylactics for the prevention of hypocalcemia. However the most advantageous timing of administration and form of vitamin D remains in question.

Intramuscular injections of 25-OH of 4 or 8 mg prevented clinical hypocalcemia when calving occurred within 10 d after injection (Olson et al., 1973). When compared to controls, cows injected with 25-OH had a 50% reduction of clinical hypocalcemia. Hodnett et al. (1992) examined the impact of injecting 25-OH plus 1α-hydroxyvitamin

D3 (1α-h) into Holstein cows within 1 to 5 d of calving. Cows were consuming a prepartum diet high in Ca (170 g/d). Serum Ca and P concentrations were higher in treated cows and incidence of clinical hypocalcemia was reduced. However, when this study was repeated at a commercial herd with a lower dietary Ca concentration there was no reduction in hypocalcemia (Hodnett et al., 1992).

Dietary Effects on Calcium Metabolism

Braithwaite (1983a) quantified utilization and metabolic fate of Ca in ewes during pregnancy and lactation by infusing animals with radiolabeled Ca. Demands increased in late pregnancy and reached a peak in early lactation. These changes in Ca demands were due to changes in the rate of passage of Ca to the fetus and the sudden need for milk production combined with constant urinary and fecal losses of Ca. Despite high Ca concentration in the diet and increased intestinal absorption of Ca, ewes fed a diet plentiful in Ca and P were not able to meet the Ca demands of late pregnancy and early lactation with dietary Ca. The ewes' skeletal reserves supplied the difference between absorbed dietary Ca and demand. Although Ca absorption continued to increase and demand for Ca began to decrease in mid-lactation, dietary Ca was absorbed in excess of demand. At that time and later in lactation the skeletal Ca reserves of these ewes were replaced because both Ca and P were plentiful in the diet (Braithwaite, 1983a). Ewes on a restricted Ca and P diet, however, were not able to replace skeletal reserves at any time during lactation. This study demonstrates the importance of having sufficient dietary Ca and P to allow for repayment of the mineral loans from the skeleton.

There have been multiple recent studies that examined metabolic effects of dietary Ca concentration in the prepartum period in lactating cows (Kamiya et al., 2005; Chan et al., 2006;; Liesegang et al., 2007) but few have tracked Ca demands and metabolism in the dairy cow throughout lactation. Calcium is required in large quantities by the lactating cow but it is difficult to determine its requirement when there are still unknowns about how it is metabolized.

Calcium Requirements of Lactating Dairy Cows

The equations for predicting Ca requirements were not modified between the 1989 and 2001 NRC. The requirement for absorbed Ca is the sum of the individual

requirements for maintenance, growth, pregnancy, and lactation. The concentration of dietary Ca required is dependant on the availability of Ca from the feed and the efficiency of intestinal absorption. In the present NRC the efficiency of absorption of Ca from forages is assumed to be 30% and from concentrates, 60%.

Age and breed affect the efficiency of Ca absorption due to changes in DHVD receptors (Horst et al., 1990). Jerseys have fewer intestinal DHVD receptors than Holsteins decreasing their ability to absorb dietary Ca efficiently (Goff et al., 1995). However a decrease in DHVD receptors also affects bone metabolism. This has not been accounted for in current mineral recommendations. It is critical to completely understand the timing of bone formation and resorption to refine Ca feeding recommendations for the lactating cow.

PHOSPHORUS FUNCTION AND METABOLISM

Environmental Issues with Phosphorus and Dairy Cows

Phosphorus is one of the leading causes of fresh water eutrophication and a major focus for nutrient management of livestock producers (Knowlton et al., 2004). Phosphorous-based nutrient management plans are mandatory in many states. One of the ways to reduce P concentration of feces is to feed less of it (VandeHaar and St-Pierre, 2006). The amount of P excreted in the feces of dairy cows is directly proportional to excess P in the diet or excess P entering the bloodstream from dietary and endogenous sources, including liberated P from bone (Wu et al., 2000). Field surveys demonstrate that P is typically fed at 20-40% above published recommended guidelines for dairy cows (Knowlton et al., 2004) and these recommendations are probably excessive because of the lack of information on bone mineral resorption.

Dietary Effects of Phosphorus in Ruminants

The small intestine absorbs dietary P in the form of phosphate in response to need by the animal (Hibbs and Conrad, 1983). Intestinal P is absorbed via a Na/P cotransporter present in the brush border membrane (Shirazi-Beechey et al., 1996). The P concentration of the diet has the biggest effect on intestinal P absorption. The composition of the diet also affects transit time through the gastrointestinal tract and

therefore can affect site of absorption (Braithwaite, 1976). When transit time is increased through the gastrointestinal tract (through decreased DMI or reduced dietary fiber) mineral absorption is negatively affected (Cragle, 1973).

In sheep, it has been demonstrated that the rate of absorption of P increased to a greater extent than demand between late pregnancy and early lactation regardless of dietary P content or the sheep's demand for P (Braithwaite, 1983b). Extra P that was absorbed from the diet was not used to meet P needs, but was excreted in urine and feces. The P demands for maintenance and lactation were met instead by mobilized P from bone and soft tissue. Bone was mobilized in response to a need for Ca in the animal due to the insufficient amount of absorbed dietary Ca to meet the Ca demand for milk production. The author concluded that Ca was mobilized from bone to meet the Ca demand and that bone P was released regardless of P supply. Sheep that were fed diets restricted in P and Ca did not replace the bone mineral reserves in mid-to-late lactation as did sheep fed diets with excess Ca and P (Braithwaite, 1983b).

It has been demonstrated that dietary P concentrations below the NRC (2001) recommendations (0.31% vs. 0.38-0.42%, respectively) do not have a negative impact on bone strength. The lower dietary P concentration appeared only marginally deficient in cows producing > 11,900 kg/305 d for a two year period (Wu et al., 2001). A study in lactating cows (Knowlton and Herbein, 2002) suggested when dietary P (0.34%) was inadequate but dietary Ca sufficient, bone was resorbed to fulfill the P deficit. However, no bone resorption indicators were measured and the conclusions were based on the assumption that P balance reflects P resorption. In contrast a study by Ekelund et al. (2006) found that dietary P at 0.32% during the first 4 months of lactation had no effect on P balance or blood markers of bone metabolism as compared to control cows fed a diet containing 0.43% P. However, the cows in this study had substantially lower milk yields (36.0 vs. 52.8 kg milk/d, Ekelund et al. 2006 vs. Knowlton and Herbein, 2002; respectively). Once again, until the metabolism of P is understood in the dairy cow it is difficult to refine dietary P requirements to reduce P excretion.

Phosphorus Requirements in Lactating Dairy Cows

Accurately predicting P requirements for ruminant animals is difficult. Phosphorus absorption and metabolism are influenced by many variables including

dietary concentrations of Ca, magnesium (Mg), and DHVD. As indicated previously, Ca and P are intertwined in bone and it is crucial to completely understand the relationship of Ca and P in the dairy cow in order to refine P feeding recommendations. Phosphorus absorption is also affected by other factors such as season of year, stage of lactation, age of animal, and pregnancy status (Church et al., 1988).

In the current NRC model (2001), the overall requirement for P is listed as the sum of the estimates of requirements for maintenance, growth, pregnancy, and lactation. The recommendation for the maintenance requirement of P is 1.0 g/kg of DMI (NRC, 2001). In the past, calculation of the maintenance requirement was expressed as a function of body weight (NRC, 1989). This approach to calculating the maintenance requirement was thought to be inaccurate by the Agriculture and Food Research Council (AFRC) based on research by Spiekers et al. (1993). Spiekers and coworkers (1993) hypothesized that it would be more accurate to base the maintenance recommendation for P on DMI as opposed to body weight. To test their hypothesis, two groups of five cows with similar body weights but different daily milk yield and DMI were used. Cows in group I consumed 6.0 kg more DM and excreted more feces (+1.5 kg/d DM) as compared to cows in group II. Consistent with their hypotheses, fecal P excretion was a constant 1.2 g per kg of DM intake in both groups. In 2001, the NRC adopted the method proposed by Spiekers et al. (1993) for recommending maintenance P levels. Even when the multiple influences on P absorption are taken into account, it is still difficult to quantify all of the sources of P that the ruminant has available.

Accurately predicting appropriate P requirements in ruminants has been investigated (Braithwaite, 1983b; Dhiman et al., 1996; Wu et al., 2000; Wu and Satter, 2000; Knowlton and Herbein, 2002) yet a consistent determination of P requirement including credit for bone resorption has not been established.

BONE STATUS

Bone and teeth contain approximately 98% of the Ca and 80% of the P found in dairy cattle. Therefore, it is important to understand when and why bone Ca and bone P are mobilized to fully understand the Ca and P status of the animal. Bone is resorbed under both normal and pathological conditions in highly regulated, hormonally-mediated

processes. Resorption of bone is not uniform across individual bones. Bones rich in cancellous tissue are heavily resorbed while bones composed predominantly of compact tissue are less extensively drawn upon (Benzie et al., 1955).

To assess bone mineral changes over time an accurate, minimally invasive, and efficient method is needed. The histomorphometric interpretation of iliac crest bone biopsy specimens is the current standard to which bone biochemical markers are compared (Lester et al., 1995). However, biopsying is invasive and variable due to biopsy site and mode of analysis; therefore it is not an appropriate method for determining bone changes over a short period of time (Lester et al., 1995).

Benzie et al. (1959) found that in sheep, when blood P concentration was low, the skeleton was severely resorbed but observed that the converse of that statement was not always true. Serum P and serum Ca values are not always indicative of adequate mineral status of the animal. Therefore, examining only blood mineral concentrations is not an accurate method to assess mineral status of the animal.

Metabolic Acidosis in Dairy Cows

As discussed in previous paragraphs, osteoclasts and osteocytes are indirectly stimulated by PTH and DHVD to degrade bone matrix thereby releasing Ca and P. If blood pH is normal (~ 7.35), PTH binds to its receptors located on the surface of bone and renal tissue. If the pH of the blood is alkaline, the receptor changes in confirmation and PTH has a reduced binding affinity. When this occurs, the target cell does not get fully stimulated and the cow is less efficient at responding to the Ca demand of the body (Goff, 2000).

Several research studies (Oetzel et al., 1990; Goff and Horst, 1998) have examined the issue of metabolic acidosis and formulating diets that induce mild acidosis in the cow by examining the dietary cation anion difference of the diet. Prepartum diets high in cations tend to induce milk fever but diets high in anions can prevent milk fever (Goff et al., 1991). Six anionic salts were evaluated and found to decrease blood bicarbonate and urinary pH which resulted in an increase in urinary excretion of Ca (Oetzel et al., 1990). The use of HCl was also found effective as feed additive in induce mild acidosis and preventing hypocalcimia (Goff and Horst, 1998). Gaynor et al. (1989) demonstrated that anion-cation content of the diet can influence hypocalcemia and the

mechanism may be linked to the influence on the production of DHVD. In ruminants it had been previously demonstrated that when high anionic diets were consumed there was an increase in bone responsiveness and in turn more Ca was released from bone stores (Vagg and Pyne, 1970). This is likely the result of the increase in DHVD and even PTH concentration in the Ca stressed animal.

Hormonal Control of Bone Metabolism

Muir et al. (1972) reported that cows induced with hypocalcemia and injected with estrogen and progesterone had no change in bone resorption as indicated by hydroxyproline (marker of bone resorption). There was a significant reduction in feed intake in the cows injected with estrogen alone. When both hormones were injected, progesterone partially counteracted the effect of estrogen on DMI. In contrast, estrogen administration beginning 14 d prior to parturition caused a reduction in bone resorption, indicated by hydroxyproline concentration (Bargeloh et al., 1975). However there was no difference in blood Ca, blood P, or milk fever incidence as compared to control cows. In humans a decrease in estrogen causes an increase in RANK concentration that promotes osteoclast cells and ultimately results in an increase in bone resorption (Rodan and Martin, 2000). This commonly occurs in postmenopausal women but this mechanism of estrogen and bone is not fully understood in the lactating cow.

Markers of Bone Metabolism

Plasma concentration of osteocalcin (OC) is correlated with osteoblast function and bone formation (Naito et al., 1990). Osteocalcin is a noncollagen protein that is specific to bone and dentin (Lester et al., 1995). If bone resorption and formation are coupled, as they normally are, concentration of OC is considered to be an adequate marker of bone turnover. High concentrations of OC are indicative of bone formation such as growth or accretion (Liesegang et al., 2000). Several studies (Naito et al., 1990; Farrugia et al., 1991; Liesegang et al., 2000) in ruminants have reported significant decreases in OC concentrations after parturition followed by an increase in concentrations from 15-60 d postpartum.

In cows, plasma OC concentrations are also influenced by milk yield and age. A study by Liesegang et al. (2000) indicated that cows with higher milk yield had higher

bone resorption and formation rates, and the two events were uncoupled in the first 14 days of lactation. Prepartum dietary Ca had no effect on OC concentration but primiparous cows had higher serum OC than multiparous cows from -13 to 3 d relative to calving (Kamiya et al., 2005). Iwama et al. (2004) showed that primiparous cows had higher plasma OC concentrations at 21 and 27 d postpartum than multiparous cows. This could be explained by bone formation being more active in primiparous cows than multiparous cows (Van de Braak and Van't klooster, 1987) which is likely due to bone growth.

Deoxypyridinoline (DPD) is a nonreducible pyridinium crosslink that stabilizes collagen chains within the mature matrix of bone and is correlated with bone resorption (Lester, 1995). Deoxypyridinoline is released in free form into the extracellular fluid after osteoclastic bone resorption and then binds to oligopeptides from the collagen α-chains. The exact mechanism of excretion is not understood. However, serum concentrations of DPD concentrations correlate well with bone turnover as measured by histomorphometric analysis of bone biopsy specimens (Lester, 1995). Urinary DPD was higher in primiparous cows as compared to multiparous cows and prepartum dietary Ca had no effect on DPD concentration (Kamiya et al., 2005). Serum DPD concentration was not affected by prepartum dietary P concentration (Peterson et al., 2005).

A study by Liesegang et al. (2000) suggested that these bone markers, among others, should be further evaluated in cows fed diets that strongly influence Ca metabolism. Formation and resorption normally occur simultaneously; therefore it is necessary to examine both OC and DPD to understand the extent and timing of bone activity and to determine net times of formation and resorption. However, despite their increased use in cow studies, bone markers have not been validated for their utility in indicating the overall mineral status of the animal.

Biopsy of Bone to Assess Mineral Status

Beighle et al. (1993) examined serial rib bone sampling for mineral analysis in over 2000 cow ribs (9th, 10th, 11th, and 12th). Results showed that each consecutive rib biopsy collected from the same site on each rib can be compared with all of the other samples over time as long as results are expressed on an ash weight basis. The authors

concluded that this bone biopsy technique allows for evaluation of changes in bone mineral content over time with only minimal discomfort to the animal.

Differences in bone mineral concentration were examined via bone biopsy in growing beef heifers (7.5 months) fed a low P or adequate P (0.12% vs. 0.20%) diet (Williams et al., 1991). There was a low P pre-treatment period of 270 d to allow for P depletion (0.10% P). At 120 and 294 d post-supplementation, a biopsy of rib bone was collected for Ca and P analysis. The low P treatment group had lower rib P content compared to adequate P fed heifers, although P content of bone increased between the first and second collection. Bones from heifers on the high P diet were denser than bones from heifers on the low P diet for both sampling times. Bone Ca content was not affected by dietary P content and did not change over time. However this study was conducted in beef heifers, dietary P was much lower than practical, and mineral changes were evaluated and detected over a 100 day period.

The concentration of minerals tells only part of the story, however. Benzie et al. (1955) concluded that weight of ash in bones is more sensitive than percentage of ash in a study conducted in sheep to examine different levels of dietary Ca on individual bones. Changes in bone mass obviated changes in bone mineral concentration.

MINERAL BALANCE

To determine mineral metabolism or requirements, total collection is considered the standard method. Balance research is expensive and labor intensive, but necessary. Major routes of P excretion are feces and milk, which vary with DMI and milk yield (Hibbs and Conrad, 1983; Horst, 1986). Major routes of Ca excretion are feces, urine, and milk (Horst, 1986). Total collection of urine, feces, and milk is used to measure Ca and P balance in the bovine (Morse et al., 1992; Martz et al., 1999; Knowlton and Herbein, 2002; Weiss and Wyatt, 2004). The use of previously described bone markers and bone biopsy has yet to be validated with the standard of mineral research, balance.

Dietary Effects on Mineral Excretion

Calcium and P absorption can be influenced by diet. The research by Martz et al. (1999) contributed to the determination of the current NRC (2001) P and Ca true

absorption coefficients. The use of ^{45}Ca and ^{32}P allowed for determination of endogenous fecal losses when cows were fed an alfalfa-corn silage or alfalfa hay diet. True absorption of Ca from an alfalfa-corn silage diet was higher than an alfalfa hay diet even though the dietary concentration of Ca and intake was not different (Martz et al., 1999). It was also determined that actual true absorption of P from an alfalfa-corn silage diet was higher than from an alfalfa hay diet.

There was no differences between diets for P balance or apparent digestibility coefficients for 4 adult (2 cows, 2 steers) Jersey cattle (Khorashani and Armstrong, 1992). There were 8 diets fed that varied in forage type and with or without silage additives. These cows were equipped with cannulas in the rumen, proximal duodenum and terminal ileum. These cannulas paired with total collection allowed for determination of site of mineral secretion and absorption and the effect of dietary concentration of the mineral. Phosphorus intake and P balance were positively correlated.   As P intake increased there was an increase in the absorption coefficient up to 19 g/d of P, beyond which there was a decline of apparent P absorption. This study concluded that the small intestine was the major site of P absorption and net secretion of P depended on mineral intake.

Morse et al. (1992) were also one of the first to demonstrate that the amount of P excreted is dramatically affected by dietary P intake. The authors demonstrated that for each gram increase in P intake, excretion of P increased by 0.80 g/d when cows were consuming a dietary P at 0.56 vs. 0.30% P.

The P in feces is derived from three fractions: (a) unavailable dietary P, which cannot be absorbed; (b) inevitable loss, which is excreted under normal physiological conditions; and (c) excess loss where supply of available dietary P exceeds the requirement of the animal (Spiekers et al., 1993). Fraction C is where improvement can be made by closely matching the available dietary P to the cow's requirement.

Ekelund et al. (2006) reported when cows were fed low dietary P (0.32%) for the first 4 months of lactation and were then switched to high dietary P (0.43%) for the remainder of the lactation, there was no difference in P retention when compared to cows that consumed high dietary P the entire lactation. Similarly, there was no difference in P or Ca retention when dietary P was fed at 0.34, 0.51 or 0.67% of the diet (Knowlton and

Herbein, 2002). However, cows on the low diet consumed 88.7 fewer grams of P/d and had 72.5 fewer grams of fecal P than the cows fed the high dietary P.

Apparent P digestibility was higher (0.52 % vs. 0.42 %) for the low fed cows during early lactation (Ekelund et al., 2006). Similar apparent digestibilities were reported by Wu et al. (2001) and Knowlton and Herbein (2002); low dietary P results in higher apparent P digestibility. Cows on all three studies were able to adapt to the lower dietary P concentrations by increased efficiency of P absorption and higher P intake did not result in increased P retention. Knowlton et al. (2000) reported that advancing stage of lactation increased P digestibility (43.5 vs. 47.8%, early vs. late lactation) and there was a greater proportion of absorbed P retained in the body. Shirazi-Beechey et al. (1996) demonstrated that the affinity of the Na transporter for P does not change but there is a clear increase in transport activity when there is low P concentration. The authors further demonstrated that the P transporter is not affected by PTH, Ca, DHVD, or growth factors.

A set of companion papers (Braithwaite, 1983 a; Braithwaite, 1983 b) examined Ca and P requirements during lactation and pregnancy in sheep. These studies utilized mineral balance and radioisotopes (^{45}Ca and ^{32}P) to test the adequacy of the recommendations for that time. Ewes that consumed a plentiful Ca and P diet were unable to absorb enough dietary Ca in late pregnancy and early lactation and mobilized bone to meet the Ca demand. In mid-to-late lactation these ewes replaced bone that was resorbed, but ewes fed a restricted Ca and P diet bone was not replaced. Therefore, when calculating Ca requirements, consideration must be made for replacement of skeletal stores. Resorption and formation are driven by changes in Ca requirements and immediate P demands do not reflect net P demands because net encompasses changes in bone. Ultimately, this author suggested feeding animals in late pregnancy and early lactation below their immediate day-to-day Ca and P requirements because of limited absorption and the inability to meet the Ca demand from diet alone. It was further suggested to provide excess dietary Ca and P in mid-to-late lactation when the animal could absorb the minerals to replace body stores that were mobilized. Even though more than 20 years have passed, this suggestion has not been evaluated in dairy cattle.

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Figure 2-1. Parathyroid hormone (PTH) and 1,25-dihydroxyvitaminD3 (DHVD) activated bone resorption.
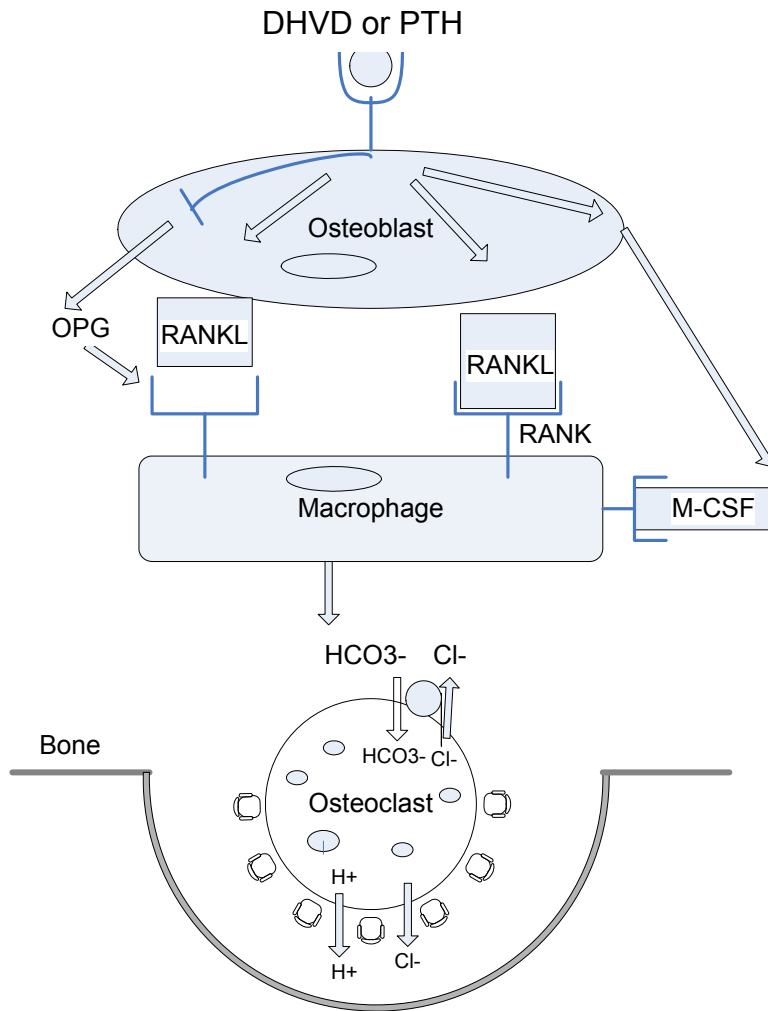
Chapter 3: Blood Mineral, Hormone, and Osteocalcin Responses of Multiparous Jersey Cows to an Oral Dose of 25-Hydroxyvitamin D_3 or Vitamin D_3 Prior to Parturition

ABSTRACT

Twenty-seven multiparous Jersey cows were randomly assigned to receive an oral bolus containing corn starch (control, CON), corn starch plus 15 mg of 25-hydroxyvitamin D_3 (25-OH), or 15 mg of cholecalciferol (D_3) at 6 d prior to expected parturition. Cows were maintained in individual box stalls from 20 d prior to expected parturition and fed a common diet. Jugular blood samples were collected at -14, -13, -5, -4, -3, -2, -1 d prior to expected calving, at calving, and 1, 3, 5, 7, 9, 11, 13, 28, 56, and 84 d with respect to calving. After calving cows were housed in one pen in a free-stall barn and consumed a common diet. Colorimetric assays were used to analyze calcium (Ca), phosphorus (P), and magnesium (Mg) concentrations in serum. Serum concentration of osteocalcin (OC), an indicator of bone formation, was determined using a competitive immunoassay. Serum 25-hydroxyvitamin D_3 and parathyroid hormone (PTH) concentrations were determined using radioimmuno assays in samples obtained from -5 through 13 d. The nine control multiparous cows and five untreated primiparous cows were used to evaluate the effect of parity on the variables that were measured. There was no effect of parity on Ca, PTH, or 25-OH concentration. Serum OC was higher in 1st lactation cows as compared to cows in their 2nd or > 2 lactations (48.3 vs. 32.0 or 22.3 ng/mL, respectively) indicating that younger animals were forming more bone. Data reported for the effect of treatment is from 27 multiparous cows. Blood Ca, P, and Mg decreased near the time of calving and then increased over time. Serum 25-hydroxyvitamin D_3 was higher for cows dosed with 25-OH (119.0 pg/ml) compared with those dosed with D_3 (77.5 pg/ml) or CON (69.3 pg/ml). Cows dosed with 25-OH tended to have lower serum PTH concentration, but treatments did not affect serum Ca, P, or Mg. Serum OC was higher in second lactation cows compared with cows entering their third or fourth lactation but OC was unaffected by treatment. Although results indicated a 60% increase in serum 25-hydroxyvitamin D_3 due to a single oral dose of 25-OH prior to calving, the amount administered in this study

apparently was not sufficient for initiation of any improvement in Ca homeostasis at parturition.

(**Key words**: calcium, cow, osteocalcin, hypocalcemia)

INTRODUCTION

Hypocalcemia is a common condition near parturition for 6% of US dairy cows (USDA, 1996). Maintenance of blood calcium (Ca) within the acceptable range of 8 to 10 mg/dl is a balancing act between the demand for Ca for milk production and the cow's homeostatic mechanisms to maintain blood Ca. Blood Ca concentration below 5 mg/dl typically results in parturient paresis, the condition more commonly known as milk fever. As cows age, Ca homeostatic mechanisms are slower to react to the Ca demands of lactation (Horst et al., 1994).

To prevent milk fever in cows, researchers (Hibbs and Pounden, 1951) often recommended feeding or injecting pharmacological doses of vitamin D 2 wk prior to calving. This practice increases intestinal absorption of Ca and helps prevent milk fever, but the recommended dose (up to 10 million units/d) of vitamin D is very close to the amount that causes irreversible metastatic calcification of soft tissue (Littledike et al., 1986).

Vitamin D is a necessary precursor for production of the Ca regulating hormone 1,25-dihydroxyvitamin D_3 (DHVD). Vitamin D can be produced within the skin of most mammals by photochemical conversion of 7-dehydrocholesterol to vitamin D_3 . The liver has a high affinity for vitamin D in circulation. In the liver, vitamin D is hydroxylated into 25-hydroxyvitamin D_3 (25-OH) by action of microsomal enzymes (Goff et al., 1991). The 25-OH then enters systemic circulation and is further converted to DHVD in the kidney.

The DHVD hormone increases active transport of Ca and phosphorus (P) across intestinal epithelial cells and is tightly regulated by parathyroid hormone (PTH). Parathyroid hormone has three main functions in cows: to mobilize Ca from bone, to promote absorption of Ca from the digestive tract through increasing DHVD concentration, and to stimulate the kidneys to excrete excess P while retaining Ca for reabsorption (Goff, 2000). As blood Ca concentration decreases, PTH concentration increases. High concentrations of PTH promote the production of 1-α-hydroxylase in the kidney, the enzyme that converts 25-OH to DHVD. When DHVD binds to osteoblasts

(bone forming cells), osteoclasts (bone resorbing cells) are stimulated indirectly to resorb bone and free bone mineral. Biochemical markers of bone formation, such as osteocalcin (**OC**), can be measured in the blood to evaluate bone metabolism. When blood Ca is within the normal range, PTH secretion decreases and DHVD is catabolized through a negative feedback mechanism and 25-OH is diverted to inactive compounds (Horst et al., 1994).

Various forms of vitamin D and their derivatives have been examined in relation to Ca mobilization and/or prevention of hypocalcemia (Olson et al., 1973a; Horst et al., 1983; Naito et al., 1990; Hodnett et al., 1992; Okura et al., 2004). Pharmacological doses are effective at preventing hypocalcemia but are close to the toxic dose. Lower doses may actually induce milk fever, depending on timing of administration, because high doses of 25-OH and DHVD suppress renal synthesis of DHVD and suppress release of PTH (Littledike and Horst, 1980). It is speculated that the Jersey breed is more prone to milk fever because they express fewer intestinal DHVD receptors and secrete more Ca in colostrum and milk (Goff et al., 1995).

The stimulation, regulation, and activation of the homeostatic mechanisms related to Ca metabolism is a well orchestrated cascade of events. One exception is the time around parturition when the animal is subject to a dramatic increase in Ca demand concomitant with a delay in the available Ca. Theoretically, exogenous manipulation of this cascade via vitamin D supplementation should be possible. Despite years of research the most advantageous form, timing of treatment, and route of administration are still in question and new commercial products such as 25-OH have been developed to address this problem. Our hypothesis was that dosing preparturient cows with exogenous 25-OH 6 d prior to parturition would allow Ca homeostatic mechanisms to be active at parturition. Further, increasing blood Ca prior to the demand for Ca by the mammary gland at parturition could attenuate the severity of hypocalcemia and prevent parturient paresis.

MATERIALS AND METHODS

Twenty nine multiparous Jersey cows were randomly assigned to one of three treatments. Treatments were oral boluses of either corn starch (CON), corn starch plus

15 mg of HyD (DSM; Parsippany, New Jersey) commercial 25-hydroxyvitamin D_3 (25-OH), or corn starch plus 15 mg of cholecalciferol (D_3) administered as two gel capsules 6 d prior to expected calving. If the cow did not calve within 6 d of bolus treatment, a second dose was administered (4, CON; 5, 25-OH; and 7, D_3 cows were dosed twice). Animals were housed in individual box stalls and fed a common dry cow diet (Ca 0.29%, P 0.33%, and Mg 0.19% DM basis; Table 3.1) to achieve 10% refusals beginning 20 d prior to expected calving until parturition. After parturition animals were relocated to a free stall facility, group housed, and fed a common lactating cow diet. All procedures used in this experiment were approved by the Virginia Tech Animal Care and Use Committee.

A Foal Alert system (Foal Alert; Acworth, Georgia) was used to alert project personnel to parturition. Two days prior to expected calving, a magnetic radio device was sewn to each side of the vulva. When parturition began, the magnetic connection linking both sides of the device was broken and a radio signal was then sent to a receiver that was linked to a telephone line. This device allowed personnel to arrive for parturition and a blood sample to be collected within minutes of parturition.

Jugular blood was collected prior to feeding on days -14, -13, -7, and everyday till expected calving; at calving (0); and 1, 3, 5, 7, 9, 11, 13, 21, and 28 d. Blood was also collected every 2 wk until 84 d of lactation. Samples were numbered after actual calving occurred and samples collected outside of specified times were discarded. If cows were diagnosed by veterinary staff with parturient paresis, a blood sample was collected and then treatment was administered. Blood was maintained on ice until centrifugation at 2200 x g for 20 min. Serum separators (Fisher Scientific, Pittsburgh, Pennsylvania) were utilized to facilitate serum separation in the centrifuge. Serum was harvested and stored frozen for future analysis.

Colorimetric methods were used to analyze all serum samples for Ca (Aresenazo Reagent Set, Pointe Scientific, Canton, MI), inorganic P (Inorganic Phosphorus Reagent, Pointe Scientific, Canton, MI), and magnesium (Mg) (Magnesium Reagent Set, Pointe Scientific, Canton, MI). Serum 25-OH and PTH were analyzed in samples from d -5, -4, -3, -2, -1, 0, 1, 3, 5, 7, 9, 11, and 13. A radioimmunoassay was used to determine 25-OH (25-hydroxyvitamin D_3, DiaSorin Saluggia, Italy) and PTH (N-tact PTH SP, DiaSorin,

Saluggia, Italy) concentrations. A competitive immunoassay was used to quantify serum OC (Meta Osteocalcin, Quidel Corporation, San Diego, CA) in samples from d -5, -2, -1, 0, 1, 3, 5, 7, 13, 28, 56, and 84.

Milk samples were collected on 1, 3, 5, 7, 9, 11 and 13 d postpartum. Milk samples from d 1, 3, and 7 were kept frozen until analysis for 25-OH concentration by Walters HPLC (Milford, MA) according to the procedure of Chen et al. (1990). There was no detectable concentration of 25-OH found in any of the milk samples analyzed.

Five primiparous Jersey cows were added as part of a second objective within the confines of the first experiment. The primiparous animals were maintained and sampled the same as the 29 multiparous animals. The primiparous animals received no treatment but were used to examine the effect of lactation number on Ca, P, Mg, PTH, 25-OH, and OC with the 9 control multiparous (3, 2nd lactation and 6, > 2 lactations) animals from the first objective.

Data for two multiparous cows were removed from analysis because one cow on the 25-OH treatment had a displaced abomasum and one cow on the D_3 treatment was removed due to breech of experimental protocol, so n = 27 (9 cows per treatment). Data for another cow on the 25-OH treatment were removed from analysis after 13 d in milk due to unexpected failure of the median suspensory ligament and a displaced abomasum (data from 28 through 84 DIM, n = 26, 9 cows on CON, 9 cows on D_3, and 8 cows on 25-OH). There were 3 cows on the D_3 treatment and 2 cows on the 25-OH treatment that were diagnosed and treated for parturient paresis within the first 48 hr after parturition.

All cows on this study were concurrently participating in a crossbreeding study. Each animal on this study was bred with mixed Holstein-Jersey semen for the other research trial. The calves from these cows were either Jersey x Jersey or Holstein x Jersey crosses. The effect of calf weight on treatment responses was examined statistically, but found to be nonsignificant.

Statistical Analysis

Serum Ca, P, Mg, and OC were analyzed using the Mixed procedure of SAS (9.1, 2003) with the model:

$Y_{ijkl} = \alpha + b_1 X_{(ij)k} + T_i + L_j + (TL)_{ij} + C_{(ij)k} + D_l + (TD)_{il} + (LD)_{jl} + (TLD)_{ijl} + E_{ijkl}$

where:

α = intercept;

b_1 = regression of Y on X;

$X_{(ij)k}$ = average pre-treatment serum concentration of cow k;

T_i = effect of oral dosing (i = 1 to 3);

L_j = effect of lactation number (j = 2 to >3);

$(TL)_{ij}$ = effect of interaction of treatment and lactation;

$C_{(ij)k}$ = random effect of cow k within treatment and lactation;

D_l = effect of day relative to calving (l = -5 to 84);

$(TD)_{il}$ = effect of the interaction of treatment and day;

$(LD)_{jl}$ = effect of the interaction of lactation number and day;

$(TLD)_{ijl}$ = effect of the interaction of treatment, lactation number and day; and

E_{ijkl} = random residual;

Several covariates were examined to account for the difference in breed of calf carried. This was examined because the variables in question could be influenced by differences in the calf. The current calf weight and previous calf weight were originally in the model as covariates to account for the potential effect of calf breed differences. Calf weights ranged from 21 to 43 kg but neither covariate was significant.

For all blood analyses, the pooled -14 and -13 d preliminary samples were used as a covariate for each measurement when significant. Cows were grouped by parity into three groups; 2nd, 3rd, or >3rd lactations; the last included both 4th and 5th lactation cows. Day was included as a repeated measure, using the ar(1) covariance structure. Preplanned orthogonal contrasts were used to compare the CON treatment to the average of 25-OH and D_3 treatments, and to compare the D_3 treatment to the 25-OH treatment. The model was also used for PTH and 25-OH analysis until day 13 of lactation. For PTH, the slice option was used to determine significant treatment differences within each lactation number and day. Results are reported as LSMeans ± SEM with differences declared significant at P < 0.05 and trends at P < 0.10.

For the evaluation of the effect of lactation number, in the primiparous and control cows, on serum Ca, P, Mg, PTH, 25-OH, and OC the model was:

$$Y_{ijk} = α + b_1 X_{(i)j} + L_i + C_{(i)j} + D_k + (LD)_{ik} + E_{ijk}$$

where:

α = intercept;

b_1= regression of Y on X;

$X_{(ij)k}$ = average pre-calving serum concentration of cow j;

L_i = effect of lactation number (i= 1 to >3);

$C_{(i)j}$ = random effect of cow k within lactation;

D_k = effect of day relative to calving (k = -5 to 84);

$(LD)_{ik}$ = effect of the interaction of lactation number and day; and

E_{ijkl} = random residual;

The pooled -14 and -13 d preliminary samples were used as a covariate for each measurement when significant. Cows were grouped by parity into three groups; 1st, 2nd, and > 2nd lactations; the last included two- 3rd, three- 4th, and one- 5th lactation cows. Group size was small. However, previous literature (Shappell et al., 1986; Hodnett et al., 1992) has shown this is acceptable when examining variables such as serum Ca, P, and PTH concentrations.

RESULTS AND DISCUSSION

Effect of Parity

Lactation number had no effect on serum Ca, P, PTH, or 25-OH concentration (Table 3.2). This is in contrast to other studies (Moore et al., 2000; Chan et al., 2006) that reported higher serum Ca concentration in primiparous cows as compared to multiparous cows. Moore et al. (2000) found lower serum PTH and 25-OH concentrations in primiparous cows as compared to multiparous cows in a study that examined dietary cation-anion difference. First lactation cows had the lowest serum Mg concentration.

Serum OC concentration was highest in 1st lactation cows as compared to all other lactations (Table 3.2, Figure 3.2), and 2nd lactation cows had higher serum OC concentration as compared to the > 2 lactation group. Liesegang et al. (2000) first reported that OC is affected by age in a study examining the effect of low and high milk yields in lactating Brown Swiss cows between the ages of 5 and 13 yr. This study did not indicate OC concentration differences between the ages. A study by Kamiya et al. (2005) reported similar results to the present study with higher concentrations of OC and a

marker of bone resorption in primiparous cows from -13 d prepartum to 3 d postpartum. Moore et al. (2000) observed that concentration of hydroxyproline, a marker of bone resorption, was higher for primiparous cows than in multiparous cows. The results from the present study and the Moore et al. (2000) and Kamiya et al (2005) studies taken together would suggest that although primiparous cows resorb more bone compared to multiparous cows, they also replace that bone throughout lactation.

Keene et al. (2004) measured bone mineral content (BMC) in cadaver Holstein metacarpal and caudal vertebrae to evaluate effects of parity. Total BMC concentration (ash, % bone DM) was not affected by parity but Ca and P concentration of the metacarpal vertebrae increased with parity. This could indicate less resorption of these two minerals in multiparous animals. However, these results are likely specific to cull dairy animals and BMC may not be the most appropriate evaluation of total bone mineral reserves due to differences in bone mineral mass over time (Beighle, 1999).

Serum Ca, P, and Mg

There were no treatment (Table 3.3) or treatment by time interaction differences for serum Ca or serum P (Figure 3.3 A and B). There was an interaction of parity and time for serum Ca concentration ($P < 0.01$). The > 3rd lactation group had lower serum Ca concentration on day 0 and 1 as compared to both 2nd and 3rd lactation groups. Serum Ca and P were both different over time and were within the normal concentration range for adult ruminants (8 to 10 mg/dL and 4 to 8 mg/dL, respectively), but were unaffected by treatment. Olson et al. (1973b) similarly found that serum Ca and P concentrations were not different in cows injected with 4.0 or 8.0 mg 25-OH in sesame oil carrier between 72 h and 10 d prior to calving as compared to controls. A study by Rivera et al. (2005) examined oral dosing of beef heifers with 0, 10, 100, or 1000 mg of 25-OH in efforts to improve meat tenderness. At daily doses of 100 or 1000 mg of 25-OH, serum Ca concentration increased in treated beef heifers as compared to controls. The oral dose of 25-OH needed to increase blood Ca was much higher than used in the present study.

The DHVD analog appears to increase serum Ca and P concentrations when administered alone or in conjunction with 25-OH. Cows injected every 5 d beginning 15 d prior to parturition with 0.5 mg of DHVD and 4.0 mg of 25-OH had higher serum Ca and P concentrations at 2, 3, 5, and 7 d post-treatment compared to control cows (Hodnett

et al., 1992). Braithwaite (1978) also found that injecting sheep with 5 µg/d of DHVD for 10 d beginning 1 wk after lambing increased serum Ca (11.6 mg/dl) and P (10.8 mg/dl) concentrations as compared to control (9.2 and 8.4 mg/dl, Ca and P, respectively) treated sheep. An intravaginal dose of 1 µg of DHVD increased plasma Ca concentration from 12 to 72 h after treatment compared to the 0 h sample (Okura et al., 2004). Taken with the present data it appears that both the timing of administration of the vitamin D analog and the form are critical factors. In the present study both the timing of administration as well as the form of vitamin D administered could be responsible for not increasing serum Ca and P concentrations.

Serum Mg concentration was not affected by treatment. The interaction of treatment and parity number tended to be significant ($P < 0.09$) for serum Mg concentration. The treatment by parity interaction can be explained by the 3rd lactation cows behaving differently on the 25-OH treatment. The 3rd lactation cows treated with 25-OH had lower serum Mg concentration as compared to the other treatments. A biological explanation for this response is not obvious.

Serum Hormones

As expected, concentration of 25-OH was higher in serum from cows dosed with 25-OH as compared to cows dosed with CON and D_3 (Table 3.3). There was no effect of time by treatment interaction (Figure 3.4) or lactation number on serum 25-OH concentration. Parathyroid hormone concentration was lower in 25-OH dosed cows when compared with the D_3 dosed cows (Table 3.3). There was a treatment by parity interaction for PTH concentration whereby cows in the $> 3^{rd}$ lactation group dosed with 25-OH had lower serum PTH as compared to the other $> 3^{rd}$ lactation cows (Figure 3.5).

Similarly, Rivera et al. (2005) observed that the blood concentration of 25-OH increased when heifers were treated with exogenous 25-OH. However, in the present study the increase in serum 25-OH in the 25-OH treated group did not result in an increase in serum Ca concentration. The lack of response in serum Ca suggests that these cows did not sufficiently convert 25-OH into the active compound DHVD, but rather catabolized the hormone. This theory is corroborated by serum PTH concentration tending to be lower in the 25-OH treated cows compared to CON and D_3 cows. High circulating PTH concentration depresses catabolic enzymes in the kidney that oxidize 25-

OH (Goff et al., 1991). In the present study where PTH concentration was low, 25-OH concentration was high, and there was no effect of 25-OH dosing on Ca concentration, the authors speculate that the catabolic enzymes were active and degraded the 25-OH.

Blood Marker of Bone Metabolism

In the present study there was no effect of treatment (Table 3.3) on serum OC concentration. In contrast, others have observed an increase in OC concentration when dosing with DHVD in humans and sheep (Markowitz et al., 1987; Fortune et al., 1989). The response of the bovine may differ from these species as Naito et al. (1990) observed no correlation between blood OC and DHVD concentration in preparturient and postparturient cows. The cows in the study of Naito et al. (1990) were not administered exogenous vitamin D in any form. In the present study, the authors suggest that although 25-OH concentration increased in cows treated with 25-OH, the 25-OH was not converted to DHVD sufficiently to stimulate bone formation.

Second lactation cows had higher serum concentration of OC than 3rd or $>3^{rd}$ lactation groups (Table 3.3). As OC is a marker of osteoblast activity resulting in bone formation (Farrugia et al., 1989), the results from the present study were expected because osteoblast numbers decrease with age (Liao et al., 1990). Also, osteoblasts are the only bone cells to contain DHVD receptors (Liao et al., 1990) and Horst et al. (1990) demonstrated that bone from older rats contain fewer DHVD receptors than bone from young rats.

CONCLUSIONS

It appears that within the control treatment the 1st lactation animals were building more bone regardless of serum Ca concentration as compared to multiparous cows.

The increase in serum 25-OH in the group treated with 25-OH did not result in an increase in serum Ca concentration. The lack of response in serum Ca suggests that conversion of 25-OH into the active compound DHVD was insufficient, perhaps due to catabolism of this compound. This theory is corroborated by serum PTH concentration tending to be lower in the 25-OH treated cows compared to CON and D_3 cows. Although a 60% increase in serum 25-OH was observed due to a single oral dose of 25-OH prior to

calving, the amount administered in this study apparently was not sufficient for improvement in Ca homeostasis at parturition. Oral dosing with 15mg of 25-OH at 6 d prior to expected calving is not justified.

ACKNOWLEDGEMENTS

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Table 3-1. Ingredient and nutrient composition of pre-partum diet

Ingredient	% of Diet DM
Corn silage	53.5
High moisture corn	22.4
Chopped orchard grass hay	13.4
Soybean meal, 44% CP	10.0
Mineral[1]	0.7
Yeast cell wall[2]	0.1
Nutrient Composition	**% of Diet DM**
CP	11.6
ADF	21.1
NDF	34.7
Ca	0.29
P	0.33
Mg	0.19
K	1.06
Na	0.11

[1]Contained 14.0% Ca; 9.0% P; 13.0% Na; 2.0% Mg; 0.2% K; 2.0% S; 24 ppm Co; 235 ppm Cu; 80 ppm I; 3,400 ppm Mn; 59 ppm Se; 895 ppm Zn; 297,000 IU/kg vitamin A; 65,250 IU/kg vitamin D-3; and 900 IU/kg vitamin E.
[2]MTB 100, Alltech, INC. (Lexington, KY)

Table 3-2. Effect of parity on serum variables from Jersey cows beginning in the prepartum period

| Variable | Parity Number | | | SEM | $P <$ |
	1st	2nd	> 2		
Number of cows	5	3	6		
Ca^1, mg/dL	9.8	9.8	9.4	0.36	0.48
P^1, mg/dL	4.7	4.8	4.4	0.18	0.13
Mg^1, mg/dL	2.4 [a]	2.5	2.6 [b]	0.05	0.01
PTH^2, pg/mL	9.7	7.8	10.3	1.0	0.13
$25\text{-}OH^2$, pg/mL	64.8	64.5	68.4	5.8	0.75
OC^3, ng/mL	48.3 [a]	32.0 [b]	22.3 [c]	3.3	0.0001

[1] Evaluated through 84 DIM.
[2] Evaluated through 13 DIM.
[3] Osteocalcin, evaluated through 84 DIM.
[abc] Numbers with different superscripts in the same column differed at $P < 0.05$.

Table 3-3. Effect of treatment on serum variables from 27 Jersey cows dosed prepartum with corn starch (CON), 25-hydroxyvitamin D_3 (25-OH), or vitamin D_3 (D_3)

Variable	Treatments					Parity					Contrasts	
	CON	25-OH	D_3	SEM	P <	2nd	3rd	$>3^{rd}$	SEM	P <	CON vs. Avg. (25-OH, D_3)	25-OH vs. D_3
Number of cows	9	9^1	9			9^1	6	12				
Ca^2, mg/dL	9.6	9.9	9.5	0.2	0.33	9.8	9.4	9.3	0.3	0.26	0.70	0.15
P, mg/dL	4.5	4.5	4.6	0.1	0.84	4.7	4.5	4.4	0.2	0.20	0.62	0.75
Mg^3, mg/dL	2.6	2.5	2.5	0.5	0.58	2.5	2.6	2.5	0.1	0.42	0.41	0.49
PTH^4, pg/mL	10.1	9.6	11.4	0.6	0.09	9.5^a	12.1^b	9.6^a	0.7	0.01	0.56	0.04
$25\text{-}OH^1$, pg/mL	69.3^b	119.0^a	77.5^b	4.0	0.0001	89.7	88.5	87.7	4.9	0.91	0.0001	0.0001
OC^5, ng/mL	25.4	25.4	28.3	2.1	0.55	32.4^a	25.4	21.3^b	2.5	0.003	0.57	0.35

[1] Evaluated through 13 DIM, 1 cow was culled after 13 DIM, 8 cows evaluated until 84 DIM.
[2] Parity number x day interaction was significant (P < 0.01).
[3] Treatment x parity number interaction tended to be significant (P < 0.09).
[4] Evaluated through 13 DIM, Treatment x parity number interaction was significant (P < 0.02).
[5] Parity number x day interaction was significant (P < 0.04).
[abc] Numbers with different superscripts in the same column differed at $P < 0.05$.

Figure 3-1. Effect of parity on serum osteocalcin (OC) concentration from -5 to 84 days relative to parturition with no treatment administered. (SEM = 17.2; P < 0.0001 for parity; parity by day P = 0.85)
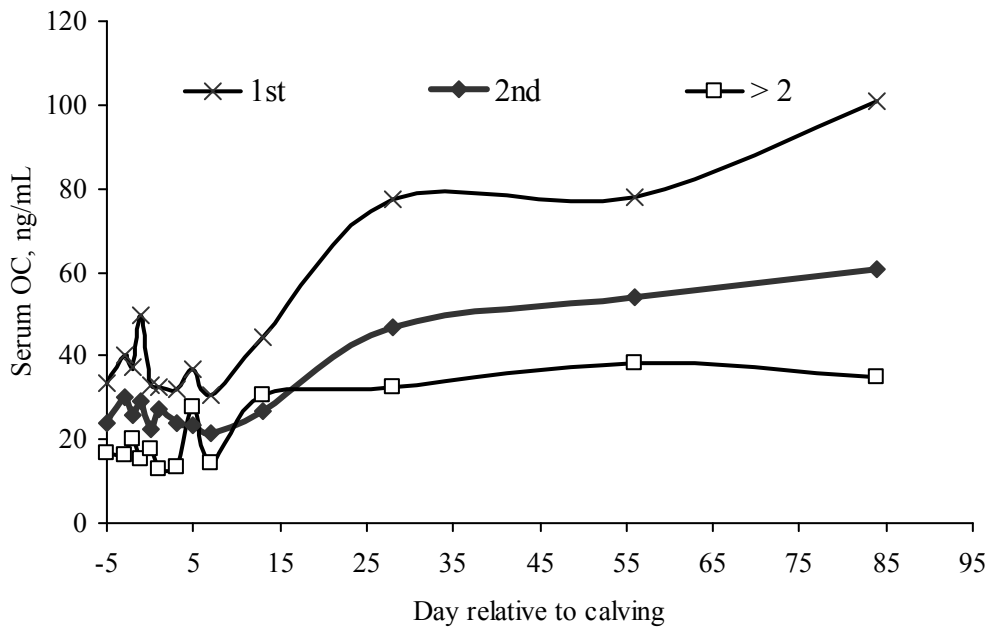
Figure 3-2 (A and B). Effect of time and treatment on serum (A) Ca and (B) P concentration in 27 Jersey cows dosed prepartum with corn starch (CON), 25-hydroxyvitamin D3 (25-OH), or vitamin D3 (D3). (SEM = 0.42)
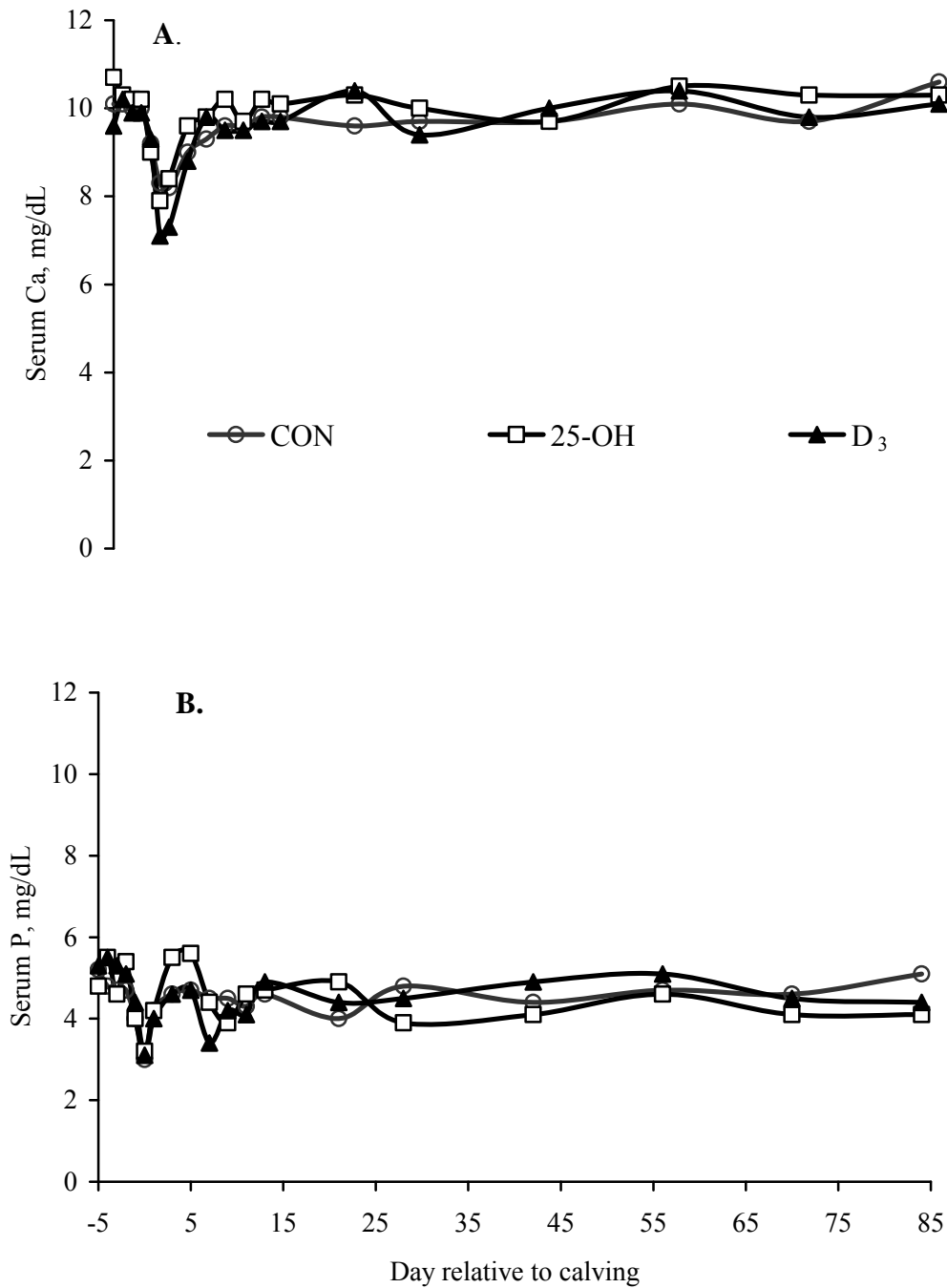
Figure 3-3. Serum 25-hydroxyvitamin D3 concentration from -5 d prepartum until d 13 of lactation in 27 Jersey cows dosed prepartum with corn starch (CON), 25-hydroxyvitamin D3 (25-OH), or vitamin D3 (D3). (SEM = 10.1)
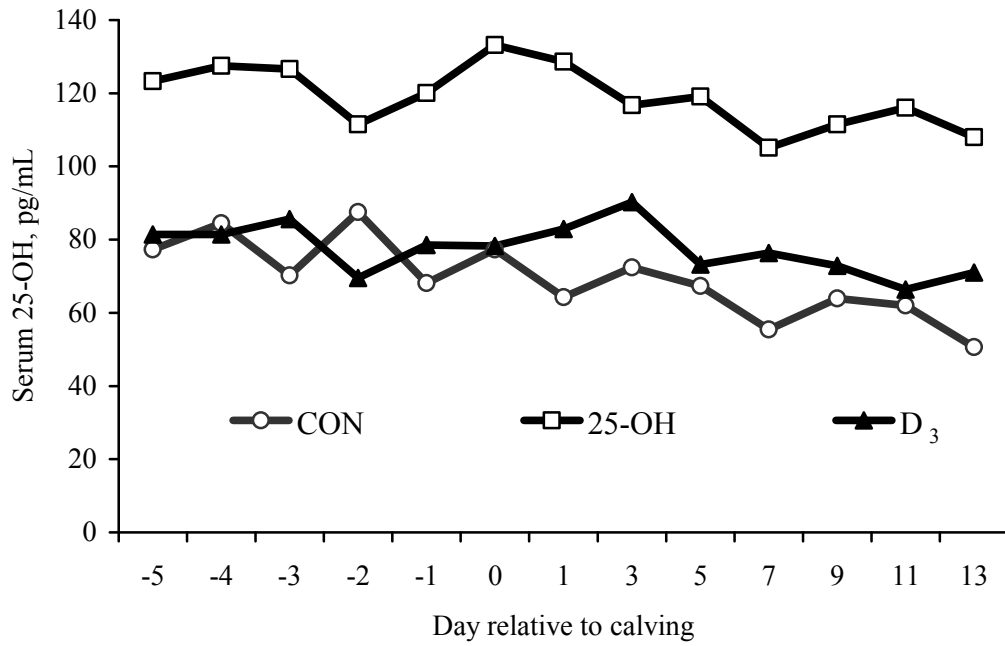
Figure 3-4. Effect of parity and treatment on serum parathyroid hormone (PTH) concentration in 27 Jersey cows dosed prepartum with corn starch (CON), 25-hydroxyvitamin D3 (25-OH), or vitamin D3 (D3). Interaction of treatment x parity, P < 0.02
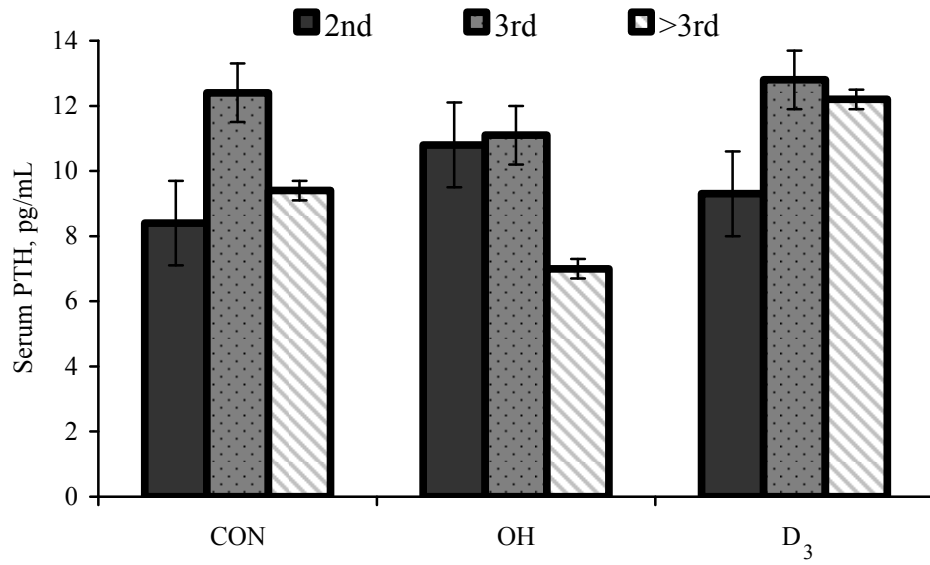
Chapter 4: Calcium and Phosphorus Balance and Bone Mobilization through Lactation with Three Dietary Calcium Concentrations

ABSTRACT

Calcium (Ca) and phosphorus (P) balance and mobilization from bone were evaluated through 20 wk of lactation to determine the timing and extent of net resorption of bone mineral and mineral balance in lactating dairy cows. Eighteen Holstein cows were blocked by parity and calving date and randomly assigned to one of three dietary treatments: high (1.03%, HI), medium (0.78%, MED), or low (0.52%, LOW) dietary Ca. Dietary P was 0.34% in all diets. Cows consumed treatment diets from calving to 140 DIM. Total collection of milk, urine, and feces was conducted 2 wk prior to expected calving and in wk 2, 5, 8, 11, and 20 of lactation. Blood samples were collected at -14 and -10 d prior to expected calving and 0, 1, 3, 5, 10, 14, 21, 28, 35, 56, 70, 84, 98, and 140 d after calving. Blood samples were analyzed for Ca, P, and parathyroid hormone concentration. Serum concentrations of osteocalcin (OC), a marker of bone formation, and deoxypyridinoline (DPD), a marker of bone resorption, were measured to assess bone mobilization. Rib bone biopsies were conducted within 10 d of calving and during wk 11 and 20 of lactation. Dietary Ca concentration affected Ca balance, with cows consuming the HI Ca diet in positive Ca balance for all weeks with the exception of wk 11. Interestingly, all cows across all treatments had a negative Ca balance at wk 11, possibly the result of timed estrous synchronization that occurred during wk 11. At wk 20, Ca balances were 61.2, 29.9, and 8.1 g/d for the HI, MED, and LOW diets, respectively. Phosphorus balances across all treatments and weeks were negative. Bone Ca content on a fat free ash weight basis was lowest in cows consuming the MED diet but bone P was not different. Serum Ca and P were not affected by treatment. Dietary Ca concentration did not affect P balance in the weeks examined for this study but there was a clear effect of parity on balance, markers of bone metabolism, and bone P. Primiparous cows had higher serum OC and DPD concentrations than multiparous cows, but multiparous cows had higher bone ash content (bone mass was not measured). Regardless of dietary treatment, serum OC concentration peaked around d 35 of lactation. Simultaneously,

DPD concentration began to decrease, which may indicate a switch from net bone resorption to net bone formation after day 35. However, this was not reflected in balance measures. This information may help refine dietary mineral recommendations for lactating dairy cows and ultimately reduce P excretion into the environment.

Key words: bone, calcium, phosphorus, cow

INTRODUCTION

Phosphorus metabolism of ruminants has been investigated by many (Braithwaite, 1983b; Morse et al, 1992; Wu et al., 2000; Wu and Satter, 2000; Knowlton and Herbein, 2002), yet estimates of bone resorption have not been fully described. The immediate Ca demand for milk production at the time of parturition through the first weeks of lactation increases the drain on blood Ca. This drain on the Ca pool is not preventable by feeding excess Ca (Horst et al., 1994), but is exacerbated when the diet is deficient in Ca (Benzie et al., 1955). A complex endocrine regulating system maintains blood Ca concentration within a narrow range (8 to 10 mg/dl, Goff et al., 1991). When this range is disturbed an array of homeostatic mechanisms are activated, including resorption of bone.

Approximately 98% of total body Ca and 80% of body P are located in the skeleton. Bone Ca and phosphorus (P) are stored as calcium phosphate $[Ca_2(PO_4)_2]$, which is amorphous, and hydroxyapatite $[Ca_{10}(PO_4)_6(OH)_2]$, a crystalline structure that provides binding locations for Ca and P. Mobilization of bone hydroxyapatite results in 10 ions of Ca and 6 ions of P being released into circulation (Goff, 2000) and is driven primarily by the concentration of blood Ca rather than blood P. The timing of bone resorption and formation are currently unknown in the dairy cow, but several blood markers of bone metabolism are available allowing bone formation and resorption to be monitored non-invasively. However, these markers have yet to be validated in dairy cows with bone samples and total collection.

By accounting for drafts on bone with different dietary Ca concentrations, the P concentration of the diet could potentially be reduced to coincide with endogenous P release from bone during times of Ca-induced bone resorption. The amount of P excreted in the feces of dairy cows is directly proportional to excess dietary P (Morse et al, 1992). Phosphorus run-off is one of the leading causes of fresh water eutrophication and is a major focus of nutrient management for livestock producers (Knowlton et al., 2004). Field surveys demonstrate that P is typically fed at 20 to 40% above published recommendations for dairy cows (Knowlton et al., 2004). If these recommendations are

excessive because of the lack of information on bone mineral resorption, the overfeeding problem is even worse than currently surmised.

Our goal was to evaluate changes in body Ca and P throughout lactation in cows fed one of three dietary concentrations of Ca based on Ca and P balance, serum markers of bone metabolism, and bone biopsy samples. Our hypothesis was that feeding a low Ca diet will cause an increase in bone mobilization in early lactation that will result in an increase in the endogenous pool of P.

MATERIALS AND METHODS

Eighteen Holstein cows (10 first, 5 second, 2 third, and 1 fourth lactation) were randomly assigned to one of three dietary treatments. The treatments diets were formulated to contain 0.45 (LOW), 0.75 (MED), and 1.1% (HI) Ca on a DM basis (Table 4.1). Dietary P concentration was 0.34% in all diets. Prior to calving all cows consumed a common dry cow diet (Ca 0.39% and P 0.38%). Treatment groups were balanced for parity and mature equivalent milk production. Treatments were applied from calving through 140 d of lactation.

Balance Periods

Total collection was conducted in six, 4-d balance periods at -14 d prior to expected calving and at 14, 35, 56, 77, and 140 d of lactation. Cows were fitted with a urinary catheter (22 French, 75 cc; C.R. Bard, Inc., Covington, GA) and moved into metabolism stalls on d 1 of each collection period for a 24 h adaptation to both the metabolism stall and the catheter. The urinary catheter was connected to tygon tubing that drained into a sealed clean plastic 12 L jug. Every 6 h, feces were removed from behind the cow and placed into sealed 130 L plastic containers, and the urine jugs were weighed and replaced with clean jugs. Urine was acidified (36N H_2SO_4) to below pH 2 and then pooled into a larger sealed plastic jug until the end of the 24 h collection. Feces and urine were weighed, pooled by day, mixed thoroughly, and sub-sampled. Urine was stored frozen until analyzed for Ca and P (Thermo electron IRIS Intrepid II XSP; Thermo Fisher Scientific, INC.; Waltham, MA). Feces samples were dried at 60^0C to a constant weight, ground through a 1 mm screen in a Wiley Mill (Arthur H. Thomas, Philadelphia, PA.)

and analyzed for Ca and P concentrations by a commercial laboratory (Cumberland Valley Analytical Services, Hagerstown MD) in accordance with AOAC approved methods.

Cows were milked twice a day at 0530 and 1730 h while standing in the metabolism stalls. Milk was weighed and sampled at all milkings; samples were analyzed for fat, protein, SCC, SNF, and lactose (Dairy Herd Improvement Association, Blacksburg, VA). A second milk sample was stored frozen for analysis of Ca and P by the method of Walter et al. (1997; CEM Corporation; Matthews, NC).

Feed offered and refused was recorded during the balance periods. Feed refusals were sampled by cow on d 3 of each balance period. When cows were not in the metabolism stalls they were group-housed in a free stall barn and fed via a Calan door system (American Calan; Northwood, NH); feed offered and refused was recorded daily. Cows were fed for 10% refusals. Individual ration ingredients were sampled weekly throughout the study and pooled by month. Feed and feed refusal samples were dried at 60°C to a constant weight, ground through a 1 mm screen in a Wiley Mill (Arthur H. Thomas, Philadelphia, PA.), and analyzed for DM, NDF, ADF, Ca, and P concentrations by Cumberland Valley Analytical Services, Hagerstown, MD in accordance with approved AOAC methods.

Blood Sampling and Analysis

Jugular blood samples were obtained at -14 and -10 d (averaged -12 and -8 d) prior to expected calving, at calving, 1, 3, 5, 10, 14, 21, 28, 35, 42, 56, 70, 84, 98, 119, and 140 d relative to calving. Samples were collected and immediately placed on ice until centrifugation (2200 x g for 20 min). Serum separators (Fisher Scientific, Pittsburgh, PA) were utilized to facilitate serum separation during centrifugation. Serum was harvested and stored frozen until analysis. Colorimetric methods were used to analyze all serum samples for Ca (Aresenazo Reagent Set, Pointe Scientific, Canton, MI) and inorganic P (Inorganic Phosphorus Reagent Set, Pointe Scientific, Canton, MI). Competitive immunoassays were used to quantify serum osteocalcin (OC; Meta Osteocalcin, Quidel Corporation, San Diego, CA) and deoxypyridinoline (DPD; Total Deoxypyridinoline, Quidel Corporation, San Diego, CA) in all serum samples. Parathyroid hormone (PTH) was analyzed in a pooled pre-calving sample (-14 and -10)

and in the 0, 1, 3, 5, and 10 d samples by a radioimmunoassay specific for the intact PTH peptide (N-tact PTH SP, DiaSorin, Saluggia, Italy).

Bone Biopsies

Rib bone biopsies were collected on d 8 ± 3.5 post calving (left side, 11th rib) and on d 77 ± 5.6 (right side, 11th rib) and d 140 ± 3.7 (left side, 12th rib) using a modification of the procedure of Beighle et al. (1993). Briefly, biopsies were performed with the animal restrained in a standing head gate. The pre-designated side for the bone sample was clipped ventrally for 25 cm beginning below the lumbar vertebrae and 20 cm caudal and cranial to the sample rib. The clipped area was cleaned and surgically prepared with alternate application of betadine scrub and alcohol. The proposed incision site was anesthetized with 2% lidocaine in an inverted L pattern. After a second surgical scrub and alcohol rinse, a 10 cm incision was made over the midline of the rib through the skin, fascia, and muscle. The periosteum was displaced from the biopsy site with a periostal elevator (Jorgensen Laboratories; Loveland, CO) and the biopsy site was exposed with a Weitlaner retractor (Jorgensen Laboratories; Loveland, CO). A power drill and sterile drill bit were used to make a guide hole for the bone trephine (16 mm, Galt cranial trephine, Mercedes Medical, Tellevast, FL). Trephine depth was restricted to allow for sampling of the exposed medial and lateral bone to the depth of the medullary cavity. Obtained bone samples weighed 700 to 1000 mg. The circular piece of bone was removed and immediately stored in a sealed plastic bag and placed on ice. The periosteal flaps were replaced loosely without suturing followed by suturing of the muscle and stapling of the skin separately. Bone samples were extracted with ether using a modified soxhlet procedure (AOAC, 2006) and subsequently ashed at 600^0C in a muffle furnace. Ash samples were then analyzed for Ca and P using a microwave digestion procedure (MARS 5; CEM Corporation; Matthews, NC).

Statistical Analysis

All data were analyzed using the Mixed procedure of SAS (2003) with the model defined in Table 4.2. Blood analyses included the -14 and -10 d samples which allowed for prepartum representation on the graphs. Two first-lactation cows consuming the MED diet did not have wk 2 balance data due to illness; one cow was treated for

pneumonia and the other cow had a displaced abomasum at 10 d postpartum. The wk -2 collection data were used as covariates for the feces and urine variables with the exception of one HI cow that calved 2 wk before her expected due date. The -2 wk data collected from the other HI cows were averaged and used as the covariate for that cow.

An autoregressive (AR(1)) covariance structure was used for DMI and milk yield data. The spatial power (SP POW) covariance structure was utilized for all other variables due to unequal spacing between sample intervals. The slice option was used when there was a significant interaction. Treatments that were different in a particular week were further analyzed with pairwise Tukey tests. Orthogonal polynomial contrasts were used to test for linear and quadratic responses across time (day or week). The NESTED procedure was used to estimate correlations among OC, DPD, Ca balance, P balance, and bone Ca and P within a cow for each treatment. Significance was declared at $P < 0.05$ and trends at $P < 0.10$.

RESULTS AND DISCUSSION

DMI and Milk Yield

Actual Ca concentration of the treatment diets were 0.53, 0.78, and 1.03% for the LOW, MED and HI diets, respectively (Table 4.3). The discrepancy is attributed to unexpectedly high Ca concentration in the cotton seed.

Dry matter intake was not affected by dietary Ca concentration (23.4 ± 0.6 kg/d; Table 4.4). Multiparous cows consumed more DM than did primiparous cows (25.4 vs. 21.4 ± 0.50 kg/d) and there was a significant parity by week interaction. The effect of week in both lactation groups was linear and quadratic over time. The multiparous cows had a more rapid increase in DMI for the first 4 wk of lactation. Intake by primiparous cows peaked around wk 14 while multiparous cows peaked during wk 11. Dry matter intake was different across week, with cows consuming an average of 11 kg/d DM during wk 1, 27.4 kg/d at peak, and 27.2 kg/d DM at the conclusion of the study.

Milk yield was not affected by dietary Ca concentration (Table 4.4). As expected, multiparous cows had higher daily milk yield as compared to primiparous cows (36.3 vs. 29.5 ± 0.86 kg/d). Diet had no effect on milk fat yield but there was a significant interaction of parity and time (Table 4.4). Multiparous cows had higher milk fat yield,

due to higher production, with the exception of wk 8 and 20 where the two parity groups were not different. There was a significant treatment by week and parity by week interaction for milk protein yield.

Serum Minerals

Serum Ca was not affected by dietary Ca concentration (Table 4.5; Figure 4.1). At calving cows were within or just below the normal serum Ca range of 8 to 10 mg/dl (Goff, 2000) with no clinical signs of hypocalcemia. Similar to our findings, dietary Ca concentration had no effect on plasma Ca concentration in sheep fed 0.47 or 0.82% dietary Ca (Takagi and Block, 1991) or in postpartum cows fed 0.99 or 1.5% dietary Ca prepartum (Chan et al., 2006). There was a linear response in serum Ca over time (Figure 4.1). First lactation cows tended to have higher serum Ca concentration than the multiparous group ($P < 0.08$; 11.45 vs. 10.76 ± 0.27 mg/dl). Chan et al. (2006) also observed that primiparous cows had higher serum Ca than multiparous cows.

Dietary Ca concentration did not affect serum P (Figure 4.1). Chan et al. (2006) also reported no difference in postpartum serum P concentration when cows were fed dietary Ca at 0.99 or 1.50% of diet in the prepartum period. Serum P concentration was higher in first lactation cows compared to multiparous cows (5.7 vs. 4.9 ± 0.13 mg/dl). However, there was a significant 3-way interaction of treatment, parity, and day for serum P concentration making main effects and two-way interactions difficult to interpret on their own.

Serum Markers of Bone Metabolism

The interaction of treatment and parity was significant for serum OC concentration (Figure 4.2). Within the primiparous group, the MED cows had lower OC concentration as compared to the other dietary treatments, while OC concentration was not affected by treatment in multiparous cows. Osteocalcin concentration reflects formation of the protein matrix; the biological explanation for this observation is not apparent.

Serum concentrations of both DPD and OC were higher in primiparous cows than multiparous cows (Figure 4.3 A and B). These results are similar to a study by Kamiya et al. (2005) that examined the effect of prepartum dietary Ca concentration (0.46% or

0.86%) on bone turnover from 13 d prepartum to 3 d postpartum in primiparous and multiparous Holstein cows. In that study, there was no effect of dietary Ca concentration on plasma OC or urinary DPD, but primiparous cows had higher concentrations of both as compared to multiparous cows (Kamiya et al., 2005). This is likely because the younger cows were still growing and had a higher rate of bone turnover than the older cows.

In the present study there was a parity by time interaction (Figure 4.3 A) for serum DPD concentrations. Both parity groups had a negative linear response in DPD over time. Primiparous cows had higher serum DPD concentration than multiparous cows from -14 d prepartum to d 3, at d 10, and again from d 35 to 77. Otherwise the two groups were not different. Kamiya et al. (2005) found no effect of parity over time on urinary DPD concentration, but that study concluded at 3 d postpartum.

Both serum OC and DPD concentrations varied with time (Table 4.3). In early lactation serum OC was low in both primi- and multiparous cows indicating relatively low formation of bone protein matrix. Osteocalcin then increased until 35 DIM where the two groups plateaued. There was a linear decrease in serum DPD concentration over time (2.8 ng/mL at calving; 1.5 ng/mL at d 140) indicating reduced bone formation immediately after calving. Liesegang et al. (2006) reported similar results for the marker of bone resorption they used, cross-linked carboxyterminal telopeptides of Type-I collagen (CTx), with an increase in serum concentration after parturition until 9 d postpartum. It was anticipated that serum DPD would be high around calving when the cows would likely be resorbing bone, but it is surprising that dietary Ca had no effect.

Ekelund et al. (2006) found an interaction of treatment and time for plasma OC from cows consuming normal vs. low to normal dietary P concentrations (0.43% vs. 0.32% for the first 4 mo of lactation then 0.43% for the remainder). These authors concluded there was net bone formation in mid lactation (wk 17 to 24) as indicated by higher concentrations of OC during those weeks. However, there was not a corresponding decrease in concentration of CTx. Also, they saw no change in P retention as measured by total collection.

Bone is a dynamic tissue constantly being resorbed and formed; the relative rates of resorption and formation determine net bone mass. Parity clearly has an effect on

bone metabolism and when the markers of formation and resorption are compared in the present study, the graphs appear to be mirror images (Figure 4.3 A and B). Serum OC concentration peaked around d 35 of lactation; simultaneously DPD concentration began to decrease. The change in direction of serum DPD and OC may provide an indicator of a net change from bone resorption to bone formation, but closer evaluation suggests that this relationship only holds in cows fed adequate Ca. In cows fed the MED and HI diets, the correlation between OC and DPD was strong and negative (-0.41 and -0.71, respectively). In cows fed the LOW diet, however, there was no correlation between OC and DPD.

Serum Parathyroid Hormone

There was no effect of dietary Ca treatment on serum PTH concentration (9.9 ± 2.9 pg/mL). Typically, serum PTH increases near calving, activating bone resorption and renal tubular reabsorption to increase blood Ca (Golf, 2000). In the present study, this was not observed in the days examined (1, 3, 5, and 10 d postpartum). Kamiya et al. (2005) also observed no effect of dietary Ca on serum PTH. However, the researchers did report an effect of parity, with PTH concentration lower in primiparous cows as compared to multiparous cows. This was not observed in the present study.

Bone Mineral Content

Bone P content was not affected by dietary Ca concentration regardless of how results were expressed (Table 4.6). Dietary Ca concentration had no effect on bone Ca content on a mass or dry weight basis, but when expressed on a % of ash weight basis, the MED cows had lower bone Ca content as compared to the LOW cows (Table 4.6). Bone Ca of cows consuming the HI diet was not different from the LOW or MED cows. The wk 11 bone sample for one cow on the LOW diet was identified as an outlier and removed from all bone analysis. Several covariates were tested to adjust for the lower bone Ca concentration in the cows on the MED diet; including the 8 d sample, the preliminary OC and DPD concentrations, or the ratio of OC to DPD but they were all non-significant. The SAS profile analysis was used to examine the difference between the samples. There were no significant differences for treatment with the profile analysis.

An unexpected result was the finding of greater bone Ca content for cows fed the LOW diet than for those fed the MED diet; nonetheless the correlation of bone Ca and Ca balance was stronger for cows consuming the LOW diet (0.56 vs. 0.37 MED and -0.06 HI). For the LOW cows, the strong correlation indicates that increased bone Ca was associated with a corresponding increase in Ca retention. However for cows consuming the HI diet, Ca content in bone was not correlated to Ca retention. This is likely because they were consuming Ca in excess of their requirement. Excess Ca was excreted rather than used to build bone (Table 4.7).

It is puzzling that there was a difference in bone Ca and not bone P, since bone mineral exists in the form of calcium phosphate or hydroxyapatite. Cohen (1973) observed that bone Ca was unrelated to pasture Ca concentration in yearling steers given no supplement or an oral drench of 35 or 70 g of P (sodium dihydrogen orthophosphate) each week. There was a negative relationship between bone Ca and P content in both studies. Cohen (1973) suggested that calcium carbonate was being removed and deposited without phosphate, resulting in an increase in the proportion of Ca in the bone. Most minerals that are combined in the body contain carbonate substitutions that are described as type A (OH^- substituted by CO_3^{2-}) and type B (PO_4^{3-} substituted by CO_3^{2-}). Type B is most prevalent (Rey et al., 1989; Penel et al., 1998). In the present study it appears cows were forming bone matrix after d 35 according to the serum markers of bone metabolism. It is reasonable to assume that cows were mineralizing the bone matrix with Ca and carbonate as indicated by the increase in bone Ca content with no change in bone P by treatment. However it is unclear why this appears to be favored in the MED treatment and not in the LOW and HI treatments.

Parity had no effect on bone Ca content but multiparous cows had higher bone P compared to primiparous cows on a % of wet and ash weight basis (Table 4.6). Wu et al. (2001) reported no differences in bone P as a percent of ash (17.6% of ash) when cows were fed dietary P at 0.31, 0.39, or 0.47% for two or three years. There was a linear increase in bone Ca content on a fat free ash basis across week (49.6, 52.8, and 54.8 ± 1.8 % of fat free ash, for 8 d, 11 and 20 wk, respectively). However, there was a quadratic response in bone Ca grams/sample (0.23, 0.17, and 0.20 ± 0.007 for d 8 and wk 11 and 20, respectively). A quadratic response across time was also observed for grams/sample

of fat free bone ash and P with the 8 d sample being highest for both. The interactions of treatment by time and treatment by parity were not significant for bone Ca or P in grams/sample, or concentration on a wet, dry, or ash basis. Bone data are presented on a gram/sample, % of wet weight, % of dry weight, or % of fat free ash weight basis for Ca, P, and ash to allow for incorporation into meta-analysis publications in the future.

Ca Partitioning and Balance

There was a linear effect of dietary Ca treatment on fecal Ca excretion (Table 4.7); cows consuming more dietary Ca excreted more fecal Ca. Fecal Ca was different by week across all treatments with linear and quadratic responses over time. There was a linear increase in fecal Ca excretion from wk 2 through the wk 11 balance. Week 11 had the highest fecal Ca excretion across all treatments. This could be caused by a decrease in Ca binding protein (CaBP) potentially associated with estrus synchronization (discussed below; Inpanbutr et al., 1994). The decline in fecal Ca excretion at wk 20 caused the quadratic response. Multiparous cows excreted more fecal Ca as compared to primiparous cows (142 vs. 119 ± 4.3 g/d). This is likely the result of increased intake and is similar to the observations of Knowlton et al. (2001).

Urinary Ca excretion was not affected by diet (Table 4.7). Irrespective of treatment all cows excreted the least urinary Ca during the wk 5 balance period but urinary Ca was less than 1 g/d throughout. Primiparous cows excreted more Ca in their urine as compared to multiparous cows (0.90 vs. 0.57 g/d). Chan et al. (2006) reported no effect of prepartum dietary Ca (0.99% vs. 1.50%) on urinary Ca excretion and no effect of parity.

Milk Ca secretion was not influenced by dietary Ca treatment (Table 4.7). Multiparous cows had higher daily milk yield therefore multiparous cows also had higher daily milk Ca secretion as compared to primiparous cows (54.2 vs. 39.5 ± 2.2 g/d). The parity by time interaction was significant with multiparous cows decreasing in milk Ca secretion from wk 8 to 20 while primiparous cows did not change. This is the result of a significant decrease in MY for multiparous cows from wk 8 to 20 where primiparous cows remained constant. Weller et al. (2006) found that primiparous cows peaked in MY lower and later but had higher persistency of milk production as compared to multiparous cows. The interactions of treatment by week and treatment by parity were not significant.

As expected, Ca balance was affected by dietary Ca concentration (Table 4.7; Figure 4.4 A). Cows consuming the LOW diet were in negative Ca balance for the entire 20 wk observed. During wk 2, cows consuming the MED diet had a negative Ca balance, but Ca balance was positive by wk 5. Knowlton and Herbein (2002) observed that all cows were in positive Ca balance by wk 5 with dietary Ca concentration at 0.72 to 0.77% of the diet DM. Cows consuming the HI diet had a positive Ca balance in most weeks except for wk 11, where all cows had a negative Ca balance due to an increase in fecal Ca excretion.

The decrease at wk 11 is not the effect of one specific calendar week because cows had a staggered start. It is also not caused by a decrease in DMI because wk 11 had higher DMI than wk 10 and was not different from wk 12. All cows in this study were enrolled in a first service synchronization program, Ovsynch (described in Cornwell et al., 2006), which was standard protocol at the Virginia Tech Dairy Center. The Ovsynch protocol was intended to align all cows to be inseminated between 75-80 d postpartum. In this study, all cows were inseminated in their respective metabolism stall during the wk 11 balance period. This synchronization may have influenced Ca metabolism. Expression of calbindin-D9k (CBP), a calcium binding protein, and CBP mRNA in the bovine uterus, has been shown to be threefold higher during the luteal phase (progesterone dominant) than during the follicular phase (estrogen dominant; Inpanbutr et al., 1994). During the wk 11 balance period all cows would have been in an estrogen dominant phase for ~ 24 to 48 h of the 120 h period, potentially explaining the dramatic drop in Ca balance observed during wk 11. The observation made in uterine tissue could also affect intestinal tissue CBP. With less CBP in the intestines to bind Ca there would be less Ca retained in the animal and therefore a lower Ca balance. This could explain the higher fecal Ca excretion during wk 11 as compared to all other weeks. Cows were likely cycling throughout the other balance weeks but it was during wk 11 that all cows were synchronized and bred for the first time.

The positive Ca balance throughout the study in cows fed HI diets contradicts earlier work. In sheep fed a plentiful Ca diet, Ca balance was negative until 63-70 d of lactation (Braithwaite, 1983a). Others have concluded that cows are unable to absorb enough Ca to meet their demands in early lactation regardless of dietary Ca concentration

(Braithwaite, 1983a; Horst, 1994). Our data at 2 wk postpartum does not support this.
Cows consuming the HI diet with 1.03% dietary Ca apparently absorbed enough dietary
Ca to meet their needs. However, our first balance period was not until 2 wk post-
calving; Ca balance may well have been negative in all diets immediately postpartum.

The interaction of treatment and time was not significant for Ca balance (Table
4.7). The main effect of week was different with a positive linear and quadratic response.
The quadratic shape to the data can likely be explained by the dramatic drop at wk 11
followed by the increase at wk 20.

Phosphorus Partitioning and Balance

There was no effect of dietary Ca on fecal P excretion (47.5 ± 2.4 g/d) and the
interactions of treatment by parity, treatment by week, and parity by week were not
significant (Table 4.8). There was however both a linear and quadratic effect of week.
Fecal P was lowest at 2 wk postpartum and highest at wk 11 and 20 for all treatments.
Apparent P digestibility was different over time with the highest value of 37.3%
occurring during wk 8 and 11 (quadratic response).

Phosphorus was included in the diet at 0.34% which is in the range of the current
NRC (2001) P recommendations. It is suggested that dairy cows have the ability to
absorb enough P to meet their needs when dietary P supplied is adequate and available
(Morse et al., 1992). The present study had lower apparent P digestibility ($35.7\% \pm 3.2$)
than observed elsewhere (Wu et al., 2000; Knowlton et al., 2001; Ekelund et al., 2006)
although all cows were in negative P balance for the entirety of the study. This may be
because the P in the diet was not entirely available, incomplete recovery of P in the
collection system, or the error inherent with the analytical methods of P analysis.

The P requirement was calculated for these cows using their actual DMI and milk
yield during each of the balance weeks and was 0.39, 0.41, and 0.42% of the diet for the
LOW, MED, and HI, respectively. It is obvious that the dietary P concentration of 0.34%
was not sufficient to meet the cows' needs. These diets were formulated for a cow
consuming 23.2 kg DM and producing 36.3 kg milk per day but the cows actually
consumed 23.9 kg DM and produced 41.0 kg milk. Although the DMI was similar
between the formulated and actual diets, the difference in milk yield was substantial and
required 4.2 g/d of P to support the additional milk produced.

Multiparous cows had higher fecal P excretion as compared to primiparous cows (53.6 vs 41.1 ± 2.0 g/d) due to higher daily P intakes. These results are similar to Knowlton et al. (2001) where primiparous cows had lower P intakes, reduced fecal P, and lower milk P secretion. In contrast to the present study, Knowlton et al., (2001) observed an effect of parity on apparent P digestibility with primiparous cows having higher apparent P digestibility than multiparous cows.

Urinary P excretion was not affected by treatment, parity, or the interactions of treatment by parity and treatment by week. The main effect of week was significant with a quadratic response for urinary P excretion (Table 4.8). The highest urinary P occurred during the 2 wk balance (1.6 ± 0.3 g/d) followed by a decrease until wk 20 (1.2 ± 0.2 g/d). It is well accepted that P absorption, and in turn excretion, are directly related to P intake but that the kidneys are a minor route of P excretion (Hibbs and Conrad, 1983; Horst, 1986; Morse et al., 1992; Knowlton and Herbein, 2002). However, when bone mineral is resorbed in support of blood Ca concentrations, the urinary excretion of P can increase to maintain P homeostasis (Todd et al., 1962) if it is not needed. Wu et al. (2001) suggested that spilling of P into urine greater than 1 g/d per cow is a reliable sign that the animal has adequate P. The present study suggests otherwise since urinary P exceeded this threshold while all cows remained in a negative P balance (data below).

Dietary Ca concentration had no effect on milk P secretion (Table 4.8). Multiparous cows secreted more daily P in milk as compared to primiparous cows across all balance weeks with the exception of wk 20 where the two lactation groups were not different (parity by time). Milk P concentration, like milk Ca, is thought to remain relatively constant throughout lactation in ruminants (Gallego et al., 2006) so this is likely due to the decrease in MY in the multiparous cows during wk 20.

Phosphorus balance was not affected by dietary Ca concentration (Table 4.8). This does not support the original hypothesis. It was postulated that dietary Ca concentration would directly affect P balance in the lactating dairy cow when dietary P was held constant.

While no effect of dietary Ca was observed, parity had a clear effect on these parameters. Multiparous cows consumed more P, excreted more fecal P, and secreted

more milk P which resulted in a more negative P balance compared to primiparous cows (-13.69 vs. -7.98 ± 1.5 g/d).

Cows were in negative P balance regardless of dietary Ca concentration for the first 20 wk of lactation (Figure 4.4 B). There was a quadratic effect of time with wk 11 approaching a 0 balance. This contrasts with the Ca balance data where the wk 11 decrease in Ca retention is the result of increased fecal Ca excretion. Knowlton et al. (2002) observed negative P balance until wk 7 when cows were fed a diet containing 0.34% P. However, over the entire 11 wk study the P retention was never above 5 g/d (Knowlton et al., 2002).

Ekelund et al. (2006) attempted to force cows to utilize mobilized bone P with low (0.31%) dietary P concentration in early lactation as compared to normal (0.43%) P concentration. In that study, however, bone resorption and overall P retention was not different between the low and normal P dietary treatment groups; low dietary P did not induce increased bone resorption. Ultimately there was no change in P excretion between the two dietary groups. This is not surprising since previous literature (Braithwaite, 1983b) reports that in sheep bone is mobilized in response to the animal's needs for Ca and is not affected by dietary P content. The present study complements the study by Ekelund et al. (2006) in that varying dietary Ca concentration also did not affect P retention, bone resorption, or bone formation during the weeks examined.

Implications for P Feeding Recommendations

This study is unique in its examination of P requirements based on dietary Ca concentration in order to account for mobilized bone minerals. Bone that is resorbed is an available source of Ca and P that has not been accounted for in current recommendations. There is not an environmental concern for Ca but excess P in the diet can increase P in the manure. Therefore, if P requirements can closely match the animal's needs and account for useable P coming from bone, the amount of P being excreted in manure and ultimately into the environment may be reduced. However, net times of bone resorption and formation need to be determined over the course of a lactation to insure adequate mineral in the diet for when the animal switches from net resorption to formation of bone.

Regardless of treatment, data collected during balance weeks suggests that net bone formation occurred after the wk 5 balance based on ratios of OC to DPD across time. Irrespective of treatment, OC concentration was lowest during the wk 2 balance period and DPD concentration was highest during wk 2 and 5. These relationships inverted by the wk 8 balance. These changes may be indicative of a switch from net resorption to net formation of bone. The apparent changes in bone metabolism did not equate to changes in Ca and P balance. Calcium balance was highest at wk 20 and was nearly double all other balance weeks. Phosphorus balance was not different across time and was in fact negative during all weeks measured. Negative P balance cannot support net bone formation unless cows deposited bone mineral primarily as Ca, such as calcium carbonate. Our data support this hypothesis; Ca, but not P, content of bone samples increased over time and were highest at wk 20 on a mass, wet weight, and ash weight basis. Also, bone Ca and P were not correlated, which further suggests that the bone mineral was something other than hydroxyapatite $[Ca_{10}(PO_4)_6(OH)_2]$.

Benzie et al. (1959) examined which bones were the most sensitive to resorption in sheep consuming diets with differing Ca concentration and determined that bone resorption is not uniform within a bone and throughout the skeleton. The relative sensitivity of bones to resorption in descending order was vertebrae > pelvis > skull > sacrum > mandible > ribs > proximal end of tibia > and scapula. In the present study, the ribs were chosen for biopsy because of their accessibility and success of previous work utilizing such an approach to assess bone mineral status (Little et al., 1972; Beighle et al., 1993). However, it appears in the present study that Ca and P were being mobilized from bones other than the ribs, especially in the LOW Ca cows. From parturition until after wk 11, cows on the LOW diet were mobilizing between 10 and 30 g/d of Ca based on balance data. Through wk 20 they mobilized between 5 and 15 g/d of P. This could be due to the cows not being severely deficient therefore the drafts on other bones were able to meet the demand for the minerals before the cow began resorbing the ribs. In contrast, Little (1972) reported when yearling cattle were fed a P deficient (P at 0.08% of diet) diet for 6 wk, rib bone had a higher bone P content at wk 1 than the 6 wk samples. Cattle were reported to be in negative P balance (1 to 2 g/d) over the entire study but the methods for determining balance were not reported.

One of our objectives was to validate the use of OC and DPD as markers of bone metabolism with total collection and bone biopsies. Total collection is often used as the standard to determine mineral requirements but it is not error-free. There is an inherent compounding of error that occurs when calculating mineral balance from intake, feces, urine, and milk each with error that may lead to an increased likelihood of committing a type I error. Variability could potentially mask the physiological activity that is implied by the serum marker data. Alternatively, the markers of bone metabolism that were utilized are simply that: markers. Alone, they may not be a perfect method for determining Ca and P metabolism either. Based on our data, validation of these bone markers of metabolism with total collection and bone biopsy is conditional. During the wk 20 balance, there was higher Ca retention, bone formation (i.e. lower DPD and higher OC), and higher bone Ca content as compared with earlier collections. This indicates that the markers of bone metabolism can be used as a non-invasive indicator of dietary effects on bone activity when the cow is in a state of metabolic stability (ie mid to late lactation).

CONCLUSIONS

Dietary Ca concentration had no effect on serum minerals, serum markers of bone metabolism, or P balance from wk 1 to 20 of lactation. However, parity and time had clear effects on these parameters. Relationship of the serum markers of bone metabolism with total collection and biopsy appears to be conditional and limited to times of metabolic steady-state. Ultimately, dietary Ca did not affect P retention in the weeks examined. Therefore the diets can be formulated for P independent of Ca concentration in the diet.

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Table 4-1. Ingredient composition of diets

	Treatment[1]		
	LOW	MED	HI
	% of diet DM		
Corn silage	39.7	39.7	39.7
Alfalfa silage	7.8	7.8	7.8
Cotton seed	2.9	2.9	2.9
High moisture corn	15.5	15.5	15.5
Concentrate mix	34.1	34.1	34.1
Concentrate Mixture	% of concentrate mixture DM		
Soybean meal, 48% de-hulled	58.3	59.1	59.9
Soy hulls, ground	35.8	32.0	28.3
Calcium carbonate	1.0	3.1	5.2
Tallow	1.5	2.1	2.8
Corn, fine ground	1.3	1.5	1.8
Salt	1.3	1.3	1.3
Sodium bicarbonate	0.6	0.6	0.6
Vitamin trace mineral mix[2]	0.26	0.26	0.26

[1] LOW 0.52%, MED 0.78%, or HI 1.03% Ca as a percent of diet DM
[2] Contained cobalt 135 ppm; copper 12,389 ppm; iodine 743 ppm; iron 61,946 ppm;
manganese 49,556 ppm; selenium 334 ppm; zinc 49,556 ppm; vitamin A 394,685 IU/kg;
vitamin D 264,819 IU/kg; vitamin E 5,460 IU/kg.

Table 4-2. Statistical model used to analyze all variables

	Collection Parameters (Week)		Blood Parameters (Day)		
Source	df	Error df	df	Error df	Error Term
Treatment	2	12	2	12	Cow (Treatment x Parity)
Parity	1	12	1	12	Cow (Treatment x Parity)
Treatment*Parity	2	12	2	12	Cow (Treatment x Parity)
Covariate	1^1	12	—	—	
Cow (Treatment*Parity)	11	—	12	—	
Time	4^2	46	15^3	174	Residual
Treatment*Time	8	46	30	174	Residual
Parity*Time	4	46	15	174	Residual
Treatment*Parity*Time	8	46	30	174	Residual
Residual	57		185		
Total	87		280		

[1] The prepartum collection week data were used as a covariate for the feces and urine variables.

[2] Milk yield and DMI were evaluated in all 20 wk. Other collection parameters were evaluated only during the 5 balance wk. The time df for DMI and MY effects was 19.

[3] Serum PTH concentration was evaluated in the first 4 post-partum samples. The time df for PTH was 3.

Table 4-3. Chemical composition of diets

	Treatment[1]		
	LOW	MED	HI
Nutrient	% of diet DM		
CP, %	16.9	16.9	16.8
ADF, %	20.4	19.7	19.1
NDF, %	31.6	30.9	30.1
Ash, %	6.12	6.82	7.51
P,%	0.34	0.34	0.34
Ca,%	0.52	0.78	1.03

[1] Based on Ca percentage of DM.

Table 4-4. Effect of dietary Ca concentration on DMI, milk yield, and milk component yields of 18 lactating Holstein cows

	Treatment[1]					Parity				Week
	LOW	MED	HI	SEM	$P <$	First	Multi	SEM	$P <$	$P <$
DMI, kg/d	24.1	23.4	22.7	0.6	0.26	21.4	25.4	0.5	<0.01	< 0.01
Milk, kg/d	32.4	34.2	31.9	1.0	0.28	29.5	36.3	0.85	<0.01	< 0.01
Milk fat, kg/d	1.55	1.53	1.41	0.11	0.66	1.27	1.71	0.09	<0.01	< 0.01
Milk protein[2], kg/d	1.17	1.17	1.16	0.07	0.99	1.00	1.33	0.06	<0.01	< 0.01
Milk lactose[3], kg/d	2.00	2.00	2.03	0.10	0.98	1.74	2.28	0.09	<0.01	< 0.01
Milk SNF[4], kg/d	3.54	3.56	3.69	0.20	0.98	3.06	4.05	0.18	<0.01	< 0.01

[1] LOW 0.52%, MED 0.78%, or HI 1.03%
[2] Treatment*Week P <0.003
[3] Treatment *Week P < 0.006
[4] Treatment*Week P < 0.002

Table 4-5. Effect of dietary Ca concentration on serum minerals and markers of bone metabolism in 18 lactating Holstein cows

	Treatment[1]					Parity				Day	Linear	Quadratic
Variable	LOW	MED	HI	SEM	$P <$	First	Multi	SEM	$P <$	$P <$	$P <$	$P <$
Cows (n)	6	6	6			10	8					
Ca, mg/dL	10.9	11.2	11.2	0.33	0.81	11.5	10.8	0.27	0.08	< 0.01	< 0.01	< 0.01
P[2], mg/dL	5.29	5.12	5.42	0.15	0.40	5.65	4.90	0.12	< 0.01	< 0.01	< 0.01	0.05
OC[3], ng/mL	56.8	50.8	56.3	3.16	0.31	68.1	41.2	2.59	< 0.01	< 0.01	< 0.01	< 0.01
DPD[4], ng/mL	2.19	2.21	2.33	0.08	0.40	2.56	1.94	0.07	< 0.01	< 0.01	< 0.01	0.08

[1] LOW 0.52%, MED 0.78%, or HI 1.03%
[2] Treatment *Parity*Day, $P < 0.04$
[3] OC, osteocalcin; Treatment*Parity, $P < 0.05$
[4] DPD, deoxypyridinoline; Parity*Day, $P < 0.01$

Table 4-6. Effect of dietary Ca concentration on bone Ca, P, and ash in18 lactating Holstein cows

	Treatment[1]			SEM	$P <$	Parity		SEM	$P <$	Week
	LOW	MED	HI			First	Multi			
Calcium										
Ca gram/sample	0.21	0.19	0.20	0.008	0.11	0.20	0.20	0.007	0.48	< 0.01
Ca, % wet weight	33.4	30.9	32.2	0.70	0.08	32.2	32.2	0.60	0.99	< 0.01
Ca, % dry weight	36.3	31.5	33.1	1.61	0.13	34.4	32.9	1.40	0.41	0.59
Ca, % ash weight	54.5[a]	49.5[b]	53.3[ab]	1.24	0.03	52.4	52.5	1.06	0.96	0.02
Phosphorus										
P gram/sample	0.06	0.06	0.06	0.003	0.98	0.06	0.07	0.003	0.24	< 0.01
P, % wet weight	10.2	10.5	10.0	0.27	0.45	9.7	10.8	0.23	0.003	0.10
P, % dry weight	11.1	10.7	10.3	0.47	0.51	10.4	11.0	0.40	0.25	0.32
P, % ash weight	16.6	16.8	16.5	0.25	0.74	15.7	17.5	0.21	< 0.01	0.80
Ash										
Ash gram/sample	0.39	0.38	0.38	0.01	0.94	0.39	0.38	0.015	0.49	< 0.01
Ash, % wet weight	61.3	62.7	60.8	1.3	0.57	61.7	61.5	1.1	0.88	0.05
Ash, % dry weight	66.7	63.7	62.3	2.8	0.54	65.8	62.7	2.4	0.35	0.40

[1] LOW 0.52%, MED 0.78%, or HI 1.03%

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Table 4-7. Effect of week of lactation and dietary Ca concentration on Ca intake and partitioning in 18 lactating Holstein cows

| | Week of Lactation | | | | | | | Contrasts (Week) | | | |
	2	5	8	11	20	SEM[2]	TRT	Week	Linear	Quadratic	TRT*WK
Ca intake, g/d							< 0.01	< 0.01	< 0.01	0.18	0.01
LOW[1]	115	120	125	124	129	11.2					
MED	118	182	191	192	229	14.8					
HI	206	247	248	248	300	11.9					
Fecal Ca, g/d							< 0.01	< 0.01	< 0.01	< 0.01	0.08
LOW	78.2	87.1	88.1	106	79.1	8.92					
MED	84.6	125	139	148	152	12.3					
HI	148	162	168	199	195	9.45					
Apparent Ca digestibility, %							0.91	< 0.01	0.60	< 0.01	0.72
LOW	32.1	27.6	29.6	13.5	38.2	4.8					
MED	36.2	30.3	26.5	22.5	32.5	6.8					
HI	26.0	34.2	30.8	19.0	34.6	5.1					
Urinary Ca, g/d							0.43	0.06	0.80	0.54	0.73
LOW	1.00	0.19	0.64	1.26	0.50	0.26					
MED	0.96	0.51	0.51	0.50	0.51	0.26					
HI	1.10	0.44	0.91	0.92	0.76	0.27					
Milk Ca, g/d							0.80	< 0.01	0.05	< 0.01	0.44
LOW	46.2	47.7	47.8	45.6	40.6	3.09					
MED	45.2	47.7	48.6	48.7	45.1	3.60					

HI	45.9	52.5	49.3	50.3	42.0	3.28				
Ca balance, g/d							< 0.01	< 0.01	< 0.01	0.02	0.85
LOW	-10.7	-14.5	-11.2	-28.8	8.10	10.9				
MED	-7.48	7.42	1.96	-6.77	29.9	15.4				
HI	10.3	31.7	29.8	-2.32	61.3	11.5				

[1] LOW 0.52%, MED 0.78% or HI 1.03%

[2] Unequal n (wk 2) on the MED diet. Largest SEM reported n = 4.

Table 4-8. Effect of week of lactation and dietary Ca concentration on P intake and partitioning in 18 lactating Holstein cows

	Week of Lactation							Contrasts (Week)				
	2	5	8	11	20	SEM[2]	TRT	Week	Linear	Quadratic	TRT*WK
P intake, g/d							0.61	< 0.01	< 0.01	<0.01	0.20
LOW[1]	61.3	70.9	82.5	85.9	79.9	4.43					
MED	47.9	72.7	80.0	84.6	79.2	5.42					
HI	58.0	64.9	78.0	81.8	73.5	4.70					
Fecal P, g/d							0.22	< 0.01	< 0.01	< 0.01	< 0.01
LOW	40.3	47.8	50.3	59.2	56.7	3.16					
MED	28.8	42.9	49.6	49.4	55.5	3.99					
HI	36.8	41.3	49.9	50.8	53.5	3.41					
Apparent P digestibility, %							0.39	< 0.01	< 0.01	0.24	0.16
LOW	34.1	32.4	38.6	31.2	26.2	4.1					
MED	46.6	39.6	36.3	41.0	28.7	5.1					
HI	39.0	37.5	37.0	39.6	28.0	4.3					
Urinary P, g/d							0.18	0.03	0.92	0.04	0.28
LOW	0.74	0.41	0.65	0.94	1.34	0.40					
MED	2.95	0.62	0.91	1.07	1.46	0.55					
HI	1.04	0.63	0.81	0.94	0.86	0.43					
Milk P, g/d							0.99	< 0.01	0.46	< 0.01	0.19
LOW	35.5	37.5	37.0	35.0	32.9	2.46					
MED	29.0	36.7	39.4	38.5	36.4	2.95					

HI	34.7	39.1	37.0	37.5	30.9	2.61				
P balance, g/d							0.89	0.08	0.55	0.01	0.44
LOW	-15.6	-15.2	-5.87	-9.58	-11.3	3.19				
MED	-12.6	-8.59	-11.0	-5.41	-15.3	4.45				
HI	-12.9	-14.7	-8.15	-6.03	-10.3	3.39				

[1] LOW 0.52%, MED 0.78%, or HI 1.03%

[2] Unequal n (wk 2) on the MED diet. Largest SEM reported.

Figure 4-1. Effect of dietary Ca concentration (LOW 0.52%●, MED 0.78%■, or HI 1.03% ◆) on serum Ca (closed symbols) and P (open symbols) concentration from -14 d prepartum to 140 d of lactation. (A: SEM = 1.61 and B: SEM = 0.62)
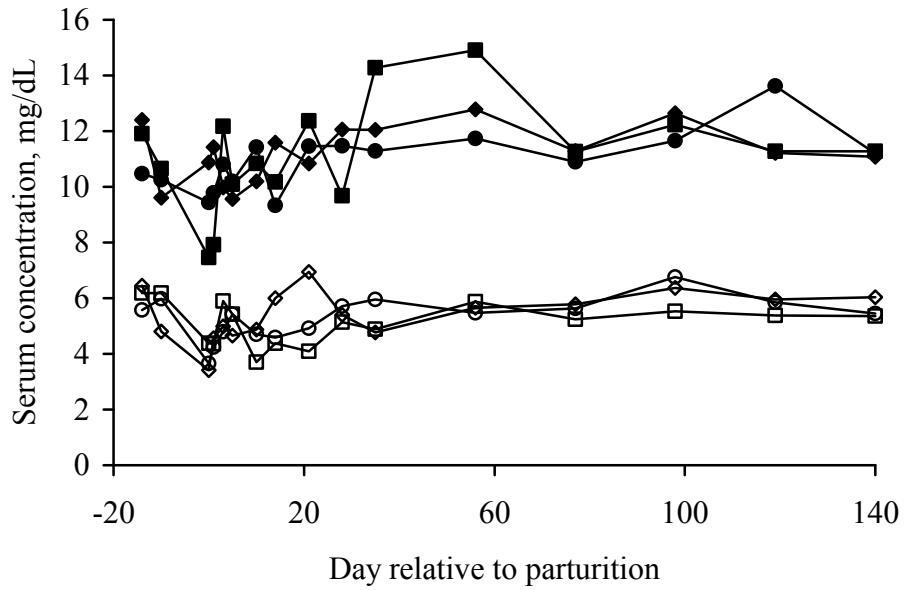
Figure 4-2. Effect of dietary Ca concentration (LOW 0.52%, MED 0.78%, or HI 1.03%) on osteocalcin (OC) in primiparous and multiparous lactating Holstein cows from -14 d prepartum until 140 d of lactation
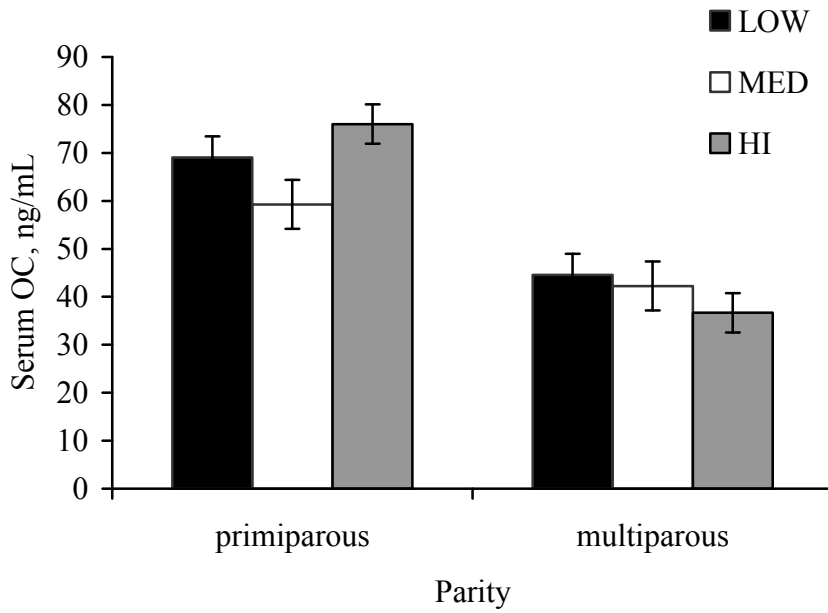
Figure 4-3. Effect of lactation on markers of bone metabolism from -14 d prepartum until 140 d of lactation in primiparous and multiparous cows. A: DPD = deoxypyridinoline, marker of bone resorption; SEM = 0.13; and B: OC = osteocalcin, marker of bone formation; SEM = 5.1
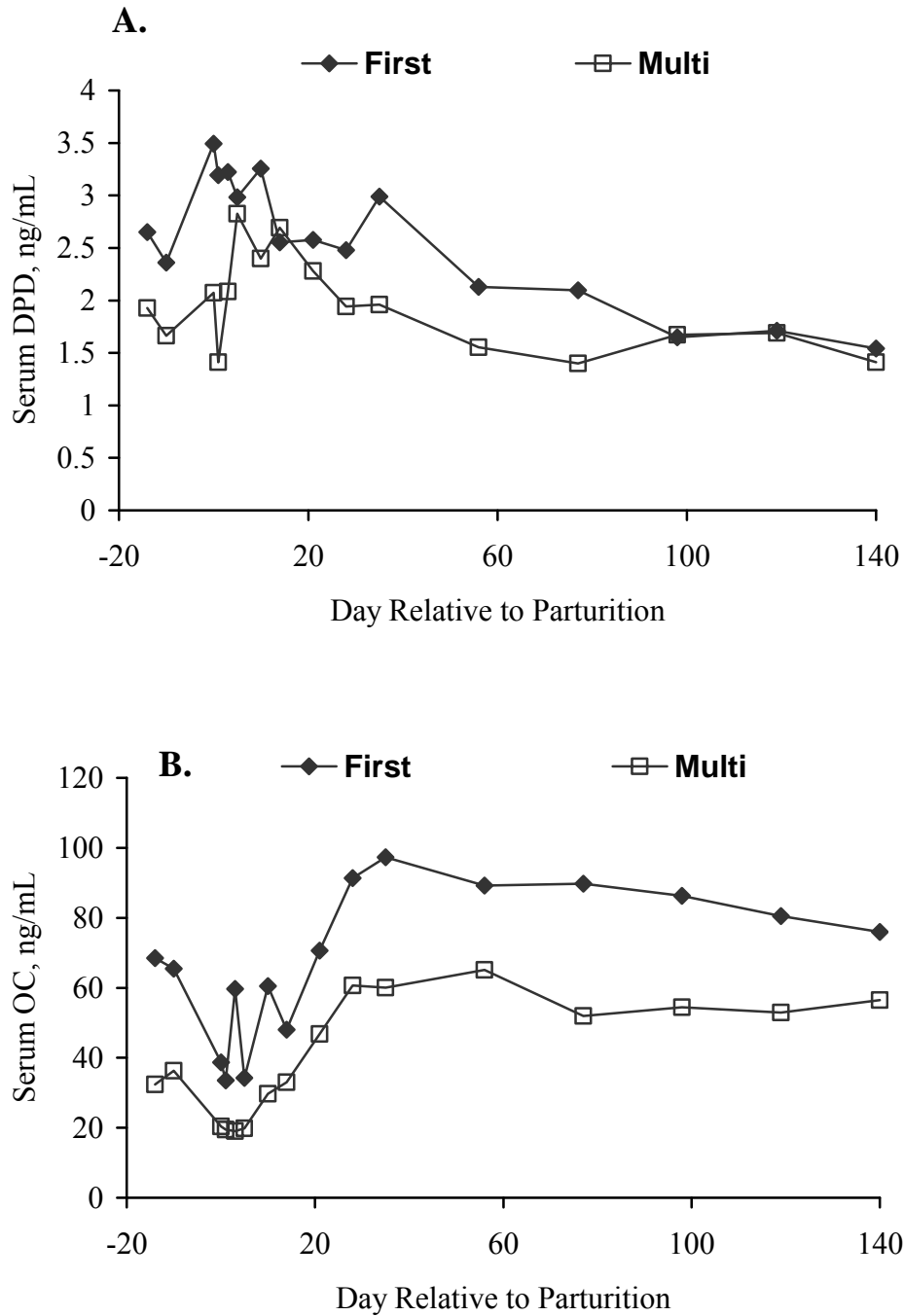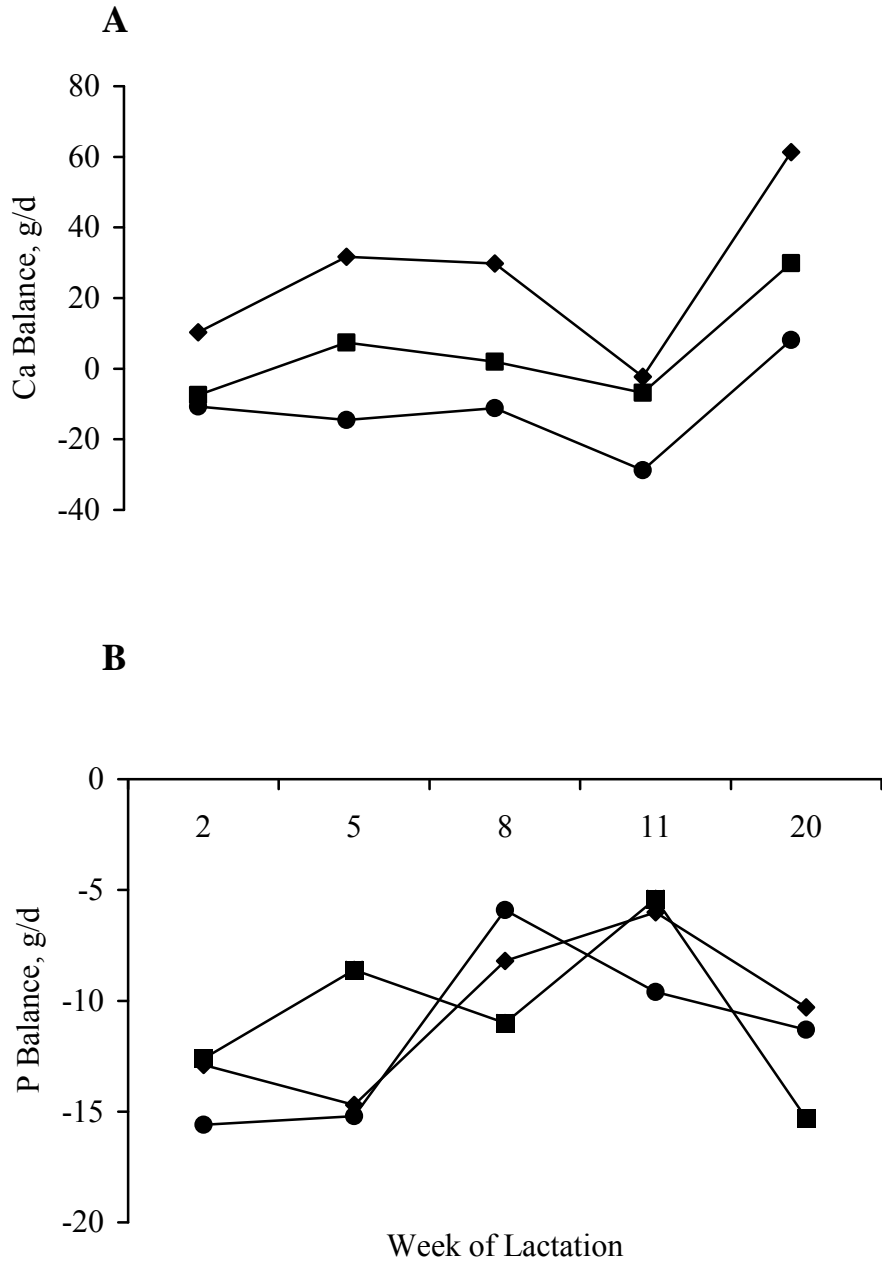
Figure 4-4. Effect of dietary Ca concentration (LOW 0.52%● , MED 0.78%■ , or HI 1.03% ◆) on Ca balance (A) and P balance (B) in lactating Holstein cows at 2, 5, 8, 11, and 20 wk of lactation (A: SEM = 11.5, treatment P < 0.001 and B: SEM = 4.5, treatment P < 0.89)

Chapter 5: Overall Conclusions

Breed and Parity Effects across both Studies

Across the two studies there were clear effects of breed and parity on OC concentration. In both breeds primiparous cows had higher OC concentration as compared to multiparous cows. Holstein primiparous cows had higher serum Ca and P as compared to multiparous cows whereas there was no effect of parity in the Jersey cows on these variables. Additionally, it appears that the Holstein cows had higher serum concentrations of Ca, P, and OC when compared to the same parity Jersey cows. The explanation for this is not apparent.

Correlations between Measures of Mineral Metabolism

We can learn about relationships between bone formation, resorption, bone markers, and mineral balance by evaluating correlations within cows on different diets. Certain logical relationships hold under specific dietary conditions. Examining these sheds new light on bone metabolism, but these are simply relationships that supplement the primary analyses.

Hypothesis 1: Bone ash content increases as the concentration of OC and Ca balance increases.

It was anticipated that bone ash content would increase when cows were in positive Ca balance and both of these would be correlated with the blood marker of bone formation, OC, but this was only true in cows with a moderate supply of Ca (cows fed the MED diet). There was no relationship between bone ash content and these variables when dietary Ca was deficient because there was a shortage of mineral to build bone and when dietary Ca was plentiful the animal had not mobilized bone stores therefore there was nothing to replace.

Hypothesis 2: Serum OC concentration will increase in times of positive Ca balance.

Osteocalcin and Ca balance had a strong positive correlation when cows were consuming the HI diet ($r = 0.44$). It appears that even if OC and Ca balance are

correlated and when cows have a positive Ca balance that does not necessarily result in bone Ca deposition which is ultimately bone ash. This could be the result of the HI cows not having to replace bone mineral because they were not in negative Ca balance at the times measured.

Hypothesis 3: Serum OC concentration increases as serum DPD concentration
                        decreases.

In cows fed the MED and HI diets, the correlation between OC and DPD was strong and negative (r = -0.41 and -0.71, respectively). The negative relationship is expected since these are markers of contrasting processes, bone formation and bone resorption. In cows consuming the LOW diet, however there was no correlation between OC and DPD. This indicates that dietary Ca must be in adequate supply for these markers to accurately indicate bone turnover. The LOW cows were in negative Ca and P balance for the majority of the study and likely were not forming bone as were the MED and HI cows, but it is important to remember that there was no effect of treatment or the interaction of treatment and time on either marker.

Hypothesis 4: Bone Ca as a percent of fat free ash is negatively correlated with serum
                        DPD concentration and positively correlated with serum OC and Ca
                        balance.

There was a negative relationship between DPD and bone Ca as a percent of fat free ash across all three diets which implies that an increase in bone resorption is associated with a decrease in bone Ca (r = -0.38, -0.47, and -0.33; LOW, MED, and HI respectively). The opposite relationship was observed with OC and bone Ca with a positive correlation in the cows consuming the MED and HI diets. This could suggest that cows consuming the diet deficient in Ca (LOW) were not forming new bone matrix to the same extent as cows on the MED and HI diets, but there was no treatment effect on OC concentration. Bone Ca as a percent of fat free ash was positively correlated with Ca balance in the cows consuming the LOW (r = 0.56) and MED (r = 0.37) diets but there was no correlation in the HI group which relates back to the statement made previously in hypothesis 2 discussion.

The relationship between bone Ca and Ca balance in the LOW fed cows is puzzling because these cows were in negative Ca balance for the entire study until wk 20. This correlation could be that when Ca balance was more negative, bone Ca was lower, but bone Ca was not different in cows fed the HI and LOW diets and was lowest in the cows fed the MED Ca diet.

Furthermore, an inference that could be drawn from the data is that cows were replacing bone mineral with Ca carbonate as opposed to hydroxyapatite. This speculation is corroborated by several points in the study. Phosphorus balance was not different across time and was in fact negative during all weeks measured. Negative P balance cannot support net bone formation with hydroxyapatite; bone mineral formed during negative P balance is more likely to be in the form of calcium carbonate. Our observation that Ca, but not P, content of bone samples increased over time and were highest at wk 20 on a mass, wet weight, and ash weight basis supports this hypothesis.

Also, bone Ca and P were not correlated, which further suggests that the bone mineral was something other than hydroxyapatite $[Ca_{10}(PO_4)_6(OH)_2]$. The negative relationship of OC and DPD was shown previously in the HI and MED fed cows. These markers are indicating the formation and resorption of bone matrix, respectively. The matrix can be mineralized with hydroxyapatite, Ca phosphate, or Ca carbonate and these markers cannot determine bone mineralization or mineral composition. The ratio of Ca to P in hydroxyapatite is 2.2 to1 but the ratio of Ca to P in bone samples in the current study was 3.5, 3.2, and 3.3 to 1 in cows fed the LOW, MED, and HI diets, respectively. This further implies formation of bone mineral compounds containing less P than hydroxyapatite.

Limitations of Both Studies

It is a limitation of the first study that there was no marker of bone resorption measured. Since bone formation and resorption are linked, at least when dietary Ca is adequate, it is necessary to consider both processes to determine what occurred. It is the relative rates of the markers that imply net formation or resorption, however due to monetary constraints a marker of bone resorption was not measured.

The primary limitation of the second study is that the diets were originally formulated to contain dietary P at 0.36%; analysis of the diet revealed a lower dietary P

content (0.34%). The P requirement was calculated for these cows using their actual DMI and milk yield during each of the balance weeks and was 0.39, 0.41, and 0.42% of the diet for the LOW, MED, and HI, respectively. It is obvious that the dietary P concentration of 0.34% was not sufficient to meet the cows' needs. These diets were formulated for a cow consuming 23.2 kg DM and producing 36.3 kg milk per day but the cows actually consumed 23.9 kg DM and produced 41.0 kg milk. Although the DMI was similar between the formulated and actual diets, the difference in milk yield was substantial and required 4.2 g/d of P to support the additional milk produced. The negative P balance observed in these cows (-11.5, -10.6, and -10.4 g/d for the LOW, MED, and HI diets respectively) were similar to those estimated by subtracting NRC requirements from actual P intake (-11.4, -14.2, and -18.3 g/d). Ultimately, the negative P balance is a limitation of the study because there was a limited amount of P available to replace bone mineral.

Implications of Ca Feeding Recommendations

The focus of this study was to assess current P recommendations with different dietary Ca concentrations. The dietary Ca concentrations used in the present study were above, within the range, and below the NRC (2001) recommendations. Since Ca is an inexpensive dietary ingredient and is not associated with negative environmental impacts, it is often included in excess of the recommendation (NRC, 2001) in lactating cow diets. The HI (>1.0%) dietary Ca diet used in the present study resembles a diet that would be fed in the industry. Given that the HI and the LOW dietary Ca diets had no effect on bone metabolism any additional research with more extreme diets would not be applicable to the dairy industry and current feeding practices. Therefore it appears unnecessary to repeat this study with more extreme dietary combinations.

These diets were designed with the goal of influencing bone metabolism by manipulating dietary Ca. Even though a treatment effect was not observed, the correlations between the variables measured and the effect of diet on those relationships, as discussed previously, was established and is useful. It is also important to remember that these relationships may have been stronger or more consistent had the bone biopsy samples been harvested from a bone that was more sensitive to mineral resorption or if the animals had been in a severe state of mineral deficiency. This study demonstrated

that the rib is not very sensitive to resorption at the times measured when cows are moderately deficient in dietary Ca.

Next Research Steps

Upon conclusion of the second study several questions remain. Two of the most interesting are 1) why was fecal Ca excretion higher during week 11 with no increase in DMI?, and 2) what was the form of the bone mineral the cows were depositing? To further examine these questions two follow up studies would need to be conducted.

The focus of the first study would be to examine CaBP in the small intestine. Three things appear to affect CaBP expression: dietary Ca concentration (high and low), estrogen, and DHVD. Sampling colon tissue may allow repeated, relatively non-invasive approach to monitoring CaBP concentration. Goff et al. (1995) observed that the concentration of vitamin D receptor (VDR) in the colon is 70% of the concentration of VDR in the duodenum. Since DHVD controls CaBP expression there is a strong possibility that the colon expression of CaBP would correlate with duodenum expression. Before beginning this next study we would confirm this phenomenon is also applicable to CaBP though slaughterhouse samples. After confirmation the study would proceed.

To evaluate CaBP expression, one might assign twenty-four multiparous Holstein cows to one of four treatments: (1) high dietary Ca with estrogen injection, (2) treatment 1 with DHVD injection, (3) low dietary Ca with estrogen injection, or (4) treatment 3 with DHVD injection. All cows will be > 60 DIM. Cows will consume their respective treatment diets for 2 wk and then colon mucosa scrapings with a bent medical spoon (Goff et al., 1995) and blood samples will be collected for 5 d consecutively. These samples will be used as a control for each of the diet combinations. At the start of wk 5 for all cows estrogen injections will be administered and cows on treatment 2 and 4 will also be administered DHVD injections be for 7 d and the same sampling routine described previously will occur for the last 5 d of that period. Through a peroxidase-antiperoxidase immunocytochemical technique (Taylor, 1982) the abundance and half life of CaBP and VDR will be determined. The cell collection for 5 consecutive days allows determination of the degradation of CaBP. Blood samples will be analyzed for

estrogen concentration and the relationship of this with CaBP concentrations will be assessed.

It is anticipated that cows in treatments 2 and 4 will have higher concentrations of intestinal CaBP because the injection of DHVD will likely mediate the negative impact of estrogen on CaBP. However, the preliminary samples in the cows consuming low dietary Ca will likely have the highest concentrations of CaBP and VDR as compared to the treatment groups after injection administration. Treatment 1 is expected to have the lowest concentration of CaBP and VDR because dietary Ca will be plentiful and estrogen appears to negatively affect CaBP.

A second study would be necessary to determine the form of bone mineral that is deposited during times of dietary Ca and P deficiency. For this study 24 multiparous cows (> 3 lactations) will be used and assigned to one of four dietary treatments: high Ca and high P, high Ca and low P, low Ca and high P, and low Ca and low P. Cows will begin consumption of their treatment diets at 10 DIM. Cows will be dosed with ^{43}Ca and ^{31}P via an indwelling cannula in the jugular vein on d 10, 20, and 40 of the study. A bone biopsy of the iliac crest will be harvested 3 d after each dosing (13, 23, and 33 d). A jugular blood sample will also be collected to examine the blood markers of bone metabolism. It is critical to sample the bone at these times (~ 23, 33, and 43 DIM) because we learned that net bone formation begins to occur at ~ 35 DIM. The use of these isotopes will allow the form of the bone mineral to be quantified using nuclear magnetic resonance spectroscopy (Myers et al., 1994).

It is predicted that cows consuming the high dietary Ca and low dietary P will produce bone containing higher concentrations of Ca carbonate as opposed to hydroxyapatite. Also the markers of bone metabolism will potentially have a strong correlation with both minerals in the diet. When Ca and P are both plentiful in the diet there will be a strong positive correlation with OC and negative correlation with DPD concentration.

Final Conclusions

Ultimately from the first study it is clear that oral dosing with 25-OH at 6 d prior to expected calving is not justified. However, we learned that parity has an effect on

bone formation and that net formation appears to occur after 30 days in milk which was corroborated in the second study.

Additionally, the second study demonstrated that dietary Ca content has no effect on P balance from 2 to 20 wk of lactation. We conclude that if cows are in negative P balance for the first 20 wk of lactation they will replace bone mineral as Ca carbonate as opposed to hydroxyapatite. The study clearly demonstrated the effect of parity on the markers of bone metabolism with younger cows resorbing and forming more bone. But this was not substantiated by the bone biopsy samples. Finally, the rib bone does not appear to be a sensitive indicator of bone metabolism or at least not at the time points we measured.

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