INFLUENCE OF SUBCLINICAL HYPOCALCEMIA ON PLASMA BIOCHEMICAL PARAMETERS, LIVER HISTOLOGIC CHANGES, AND COMMON POSTPARTUM DISEASES IN DAIRY COWS

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

INFLUENCE OF SUBCLINICAL HYPOCALCEMIA ON PLASMA BIOCHEMICAL PARAMETERS, LIVER HISTOLOGIC CHANGES, AND COMMON POSTPARTUM DISEASES IN DAIRY COWS

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This research trial was conducted to evaluate the association between calcium status at calving and postpartum energy balance, liver lipid infiltration, disease occurrence, milk production parameters, and fertility in Holstein cows. To analyze this association, 100 cows were assigned to one of two groups based on whole blood ionized calcium concentration [iCa] on the day of calving (hypocalcemic [iCa] < 1.0 mmol/L (n=51); normocalcemic [iCa] ≥ 1.0 mmol/L (n=49)). Cows were then assigned to blocks based on calving date and parity. Cows were fed a balanced dry cow diet without inclusion of anionic salts, and all cows were fed a balanced lactating cow diet during the postpartum period based upon NRC requirements. Blood samples were collected approximately 14 days before expected calving date, and again on the day of calving and on days 3, 7, 14, 21, and 35 postpartum for measurement of nonesterified fatty acids, whole blood ionized calcium, plasma total calcium, glucose, aspartate aminotransferate, gamma glutamyl transferase, and total and direct bilirubin. Liver biopsies were obtained on the day of calving and on days 7 and 35 postpartum for quantification of lipid content. Milk samples were also collected on days 3, 7, 14, 21, and 35 postpartum for the measurement of somatic cell count, milk protein concentration, milk fat concentration, and solids-not-fat. Data for peak test day milk yield, for calculating 305 day ME 4% fat corrected milk,

services per conception, and days open were obtained from DHIA herd records. Disease occurrence was determined based on herd treatment records.

There was an association between calcium status at calving and energy metabolism as demonstrated by significantly higher nonesterified fatty acids on the day of calving and on day 21 postpartum for hypocalcemic cows, indicating a more severe negative energy balance for the hypocalcemic cows. Additionally, hypocalcemic cows had significantly more lipid in the hepatocytes on day 35 postpartum, possibly as a result of mobilizing more body fat during early lactation. However, there were no indications that there was a difference in liver health or function between groups as there were no differences between groups for aspartate aminotransferase, gamma glutamyl transferase, or total or direct bilirubin.

Normocalcemic cows did have significantly higher milk protein concentration on days 21 and 35 postpartum, however no other parameters for milk quality and production (somatic cell count, milk fat concentration, solids-not-fat, peak test day milk yield, and 305 day mature equivalent 4% fat corrected milk yield) differed between groups. Also there were no differences between groups for occurrence of ketosis, displaced abomasum, retained placenta, metritis, or mastitis. There were no differences between groups for fertility measures (percent cycling at 50-60 days postpartum, services per conception, or days open). This suggests that there is an association between calcium status at calving and early lactation energy balance, but not between calcium status at calving and milk production, disease occurrence, or fertility.

Chapter 1

Literature Review

The Transition Period

The transition period is defined as the period from 3 weeks before to 3 weeks after parturition (Grummer, 1995; Drackley, 1999). The transition from a pregnant, non-lactating state to a non-pregnant, lactating state incurs stress because of the abrupt, extreme changes in metabolism and physiology of the dairy cow (Goff and Horst, 1997b). The rapid initiation of lactogenesis near parturition requires very large amounts of nutrients, but unfortunately feed intake is often depressed during the prepartum transition period (Grummer, 1995). This forces the cow to draw nutrients from body stores and increases stress on homeostatic mechanisms of the cow. As a result, disorders such as milk fever, ketosis, hepatic lipidosis, retained placenta, rumen acidosis, and displacement of the abomasum manifest themselves during the transition period. Many times diseases with later onset, such as laminitis, may also be traced back to disturbances occurring during the transition period (Goff and Horst, 1997b). Additionally, infectious diseases, mastitis in particular, may become clinically apparent during the transition period as a result of impaired immune function (Wagter et al., 2003). Many of these diseases are influenced by nutrition and are said to be interrelated as can be seen in figure 1.1, adapted from Goff (2006). Curtis et al. (1983) stated that cows that develop milk fever are eight times more likely to get mastitis. Furthermore, cows with prepartum

negative energy balance are more likely to develop a displaced abomasum (LeBlanc et al., 2005).

Another major adjustment that transition cows encounter is the change in nutrient composition of diets. Lactation diets contain much higher amounts of energy in the form of non-fibrous carbohydrates compared to diets fed to dry cows. The risk of developing ruminal acidosis increases when there is a sudden increase in starch in the diet. This is because the ruminal microbial population is poorly suited to starch fermentation (Mulligan et al., 2008). Additionally, there is a reduced ability of the rumen to absorb volatile fatty-acids (Dirksen et al, 1985), which may result in VFA build up and consequently decrease rumen pH**.**

A cow's overall health during the transition period impacts longevity and performance during the ensuing lactation. There are many examples of how poor health during the transition period can have immediate and long-term effects on productivity of a cow. Block (1984) showed that feeding a diet with anionic salts, resulting in a diet higher in anions than cations, compared to a cationic diet could significantly improve calcium status, which resulted in a lower incidence of milk fever. Additionally, the cows fed the anionic diet produced significantly more milk during the ensuing lactation. A long term effect of transition health on productivity was shown by Walsh et al. (2007) who found that cows with subclinical ketosis during the first two weeks postpartum were 50% less likely to become pregnant after the first insemination than their nonsubclinically ketotic counterparts. Another long term consequence of poor transition cow health was described by Goff (2006) who explained how rumen acidosis causes endotoxemia, which

can lead to vascular changes in the hoof wall affecting the growth rate of hoof wall cells, and ultimately manifest as laminitis.

Jordan and Fourdraine (1993) indicated the incidences of several common diseases in 61 of the highest producing herds in the country, covering approximately 14,600 cows in the early 1990's as indicated in table 1.1. More recently, McLaren et al. (2006) looked at transition disorders in 48 herds, covering approximately 3,500 cows in Ontario, Canada. The incidence and range for five disorders are also presented in table 1.1. It can be seen that the mean incidences and ranges are similar between studies. The incidence of the disorders was not surprising, but it was the range at which the disorders were found between farms that illustrates the opportunity for improvement of transition cow health in the dairy industry.

Work from Dechow and Goodling (2008) in Pennsylvania indicated that 42% of all mortalities of dairy cows occurred between the day of calving and 60 days postpartum. Furthermore, the proportion of dairy cow mortalities from 21 days prepartum through 60 days of lactation was 52%. Also, 26.2% of culls occurred between 21 days prepartum and 60 days postpartum. Since approximately half of deaths and one-fourth of all culls occur in such a narrow time frame it is evident that the transition cow is a high risk animal in terms of affecting the producer's profitability. This indicates that the transition period is an area in need of management and nutritional improvement.

Proper management and nutrition are vital to supporting cows during the transition period. Minimizing infectious and metabolic disorders during the transition period increases the productivity of the cow. Much research to date has investigated the transition period, but is typically focused on individual disorders. The purpose of this

review is to address the changes that the dairy cow undergoes through the transition period and to associate calcium metabolism and energy metabolism during the transition period.

Endocrinology and physiology of the transition cow

Numerous hormones from various different systems in the body of the dairy cow are changing during the transition period. Reproductive hormones are shifting through late gestation and early lactation. Hormones for the production of milk, energy and mineral metabolism, and stress hormones are all changing and having effects on the cow as she goes through the transition period. Progesterone is the main hormone responsible for the maintenance of pregnancy. Plasma progesterone concentrations rise to a peak of 7 to 8 ng/ml around day 250 of gestation then gradually begin to fall until the day before calving. At calving, progesterone decreases markedly to nearly undetectable levels (Goff and Horst, 1997b). Estrogen remains relatively low throughout the first part of gestation at approximately 20 pg/ml. This concentration is similar to levels during the luteal phase of the estrous cycle (Goff and Horst, 1997b). Estrogen increases to approximately 300 pg/ml by mid-gestation, which is ten-fold higher than estrogen during the follicular phase (Goff and Horst, 1997b). Plasma estrogen begins to increase around the same time progesterone starts to decrease, and by 7 days prepartum is already at 2000 pg/ml. Just prior to calving, estrogen increases very rapidly and peaks at 4000 to 6000 pg/ml (Chew et al., 1977), compared to just below 8 pg/mL during estrus (Chenault et al., 1975). Cortisol concentrations also increase from 4 to 8 ng/ml 3 days prior to calving to 15 to 30 ng/ml on the day of calving and the day after (Goff and Horst, 1997b).

During mid and late gestation, hormonal influences prepare the cow for the initiation of lactation. Around mid-gestation, prolactin and growth hormone concentrations increase, and result in synthesis of enzymes needed for milk production. However, the high levels of progesterone and estrogen during late gestation prevent the stimulatory effects of prolactin on milk secretion. It is not until parturition, when abrupt changes occur in progesterone and estrogen concentrations, that milk synthesis is fully stimulated by prolactin. Oxytocin from the posterior pituitary is also released at parturition, and its function is two-fold: to stimulate uterine contractions for expulsion of the fetus and contraction of the myoepithelial cells surrounding the alveoli for milk ejection (Sherwood et al., 2005).

During the dry period, the mammary gland involutes, giving it an opportunity to recover from the previous lactation and prepare for the next one. Mammary gland involution allows the secretory epithelium to undergo apoptosis (Strange et al., 1995) under the influence of decreasing concentrations of prolactin, growth hormone, and insulin-like growth factor-1 (IGF-1) (Svennersten-Sjaunja and Olsson, 2005). Within the first week of being dry, a keratin-like protein substance develops and plugs the streak canal to prevent any bacterial entry into the mammary gland. Other defense mechanisms during this time include increased neutrophils and macrophages and increased concentrations of lactoferrin in the mammary secretions. Lactoferrin binds iron, making it unfavorable for Gram-negative bacteria (primarily *E. coli*) to grow if present in the mammary gland (Goff and Horst, 1997b). During the first week of the dry period, defense mechanisms are still increasing and cows are no longer being milked. Consequently, bacteria are no longer being evacuated from the gland, so they have a

better opportunity to multiply (Smith, et al., 1985). As a result, this period is when the majority of dry period intramammary infections (IMI) occur. These infections are often held in check during the dry period by immune cells, but may often become apparent soon after parturition. Clinical manifestation of IMI during the periparturient period is multifactorial. First, progesterone, estrogen, and increases in IGF-1 stimulated by estrogen cause proliferation of the mammary epithelial cells, and then increasing concentrations of prolactin, growth hormone, and IGF-1 stimulate colostrum synthesis as parturition nears (Tucker, 2000). As colostrum production begins, lactoferrin concentrations in the milk decrease (Todhunter et al., 1990). Decreased lactoferrin allows more free iron in milk, creating a more favorable environment for growth of Gram negative bacteria (Goff and Horst, 1997b). Additionally, the keratin plug begins to break down 7 to 10 days prepartum allowing easier bacterial infiltration (Smith et al., 1985). Finally, if cows become hypocalcemic at calving, the smooth muscle in the teat sphincter may not contract quickly after milking to prevent bacterial entry (Goff and Horst, 1997b).

Mastitis is an inflammation of the mammary gland, typically caused by bacterial infection. It is the costliest disease to dairy producers in the United States not only because of the lost milk during the infection and treatment but also because of the damage it causes to the mammary tissues resulting in permanent production losses (Zhao and Lacasse, 2008). Wilson et al. (1997) estimated the cost per case of mastitis, subclinical or subclinical, to range from $155 to $350. More recently, Bar et al. (2008) estimated a case of clinical mastitis to average $179. Issues with mastitis can start either in the dry period or early in lactation. New intramammary infections (IMI) occurring during the dry period are normally eliminated, however, some are merely held in check.

These infections usually appear as clinical mastitis within the first month after calving due to decreased immunity during the transition period (Goff and Horst, 1997b).

Neutrophils are the first line of defense inside the mammary gland, and they will constitute greater than 90% of the leukocytes during inflammation (Sordillo, 2005). However, neutrophil numbers and function are decreased during the periparturient period, leading to a greater percentage of infections developing into clinical mastitis (Kehrli and Shuster, 1994). Effective prevention and treatment of mastitis have been the focus of a great deal of research for many years. Dry cow therapy with antibiotics may decrease the new IMI's during the dry period by as much as 45% and additional gains are made when a teat sealer is used (Robert et al., 2006). Ensuring adequate vitamin and mineral content in the diet of the transition cow is imperative, particularly those vitamins and minerals involved in the antioxidant system, such as vitamin E, selenium, ß-carotene, copper, and zinc. Making proper supplementation when necessary is also very important to prevent mastitis during early lactation (Spears and Weiss, 2008). The high metabolic demands during the initiation of lactation increase production of reactive oxygen species that can cause immune cell damage when in excess of antioxidant defense mechanisms (Sordillo, 2005). When the preventative mechanisms fail, early diagnosis of mastitis and treatment with the proper antibiotic and proper supportive therapy when necessary are key to reducing the severity and duration of infection.

The risk of developing IMI's is not the only challenge to the mammary gland of the transition cow. The initiation of lactation requires great amounts of nutrients to be partitioned to the mammary gland. The maximally secreting mammary gland may account for 80% of the body's glucose demand (Bauman and Currie, 1980). Most

notable from the work of Davis et al. (1979) was that glucose flow into the mammary gland one day postpartum was nine times that of seven to nine days prepartum and five times that on two days prepartum. Davis et al. (1979) also reported that mammary blood flow, oxygen consumption, and uptake of glucose and acetate in goats all increased markedly between two days and one day prepartum with further increases by one day postpartum. Other studies have shown that dairy cows may use as much as 27% of total body protein, primarily from muscle, for production of milk protein (Botts et al., 1979). Ellenberger et al. (1931) suggested that 800 to 1300 grams of calcium were removed from bone to support milk production during early lactation, and Ward et al. (1972) predicted that cows should be fed 5 grams of calcium per kg of milk produced to avoid a negative calcium balance.

Like the mammary gland, the uterus also experiences a transformation during the periparturient period. As previously discussed, the drop in progesterone and coinciding rises in estrogen, cortisol, and prostaglandin F_{2alpha} result in stimulation of delivery of the fetus (Goff and Horst, 1997b). After the fetus is delivered, the placental caruncles detach from the uterine wall, allowing for myometrial contractions to expel the placenta from the uterus (Kelton et al., 1998). After the placenta is passed, the superficial layers of the cotyledons begin to break down and are passed with increased fluid production (Roche, 2006). Within the first 16 hours postpartum, the cervix has contracted to only allow a small opening to allow passage of tissue and fluid (Wehrend et al., 2003). This lochia is commonly passed until 15-20 days postpartum via presumed prostaglandin F_{2alpha} stimulated contractions (Roche, 2006), and it changes from a red-brown fluid to a more viscous yellow-white material (Sheldon et al., 2006). The cervix continues to contract

until seven days postpartum (Wehrend et al., 2003), and then the cervix and uterus more slowly involute until 30-40 days postpartum, when they reach their normal, non-pregnant size (Roche, 2006). During uterine and cervical involution, the hypothalamo-hypophyseal-ovarian axis is resuming its normal cyclical production of gonadotropic hormones (Peter et al., 2009). Follicle stimulating hormone increases occur in the first week postpartum, and result in the first postpartum follicular wave by 10-14 days postpartum (Beam and Butler, 1999). From this first follicular wave, a dominant follicle is selected which leads to the first ovulation for 90% of cows around the same time that involution is complete (Peter et al., 2009). However, the endometrium is not typically ready for normal embryonic development and maternal recognition of pregnancy until approximately 60 days postpartum (Roche, 2006).

One postpartum disorder associated with the reproductive tract is retention of the placenta. After parturition, the placental caruncles normally detach from the uterine wall and the placenta is expelled from the uterus within 24 hours after calving (Kelton et al., 1998). Sometimes this does not occur and the fetal membranes are retained in the uterus for various reasons. Like other postpartum diseases, retained placentas have been shown to have multiple factors associated with their occurrence. Suppressed immune function has been described as a contributing factor to retained fetal membranes (Mulligan and Doherty, 2008). Gunnik (1984a, b) proposed that after parturition, the placenta becomes a dead, foreign body that the immune system should recognize as foreign, and then attack to facilitate detachment of the caruncles and successful expulsion from the uterus. In his experiments, Gunnik (1984a, b) demonstrated that neutrophils from cows that expelled the placenta normally had a strong chemotactic response to cotyledons. However,

neutrophils from cows that had retained placentas had decreased chemoattraction to the caruncles. He also demonstrated that this was evident for several days prior to calving (Gunnik, 1984a, b). More recently, Kimura and colleagues (2002) demonstrated a very similar response of decreased chemotactic activity of neutrophils as early as one week prepartum, and this continued until the day after calving. Cai et al. (1994) also reported that neutrophils had suppressed function when exposed to placental tissue, but this only occurred after parturition, and not before. It has also been shown that cows with milk fever are three times more likely to have retained fetal membranes (Curtis et al., 1983). It was initially thought that this was possibly due to the function of calcium in muscle contractions of the uterus. Confounding this is the fact that other investigators have shown that cows with retained placentas often have stronger uterine contractions than cows without retained placenta (Burton et al., 1987). In fact, later work completely disproved this theory because Eiler (1997) revealed that uterine motility played little or no role in the occurrence of retained placenta. One possible explanation is the increased levels of cortisol seen with cases of milk fever (Horst and Jorgensen, 1982) and the immunosuppressive effects that cortisol exhibits.

Another possible explanation is that the demand for calcium for lactogenesis puts a strain on the intracellular calcium stores of immune cells (Kimura et al., 2006). Intracellular calcium is important in signaling for immune cell activation, and if there is inadequate intracellular calcium, the signal may not be able to activate the immune cells. Julien et al. (1976a, b) found that supplementing cows with vitamin E and selenium either via intramuscular injection 21 days prepartum or by incorporating it into the ration during the dry period can significantly reduce the number of cases of retained placentas. This is

likely due to the effects of vitamin E and selenium on the immune system. Polymorphonuclear neutrophils from selenium deficient cows have been shown to have a decreased killing ability when compared to normally functioning neutrophils (Boyne and Arthur, 1979). Vitamin E deficiency can also lead to reduced immune function possibly due to an antioxidant effect of immunopoietic cells (Sunde and Hoekstra, 1980). LeBlanc et al. (2004) reported that as alpha tocopherol increased in the week before parturition, the risk of developing retained placenta decreased.

One final risk factor for retained placenta is energy balance, more specifically NEFA concentrations. LeBlanc et al. (2004) showed that cows with elevated NEFA levels (≥0.5 mEq/L) during the last week prepartum tended to have an increased risk of retained placenta. Goff and Horst (1997b) indicated that negative energy balance is one of the elements in peripartum immune suppression. This is possibly due to the role of cortisol signaling lipolysis during negative energy balance, as cortisol is known to suppress immune function (Lippolis et al., 2006).

Another reproductive disorder occurring during the transition period is metritis. Metritis is an inflammation of the uterus caused by bacterial infection, typically within ten days after calving. It is characterized by fever, red-brown foul smelling, watery uterine discharge, inappetance, lethargy, and decreased milk production (Sheldon et al., 2006). Metritis is often associated with retained placenta, dystocia, stillbirth or twins, and has also been linked to decreased dry matter intake and behavioral changes during the prepartum period (Huzzey et al., 2007). For every 1kg decrease in DMI prepartum, a cow is three times more likely to be diagnosed with metritis. Furthermore, the cows diagnosed with metritis appeared to be more subordinate cows, engaging in fewer

aggressive interactions at the feed bunk (Huzzey et al., 2007). Dohmen et al. (2000) reported that cows with retained placenta may have *E. coli* present in the uterus immediately after calving, and those cows are more likely to develop metritis from *Arcanobacterium pyogenes* later postpartum. Metritis has been associated with decreased postpartum dry matter intake, milk production (Sheldon et al., 2006), and development of endometritis (LeBlanc et al., 2002). Indirectly, through increased likelihood of developing endometritis, metritis can also increase the number of days until a cow becomes pregnant (LeBlanc, 2008). Prevention of metritis entails maintaining proper DMI through the transition period in an attempt to facilitate adequate immune function because cows in negative energy balance will have elevated cortisol signaling lipolysis, and cortisol suppresses immune function (LeBlanc, 2008). Additionally, prevention of the main risk factor for metritis, retained placentas, by methods listed previously is very important.

A properly functioning immune system is key for the prevention of infectious diseases, such as metritis and mastitis. During the transition period, there are hormone interactions and nutrient deficiencies that may occur which have negative impacts on the ability of the immune system to properly function. Progesterone has been shown to decrease blood polymorphonuclear leukocyte (PMN) oxidative burst capacity (Moreira da Silva et al., 1998), which is accepted as the main method to prevent rejection of the fetus as foreign during pregnancy (Weinberg, 1987). However, as stated above, progesterone concentrations decrease as parturition approaches, so this is not likely to contribute to the severe immune suppression following calving. Estradiol influences some functions of PMN, such as chemotaxis, phagocytosis and oxidative burst activity

(Lamotte, et al., 2006). Similarly, glucocorticoids have been shown to cause some of the same protein expression changes in neutrophils that are caused naturally in immunosuppressed parturient cows (Lippolis et al., 2006). Therefore, it is reasonable to assume that since estrogen and cortisol are increasing around the time of parturition, they may be major contributing factors to immune suppression.

In addition to the hormonally induced immune suppression, nutritional balance plays an important role in affecting the immune system. Deficiencies in energy, protein, vitamins, or minerals can impair immune cell function, and since cows have depressed feed intake around calving, these deficiencies are likely to occur. Negative energy balance results in increased cortisol, which initiates lipolysis in an attempt to maintain energy balance, and as stated by Lippolis et al. (2006), glucocorticoids are immunosuppressive. Additionally, negative energy balance can result in the formation and release of ketoacids as a result of fat metabolism, and these ketoacids can impair lymphocyte function (Franklin et al., 1991). Vitamins A and E are used for incorporation into colostrum and by the stressed immune and metabolic systems around calving. Plasma levels of these vitamins have been shown to decrease by 38% and 47%, respectively, to levels of chronic deficiency during this time period (Goff and Stabel, 1990). Additionally, these authors reported that plasma zinc levels also decreased on average by 22% at calving, and by as much as 49% in cows affected by milk fever. Zinc plays important roles in the antioxidant system and in immune cell proliferation (Spears and Weiss, 2008). Calcium also has an important influence on immunity. Lymphocytes use calcium as a messenger to carry out different signaling processes within the cell (Gallo et al., 2006). Calcium depletion can contribute to an impairment of lymphocyte

function (Kimura et al., 2006). Therefore, since an estimated 5 to 10% of cows suffer from clinical milk fever (Houe et al., 2001) and upwards of 50% of cows can experience subclinical hypocalcemia (Horst et al., 2003), immune suppression due to hypocalcemia is likely to occur during the periparturient period. Additionally, cows with milk fever have been shown to possess elevated levels of cortisol (Horst and Jorgensen, 1982; Goff et al., 1989), and since glucocorticoids can have a negative impact on neutrophil function (Lippolis et al., 2006), hypocalcemia can also indirectly affect the immune function of cows.

Behavioral and Intake Changes During the Periparturient Period

Unlike many aspects of dairy cow management and nutrition, which have been studied for many decades, behavior has only more recently been studied in a quantifiable manner, and has been shown to be important for indicating increased risk for diseases in the future. Certain aspects such as the decrease in dry matter intake (DMI) beginning three weeks prepartum (Drackley, 1999) are generally accepted, but aspects of the behavioral changes leading to decreased DMI have received little attention until the last decade. Producers must work to increase cow comfort and find ways to promote dry matter intake and water consumption throughout the transition period to maximize health and production of the transition dairy cow. Huzzey et al. (2005) found that the frequency of trips to the feed bunk was higher during the first ten days postpartum than the last ten days prepartum. However, the average time spent feeding per day was 25 minutes less postpartum compared to prepartum, but increased by 3.3 min/day over the first ten days postpartum. The increased meal frequency may be, in part due to increased competition as reported by Olofsson (1999). Friggens et al. (1998) reported that increasing the

concentrate portion of the diet increased the rate of feeding, so while Huzzey et al. (2005) did not record DMI, the likelihood of increased feeding rate may compensate for the decreased total feeding time. Osborne et al. (2002) found that the amount of water consumed by cows averaged 57 L/d during the week before calving and 73 L/d during the week after calving. Huzzey et al. (2005) reported that there was an 80% increase in the number of standing bouts on the day of calving compared to prepartum standing, and that the total time standing on day of calving was two hours longer than during the pre-calving period and one hour longer than during the post-calving period.

These studies point to a few key areas of management that should be carefully considered during the transition period. They indicate the importance of decreased stocking rate in the fresh pen to reduce competition in an effort to increase feeding time, and hopefully increase DMI (Oetzel, 2001). They also point to the importance of cow comfort because restless cows are standing and shifting around more, therefore stalls and flooring should be non-abrasive and comfortable (Cook and Nordlund, 2004) to promote adequate resting time because increased lying time has been suggested to be related to increased milk production (Grant, 2003). Furthermore, cows housed in sand bedded stalls have been shown to be at significantly lower risk of developing lameness compared to cows housed on mattress stalls (Cook et al., 2004), and lameness would also lead to decreased production. Since water consumption did not appear to decrease, it does not seem to be an issue to try to promote extra water intake, but it is very important to note that water is the most important nutrient a cow requires, especially a lactating cow, as milk is approximately 87% water. Additionally, water intake has been shown to be

positively correlated with DMI (Dado and Allen, 1994) so as DMI is increased, water consumption will also be increased.

Matching nutrient intake with nutrient requirements is very important for maximizing the production, reproduction, and health of animals. In most cases this is possible, unless the animal is sick. However, maintaining dry matter intake during the transition period is a very challenging issue. The goal of transition cow nutrition is to maintain proper body condition, enable rapid fetal growth near the end of gestation, ease the metabolic transition from pregnancy to lactation, and adjust the rumen microflora to lactation diets (Hayirli et al., 2002) in an effort to promote health and maximum production. It is well known that cows will start decreasing feed intake around three weeks prepartum, and that they have major decreases in intake a few days before and after calving (Drackley, 1999) with ultimate decreases as high as 40% (Hayirli et al., 2002). However, dry matter intake does not peak until eight to 22 weeks postpartum, whereas milk production peaks around five to seven weeks postpartum (Ingvartsen and Andersen, 2000). Decreased DMI will result in a negative energy balance, so maximizing energy intake may help offset the negative energy balance (Grummer, 1995). However, beneficial effects of limiting energy intake during the far off dry period have also been shown (Dann et al., 2006). For these reasons it is important to understand the mechanisms influencing intake and energy balance and use this knowledge to promote appropriate nutrient intake for transition cows.

Decreased DMI around parturition is multifactorial. It was speculated that one contributing factor is the increasing size of the gravid uterus compressing the rumen, decreasing its size (Lagerlof, 1929). Forbes (1969) found that rumen volume of pregnant

ewes was positively related to hay intake two weeks prior to slaughter. This indicates a relationship between DMI and rumen volume, but does not confirm pregnancy actually decreases rumen volume. Coppock et al. (1974) found, however, that the decline in DMI was more pronounced in high concentrate versus low concentrate diets. Also, Stanley et al. (1999) found that rumen volume only increased by 5% at 22 days postpartum compared to 61 days prepartum, whereas DMI increased by 69% over the same period, so it is unlikely that the decreased size of the rumen is the sole cause of decreasing DMI near parturition. Furthermore, once the cow calves, the fetal and placental mass is immediately removed, allowing more room for the rumen, so intake should increase rapidly, but it does not. This provides further evidence that rumen compression is not controlling intake.

Endocrine factors have also been suspected to contribute to intake regulation. Intravenous injections of 17-ß estradiol have been shown to decrease feed intake, and consequently milk production in dairy cows (Grummer et al., 1990). Similarly, intravenous injections of 17-ß estradiol at levels similar to estrus and late pregnancy in whethers and goats have been shown to reduce intake (Forbes, 1986). However, progesterone has been shown to block the effects of estrogen on intake (Muir et al., 1972). Since estrogen is rapidly rising and progesterone is rapidly decreasing at parturition, this is likely to be a contributing factor to the decrease in feed intake around the time of calving. Corticotrophin releasing factor (CRF) has been shown to cause decreased intake in cattle (Ruckebusch and Malbert, 1986). Corticotrophin releasing factor is responsible for stimulating adrenocorticotropic hormone (ACTH), which consequently causes the release of cortisol. It is known that cortisol levels are high

around the time of calving (Goff and Horst, 1997b). While some cortisol comes from the fetus, some also comes from the cow via the actions of CRF and ACTH, which may identify another possible factor to explain the decrease in intake around calving. This argument is also supported by the fact that cortisol levels return to normal a few days after calving, and intake begins to increase at that time as well (Ingvartsen and Andersen, 2000).

Leptin is a hormone produced primarily by adipose cells (Masuzaki et al., 1995) that has been shown to decrease intake in ovariectomized ewes when given over a three day period (Henry et al., 1999). Chilliard et al. (1998) referred to studies that showed positive correlations between leptin and body fatness in cattle, indicating a possible reason that fat cows typically have a more severe decline in DMI at calving than thinner cows. Block et al. (2001) reported that the decrease in leptin at parturition coincided with the onset of negative energy balance, and Henry et al. (1999) also reported that leptin treated ewes had a more severe negative energy balance. The role of leptin in regulating intake appears to be controversial, however, because if leptin is falling near parturition, then intake should increase, but this is not the case. Therefore it seems that leptin may not be contributing to increased DMI in periparturient dairy cows.

Bines and Morant (1983) noticed that body reserves have an effect on feed intake because thin cows (BCS <2.5) had greater intake than overconditioned (BCS > 4.0) cows when fed the same diet. Therefore, keeping cows at ideal body condition is critical for maintenance of proper intake, specifically, minimizing the drop in DMI around the time of parturition. Elevated NEFA levels are common among dairy cows through the transition period, and they have been shown to reduce feed intake in rats (Scharrer and

Langhans, 1988). This is probably linked to mitochondrial oxidation of NEFA providing satiety signals through vagal afferents (Scharrer and Langhans, 1988). This was supported by earlier work of Langhans and Scharrer (1987) because a hepatic branch vagotomy caused a partial block of the effect of decreased intake by elevated fatty acid oxidation. However, this has yet to be proven as an acceptable model in the periparturient dairy cow. In fact, cortisol released during negative energy balance which signals the breakdown of triglycerides and release of NEFA may be the link between elevated NEFA levels and decreased DMI.

Some researchers have focused on increasing energy intake during the close-up dry period. Grummer (1995) stated that prepartum DMI was positively correlated to postpartum DMI and for this reason, he suggested that efforts to maximize DMI should begin in the prepartum period in order to increase productivity and performance in the postpartum period. Grummer (1995) also indicated that increasing nutrient concentration could help increase DMI and consequently, nutrient intake. Increasing energy concentration should help the transition cow in multiple ways. First, it should help offset the negative energy balance that is the result of a decline in DMI around parturition (Mashek and Beede, 2000). Moreover, it acclimates the rumen microbes to the highly fermentable, high concentrate diets of lactating cows (Mackie and Gilchrist, 1979). Also, increased propionate production from starch fermentation is used for gluconeogenesis, thereby increasing glucose concentrations for the production of lactose. This increased propionate has also been shown to stimulate ruminal papillae development (Dirksen et al., 1985), which increases nutrient absorption, volatile fatty acids in particular. For these reasons, many studies have focused on offering a more nutrient dense diet or force-

feeding through rumen fistulas in the close-up dry period in an effort to increase nutrient intake and evaluate the cow's subsequent performance (Dann et al., 1999; Mashek and Beede, 2000; Rabelo et al., 2003). However, these studies have ended with mixed results. Rabelo et al. (2003) found no effect on performance based on prepartum diets of differing energy density. Mashek and Beede (2000) found that there was also no difference in lactational performance for first and second parity cows, but did see an increase in production in third or greater parity cows fed supplemental corn during the prepartum period. Dann et al. (1999) showed that feeding a diet with higher carbohydrate availability decreases NEFA concentrations and improves milk production compared to a diet with lower carbohydrate availability.

Maintaining ideal body condition of dry cows is important to minimize decreases in intake as a result of being over weight (Hayirli et al., 2002). Some researchers have focused on limited energy intake during the far-off dry period (from dry-off until 3 to 4 weeks prepartum) in an attempt to prevent over-conditioning of dry cows and the subsequent decline in DMI near parturition associated with over-conditioned cows (Holcomb et al., 2001; Douglas et al., 2006; Dann et al., 2006; Janovick and Drackley, 2010). Holcomb et al. (2001) found no advantage to restricting energy intake during the dry period. However, Dann et al. (2006) showed that restricting energy intake of far-off dry cows resulted in higher DMI and lower NEFA and BHBA concentrations during the first ten days in milk. Similarly, Douglas et al. (2006) and Janovick and Drackley (2010) also reported that restricting energy intake of dry cows led to greater DMI and lower NEFA, BHBA, and liver triglyceride content in the early postpartum period. Janovick and Drackley (2010) showed that adding wheat straw (approximately 30% DM basis) to a

dry cow diet provides a practical method for effectively limiting energy intake while allowing for ad libitum dry matter intake. Beever (2006) summarized studies restricting energy intake during the far-off dry period and showed where limit feeding had benefits of moderating body condition gain, thereby preventing insulin resistance, resulting in shorter duration of elevated NEFA postpartum, and increasing the ability of the liver to oxidize mobilized fatty acids postpartum. Preventing overconditioning of dry cows (BCS > 4.0) is crucial to preventing insulin resistance because overconditioned cows eat less, leading to hypoglycemia and hypoinsulinemia. Furthermore, with the decrease in DMI, comes increased NEFA levels in the blood, which inhibits insulin-stimulated glucose uptake in peripheral tissues, decreases glucose transporters, and disrupts intracellular insulin signaling pathways in the liver and peripheral tissues (Hayirli, 2006). This has been shown as dry cows fed a higher energy diet had 2.5 times the insulin concentration during the close-up dry period as cows fed a diet to meet NRC requirements (Dann et al., 2003).

Through the transition period, adaptation of the rumen environment to appropriately handle the change in diet composition between dry cow diets and lactating cow diets must occur. Typically, dry cows are fed low energy, high fiber diets, and on the day of calving, they are abruptly moved to the lactating pen with a diet that is lower in fiber and much higher in non-fibrous carbohydrates (NFC). Dry cow diets result in abundant cellulolytic bacteria and protozoa for fiber digestion and relatively few amylolytic bacteria for starch fermentation and also few lactate utilizing bacteria due to a lack of lactate production via NFC fermentation (Yokoyama and Johnson, 1988). Lactation diets that are higher in concentrate, lower in roughage, and have shorter particle

length do not promote chewing and rumination as well as high forage, coarse diets, and as a result, salivation and buffer inflow to the rumen is decreased in the early postpartum period compared to the dry period (Allen, 1997; Beauchemin and Yang, 2005). Furthermore, Dirksen et al. (1985) showed that low energy diets allow the rumen papillae to reduce in size, decreasing absorptive capacity of the rumen by as much as 50%. Therefore, the dry cow's rumen is not fully capable of absorbing the increased VFA production due to NFC fermentation that will take place after parturition. When high amounts of grain enter the rumen, lactate producing bacteria, which were in low numbers during the dry period, respond quickly by producing large amounts of lactate. It takes lactate using bacteria (primarily *Megasphaera elsdenii* and *Selenomonas ruminantium*) several weeks to build up to levels adequate to convert excess lactate to acetate, propionate, or long chain fatty acids (Goff and Horst, 1997b).

Due to these risk factors, subacute rumen acidosis (SARA) (pH between 5.0 and 5.5), or clinical rumen acidosis (pH<5.0) may develop (Nocek, 1997). When acidosis occurs, rumen pH becomes so low that the microflora of the rumen are killed, releasing endotoxins into the bloodstream. Acidosis in dairy cows is most often different from acidosis described in beef feedlot cattle. In dairy cows, it is the total organic acid load that causes the acidosis, not only lactic acid built up (Goff, 2006). The increasing VFA concentration coupled with increasing lactic acid concentration, causes a rapid drop in rumen pH, which may result in acidosis. Subacute rumen acidosis arises by the same mechanism as clinical acidosis, however, it is not characterized by as severe of a pH drop as clinical acidosis. Subacute rumen acidosis is more common than its clinical counterpart, and has been implicated with problems such as laminitis, reduced and erratic

feed intake, decreased BCS (Oetzel, 2000), low milk fat syndrome, and displacement of the abomasum (Olson, 1991). Rumen acidosis results in laminitis later on in lactation. Laminitis occurs as a result of the endotoxins released into the bloodstream after rumen microbes die, which cause vascular changes in the small capillary beds in the hoof. This causes differences in growth rates of cells of the corium, which can ultimately weaken adhesion between layers of the hoof wall (Goff, 2006). Finally, the third phalanx changes its position as a result of breakdown between hoof layers, and it compresses the soft tissue below it. This not only results in extreme pain for the cow, but also in hemorrhage, thrombosis, and may manifest as ulceration of the sole (Nocek, 1997).

Prevention of rumen acidosis continues to be a main priority for early lactation cows and cows at peak DMI. Initiating grain feeding prior to parturition can both help the microbial population of the rumen adjust, and can give the rumen papillae time to grow enough to facilitate proper absorption of VFA. Both of these things take three to five weeks to occur, so initiating grain feeding will not be as effective unless implemented in advance of the designated risk period (Goff and Horst, 1997b). Furthermore, providing forages in the diet that are physically coarse enough to stimulate adequate rumination, salivation, and formation of the rumen mat are key for buffering of the rumen contents (Heinrichs et al., 1999). There are several feed additives that act to buffer the rumen, such as sodium bicarbonate, sodium sesquicarbonate, potassium bicarbonate, magnesium oxide, or limestone, and their use is common on dairies (Erdman, 1988). For example, Hu and Murphy (2005) showed that by supplementing cows with sodium bicarbonate, when their only forage source was corn silage, DMI and milk fat percent significantly increased compared to cows that did not receive the buffer.

This effect was not seen with alfalfa hay and haylage because they stimulate sufficient chewing and salivation to maintain salivary buffer secretion. It is thought that dietary buffering ability is explained by the dietary cation-anion difference (DCAD) (Krause and Oetzel, 2006). Therefore, diets high in cations, such as sodium and potassium have high DCAD (> 100 mEq/kg), which promotes higher rumen pH by decreasing the molar proportion of H^+ and increasing the molar proportion of bicarbonate ions (Stewart, 1983).

Another disorder commonly implicated with the dietary and physiological alterations during the transition period is displacement of the abomasum. Abomasal displacement occurs when the abomasum floats out of its normal resting position either up to the left (90%) or right (10%) side (Shaver, 1997), resulting in a cessation of feed flow through the abomasum. Displacement of the abomasum, as shown in table 1.1, occurs in approximately 7% of cows, and a majority of these cases are within the first four weeks postpartum (Van Winden, 2002). It is thought that there are three things that must occur to allow abomasal displacement. First, during late gestation, the uterus and fetus occupies some of the space normally taken up by the abomasum and rumen, reducing rumen volume by as much as one-third. When the calf is expelled at birth, there is a void of space that must be taken back up by the rumen. Typically, however, intake is depressed around calving, so rumen fill does not occur, leaving room for the pylorus of the abomasum to slide under the rumen to the left (Goff and Horst, 1997b). Next, these authors suggest that during pregnancy the uterus and fetus may have pushed on the abomasum so hard that it stretched the omentum attached to the pylorus that normally assists in holding it in place. Since it is stretched, it allows the pylorus to move around more freely. (Goff and Horst, 1997b). Finally, gas must be present in the abomasum to

increase its buoyancy to the point that the pylorus actually floats up out of place (Van Winden, 2002). This is the result of abomasal atony, because gases in the abomasum are normally pushed back into the rumen by waves of contractions (Goff and Horst, 1997b). Decreased calcium concentration in early postpartum cows is thought to contribute to atony of the abomasum because of calcium's role in muscle contraction. It has been shown that at plasma calcium concentrations of 5mg/100ml, abomasal contractility is reduced by 70% and strength of contraction by 50% (Daniel, 1983). Hypocalcemia, subclinical or clinical, as a risk factor for displaced abomasum has been both supported (Curtis et al., 1983; Massey et al., 1993) and refuted (Leblanc et al., 2005), however. To explain the previously assumed relationship, LeBlanc et al. (2005) suggested that hypocalcemia may be related to decreased DMI prepartum, and decreased DMI has been associated with significant risk factors for displaced abomasum such as elevated NEFA and subclinical ketosis. Another factor contributing to reduced abomasal contractility is high concentrations of VFA in the abomasum (Breukink, 1991), which is promoted by the high concentrate:forage ratio in diets fed to postpartum cows. High amounts of concentrates reduce the depth of the rumen mat, where carbohydrates are normally fermented and VFA are absorbed through the rumen wall and never reach the abomasum (Goff and Horst, 1998). Furthermore, the rumen papillae are often underdeveloped in early lactation (Dirksen et al., 1985), resulting in slower rates of VFA absorption, allowing more to pass to the abomasum. Negative energy balance has also been implicated as a risk factor for displaced abomasum. Serum NEFA concentrations began to increase approximately two weeks prepartum and serum BHBA concentrations began to increase around calving and afterward, both being higher for cows developing a

displaced abomasum (Leblanc et al., 2005). Cows that are over conditioned at calving (BCS ≥ 4) are also at an increased risk of developing a displaced abomasum, however, this may be due to the greater decrease in intake and increased likelihood of becoming ketotic associated with fat cows (Shaver, 1997). Ramifications of displaced abomasum include the direct treatment cost (typically surgical correction), lost milk production because of decreased intake, and increased culling rates (LeBlanc et al., 2005). Prevention of displaced abomasum is multifaceted, just like the risk factors for disease. It starts in the dry period with managing cow body condition score, preventing hypocalcemia, and adjusting the rumen to higher concentrate diets for lactation. If a cow calves and maintains normal DMI of a diet that is similar to what was previously being fed, the cow will have more proper rumen fill, a lower spike in VFA, and a less severe negative energy balance, all of which should decrease incidence of developing a displaced abomasum.

Energy Metabolism

The transition from pregnant, non-lactating to becoming a non-pregnant, lactating animal requires great changes in metabolism. According to Bell (1995) the late gestation fetus may require about half of the dam's circulating glucose and upwards of 72% of the circulating amino acids. However, it should be noted that Bell stated the amino acid uptake is probably overestimated. Since the late gestation cow does not require much energy, she does not have to make very many changes to deal with the draw of nutrients by the conceptus. The late gestation cow can mobilize body fat to utilize for energy without losing substantial amounts to the conceptus because it is unable to utilize lipid for energy. Bell's work (1995) indicated that at 4 days postpartum, mammary requirements

for energy, glucose, amino acids, and fatty acids are, respectively, 3.0, 2.7, 2.0, and 4.5 times greater than requirements by the late gestation gravid uterus, as seen in figure 1.2. With this increase in nutrient demand in only a few days and the decline in dry matter intake seen around calving, it is apparent that there must be drastic changes in metabolism to compensate for the negative energy balance that is inevitable. The liver increases gluconeogenic activity and utilizes substrates such as intestinally absorbed amino acids, endogenous amino acids, lactate, and glycerol (Drackley et al., 2001) in an effort to meet the increased demand for glucose by the mammary gland. The hallmark of the dairy cow's negative energy balance is mobilizing fat from body stores as non-esterified fatty acids (NEFA) to utilize for energy, and NEFA monitoring has been widely accepted as the method of evaluating negative energy balance for over 20 years (Kunz et al., 1985). NEFA concentrations typically begin to rise two to three weeks prepartum and peak within the first week after calving (Ingvartsen and Andersen, 2000). Periparturient disorders associated with elevated NEFA levels include ketosis, fatty liver disease (Grummer, 1993), displaced abomasum (LeBlanc et al., 2005), and retained placenta (LeBlanc, 2010).

Negative energy balance results in the use of body stores of different substrates for energy. The mammary gland demands glucose in order to produce lactose, the main sugar of milk, in very large quantities (Guo et al., 2008). Overton (1998) has reported that the demand for glucose in the last 21 days of gestation is around 1000 to 1100 g/d, whereas 21 days postpartum, the demand has risen to 2500 g/d. Estimates for glucose available from digestible energy of the diet indicate that there is a lack of approximately 500 g/d in glucose availability, so the liver must compensate for this shortfall with de

novo synthesis of glucose (gluconeogenesis) from other substrates such as intestinally absorbed amino acids, endogenous amino acids, lactate, and glycerol (Drackley et al., 2001). Insulin is a hormone that stimulates nutrient storage, primarily glucose, which may be used by the animal at a later time. Typically, in monogastric species, insulin concentrations increase after a meal when blood glucose levels are high. However, in ruminants, most nutrients are fermented by rumen microbes to volatile fatty acids (VFA) and little glucose makes it out of the rumen to be absorbed. Consequently, basal concentration of insulin in ruminants is low, and most glucose used by dairy cattle is derived through gluconeogenesis (Young, 1976). This insulin "deficiency" is compounded with insulin resistance during the periparturient period because increased progesterone in late gestation and increased cortisol around parturition decrease both glucose transport and insulin binding (Hayirli, 2006). As a result, the cow is incapable of storing enough glucose to meet the demands for lactation by the mammary gland. This is why gluconeogenesis is of vital importance during the periparturient period. Insulin has also been shown to decrease NEFA uptake by the liver by stimulating lipogenesis and inhibiting lypolysis, and it enhances utilization of ketones by extrahepatic tissues (Hayirli, 2006). Given insulin concentrations are low during the periparturient period, the cow has reduced ability to minimize lipid mobilization and the negative consequences that can occur as a result. Finally, insulin promotes VFA absorption by stimulating growth of rumen papillae (Sakata et al., 1980), which stabilize rumen pH by removing lingering VFA and may consequently increase feed intake, decreasing the negative energy balance.

Protein catabolism is common in the early postpartum period both as a mechanism to compensate for negative protein and energy balance and to handle the increased demand for milk protein production. It is known that all amino acids except for leucine and lysine can contribute to gluconeogenesis (Drackley et al., 2001), but the greatest contribution is from alanine and glutamine at 40% to 60% respectively, of the total glucogenic capacity of amino acids (Bergman and Heitmann, 1978). Bell (1995) initially hypothesized that skeletal muscle might serve as a pool of amino acids that can be mobilized to support gluconeogenesis in the periparturient period. Bell et al. (2000) later proved that skeletal muscle indeed undergoes adaptations to provide amino acids for gluconeogenesis. Overton (1998) used alanine as an indicator of amino acid conversion to glucose and compared this to propionate conversion to glucose. They found that propionate conversion to glucose at days one and 21 postpartum was 119% and 129% of that at 21 days prepartum, but alanine conversion was at 198% and 150% for the same time period compared to 21 days prepartum. This indicates that while propionate (primarily from diet fermentation) use increased, alanine use for gluconeogenesis increased at an even greater rate, highlighting the importance of amino acids in gluconeogenesis during the periparturient period.

When the gluconeogenic capacity of the liver is maximized and still cannot meet the energy demands for lactation, lipolysis occurs in adipose tissue and glycerol and NEFA are mobilized from triglycerides in adipose stores. In hepatocytes, NEFA are transported from the cytoplasm into the mitochondria by carnitine palmitoyltransferase-1, where they are either re-esterified or oxidized (Hayirli, 2006). However, under situations of adequate carbohydrate feeding, mitochondrial oxidation of carbohydrates results in an

efflux of citrate, which is converted to malonyl Co-A under the influences of ATP:citrate lyase and acetyl Co-A carboxylase (McGarry and Foster, 1980). Malonyl Co-A inhibits CPT-1 and therefore prevents mitochondrial ß-oxidation and directly promotes lipogenesis by being a substrate for fatty acid synthase (McGarry and Foster, 1980). The process of mitochondrial ß-oxidation is outlined in figure 1.3 from Eaton et al. (1996). Ultimately, ß-oxidation in the mitochondria yield Acetyl Co-A, which enters the TCA cycle with the end result being combustion to carbon dioxide and water, yielding adenosine triphosphate (ATP) (Eaton et al., 1996). Additionally, when large amounts of NEFA are entering the hepatocytes, lysosomal ß-oxidation can be upregulated to assist in meeting the demand (Drackley et al., 2001). In fact, Grum et al. (1996) noted that there was a 12% increase lysosomal ß-oxidation at one day postpartum compared to 21 days prepartum. If hepatic NEFA influx is too great, the TCA cycle cannot oxidize all of the acetyl Co-A generated from ß-oxidation of NEFA, so ketogenesis is stimulated (Grummer, 1993). Acetyl Co-A from fatty acid ß-oxidation can be used for ketone body production by the enzyme 3-hydroxy-3-methylglutaryl-CoA synthase (HMG-CoA) (Hayirli, 2006) as shown in figure 1.4 from Hegardt (1999). Succinyl Co-A can either come directly from the TCA cycle or can be produced from propionate (Drackley et al., 2001), and it covalently binds to HMG Co-A to inhibit ketogenesis when sufficient energy exists from carbohydrate intake (Hegardt, 1999). Ketone bodies (acetoacetic acid and ß-hydroxybutyrate) are used for energy by the heart, skeletal muscle, kidney, mammary gland, and intestinal tract of ruminants (Rukkwamsuk et al., 1999a). An increase in ketone bodies may become evident in the blood, milk, and urine, and is often used as the primary indication of ketosis (Goff and Horst, 1997b), many times occurring

by as soon as ten days postpartum (Goff, 2006). When NEFA influx to the liver is too great for ß-oxidation to keep pace, reesterification to triacylglycerides (TAG) occurs, and TAG can be exported in very low-density lipoproteins (VLDL) or stored in the hepatocytes (Hayirli, 2006). PARAGRAPH

The final option for NEFA mobilized during negative energy balance is to be incorporated directly into milk. Commonly this accounts for less than 10% of milk fat, however as negative energy balance becomes more severe, the amount of NEFA incorporated into milk increases proportionally (Bauman and Griinari, 2003). A schematic representation of lipid metabolism in the adipose tissue, liver, and mammary gland is illustrated in figure 1.5 from Drackley (1999).

Ketosis often occurs in U. S. dairy cattle within the first ten days of lactation, and is attributed to severe negative energy balance. As discussed above, NEFA mobilized in response to negative energy balance can ultimately be converted to the ketone bodies acetoacetic acid and ß-hydroxybutyrate (Goff and Horst, 1997b). Clinically ketotic cows will have blood glucose concentrations that are too low to support normal nerve and brain function, so they typically exhibit normal signs of central nervous system impairment such as stumbling and head pressing. They are also inappetant, which further complicates the problem by making negative energy balance more severe. Ultimately, cows will decrease production in an attempt to decrease energy expenditure to cope with ketosis (Goff, 2006). Treatment of ketosis can be difficult as it is often accompanied by some degree of fatty liver, but typically oral glucose precursors or intravenous infusion of glucose is the accepted method of treating ketotic cows.

Methods to prevent ketosis are similar to prevention of hepatic lipidosis. Limiting the decrease in feed intake, or more importantly energy intake, around parturition is paramount for successful prevention of this disorder. Diets with increased amounts of nonfibrous carbohydrates will increase insulin concentrations, which consequently decrease ketogenesis (Hayirli, 2006). Feeding monensin may have beneficial effects as it shifts rumen microbial fermentation toward increased propionate production, which precedes glucose production via gluconeogenesis (Duffield et al., 1998), but it is important to maintain intake if it is added to the ration. Oral administration of propylene glycol has shown to be effective in preventing ketosis, however the requirement for daily administration is inconvenient (Sauer et al., 1973). A management factor that is paramount to prevention of ketosis and fatty liver development is maintaining ideal body condition score of cows during the dry period. Overconditioned cows have a greater decrease in intake around parturition (Stockdale, 2001), more severe negative energy balance and increased NEFA mobilization (Kokkonen et al., 2005). Additionally, cows with lower prepartum DMI have greater lipid deposition in the liver (Bertics et al., 1992), and consequently a decreased ability to produce glucose via gluconeogenesis and oxidize NEFA for energy (Grum et al., 2002). Cows should be fed in the far-off dry period to either obtain or maintain ideal body condition in order to minimize the risk of ketosis and fatty liver associated with overconditioned cows.

Hepatic lipidosis, more commonly referred to as fatty liver disease, is a metabolic disorder that occurs when the rate of triglyceride (TG) synthesis exceeds the rate of TG hydrolysis and TG export as VLDL (Grummer, 1993). This generally occurs within the first four weeks postpartum (Grummer, 1993) when as many as 50% of cows can be

affected by lipid deposition in the liver (Jorritsma et al., 2001). Liver TG accumulation has been shown to be proportional to the load of NEFA circulating in the blood (Heimberg et al., 1978). Another factor contributing to fat deposition in the liver during the periparturient period is the rising level of estrogen near parturition (Grummer et al., 1990). Estrogen has been shown to be a potent regulator of fatty acid metabolism in nonruminants by stimulating esterification of NEFA in the liver and also promotes export via VLDL (Park and Cho, 1988). However, since VLDL secretion is very low in ruminants, the increased esterification to TG may be greater than the increase in VLDL export, resulting in increased hepatic deposition of lipid (Grummer et al., 1990). Typically, these problems are further exacerbated in overconditioned cows because of the more pronounced decrease in DMI and greater lipid mobilization compared to ideally conditioned cows (Hayirli, 2006). Increased lipid deposition in hepatocytes has been shown to decrease hepatic gluconeogenic capacity (Rukkwamsuk et al., 1999b), which may lead to decreased production (Gerloff et al., 1986). Hepatic lipidosis has been associated with decreased immune function (Ropstad et al., 1989; Kaneene et al., 1997), which may be related to the negative ramifications of ketonemia and increased cortisol, due to negative energy balance, on immune function. Fatty liver disease has also been associated with decreased fertility (Reid et al., 1979a,b) and increased likelihood of ketosis (Drackley et al., 1992).

Attempts to prevent fatty liver disease have focused on either preventing fat mobilization and improving fatty acid metabolism or increasing VLDL export. Prevention of fat mobilization is typically done by either increasing the amount of grain in the diet or by increasing fat in the diet to try to prevent negative energy balance during

late gestation and early lactation (Grummer, 2008). Propylene glycol has been well documented to reduce plasma NEFA and prevent liver TG accumulation when given as an oral drench for seven to ten days prepartum (Studer et al., 1993; Rukkwamsuk et al., 2005). Propylene glycol is a glucose precursor, so it increases glucose concentrations and presumably insulin as well. Insulin as discussed above has preventative effects on lipolysis (Grummer, 2008). Niacin has been used as a feed additive to alleviate ketosis and fatty liver disease because of its antilipolytic effects. While it is commercially used, the only proven effectiveness is when it is given in large enough doses or is rumen protected so that it reaches the intestine (Pires and Grummer, 2007). However, it must be given through the duration of decreased feed intake (Grummer, 2008), so maintaining adequate intake to ensure consumption of the niacin is critical to the efficacy of added niacin.

Rumen bypass choline has been used as a feed additive because of its ability to increase VLDL export by the liver. Choline serves as a substrate for the synthesis of phosphatidyl choline, which is a component of VLDL (Grummer, 2008), so if feed intake caused a deficiency in choline, VLDL clearance could be reduced. Additionally, choline is degraded by rumen microbes, so speculation of choline deficiency by this mechanism has been proposed (Cooke et al., 2007). The efficacy of choline has been disputed, however due to differences in rumen degradability of additives from different sources. Some research has not seen statistically significant benefits from feeding choline prepartum (Zahra et al., 2006) or through late gestation and early lactation (Piepenbrink and Overton, 2004). However, others have seen significant decreases in liver TG when feeding choline through the periparturient period (Elek et al., 2004; Cooke et al., 2007).

It appears that if adequate quantities of choline are fed in rumen protected form, there is a decrease in liver TG deposition.

Negative energy balance is unavoidable during early lactation as the drive for milk production requires more energy than cows are capable of consuming. It is important to limit, not eliminate negative energy balance because some lipid mobilization is necessary to maintain high milk production, but when extreme amounts of lipid are mobilized, disorders such as ketosis or hepatic lipidosis may prevail (Grummer, 2008). Strategies such as preventing the overconditioning of dry cows, minimizing the decline in dry mater intake around parturition, and using nutritional supplements such as choline or propylene glycol are crucial for avoiding the most severe ramifications of negative energy balance.

Calcium balance, metabolism, and homeostasis

Calcium is necessary for proper function of a wide variety of systems in the body from structural functions such as bone and other tissues to intracellular processes as a second messenger. Extracellular calcium is necessary for muscle contraction, nerve impulses, blood clotting, and is incorporated into milk and bone (NRC, 2001). Intracellularly, calcium is involved in second messenger systems for a wide variety of processes (NRC, 2001). Of particular importance to transition cows is the function of calcium in hepatocytes and adipocyte metabolism and immune cell function.

Calcium and energy metabolism are two major focuses of transition cow research and have been shown to be interrelated. It has been shown that increases in cytosolic calcium concentration lead to parallel increases in the concentration of calcium in mitochondria, and that this is important for the stimulation of mitochondrial oxidative

metabolism (Hajnoczky et al., 1995). Calcium flux into the mitochondria phosphorylates pyruvate dehydrogenase to its active form (Denton et al., 1972), which converts pyruvate to Acetyl CoA to enter the TCA cycle. This also causes an increase in NADH that is responsible for donating electrons during oxidative phosphorylation (Robb-Gaspers et al., 1998). A schematic representation for this process can be seen in figure 1.6 (Peters and LeBlanc, 2004). Kimura et al. (2006) demonstrated that hypocalcemia depletes intracellular stores of calcium in lymphocytes. Therefore, if a cow is hypocalcemic, it may be possible that there is also a decrease in intracellular calcium in the hepatocytes. If this is true, pyruvate dehydrogenase may not be activated as efficiently. As a result, carbohydrate metabolism via the TCA cycle would be decreased, resulting in a more severe negative energy balance than a cow would see without hypocalcemia.

Calcium is also important in adipocytes for regulating lipid metabolism and triglyceride storage (Zemel, 2004). In studies with rat and human adipocytes, increased intracellular calcium has been shown to have antilipolytic effects (Tebar et al., 1996; Xue et al., 2001; Cifuentes and Rojas, 2008). This occurs by activation of adipocyte phosphodiesterase and a reduction in cyclic AMP levels, leading to a decrease in hormone sensitive lipase phosphorylation and, consequently, inhibition of lipolysis (Xue et al., 2001). Following the concept of hypocalcemic depletion of intracellular calcium stores put forth by Kimura et al. (2006), it can be speculated that hypocalcemia may deplete adipocyte calcium stores, resulting in increased lipolysis.

These concepts offer possible explanations of why subclinical hypocalcemia may result in greater circulating non-esterified fatty acid levels, which have been observed by Horst et al. (2003) and Spain et al. (2004).

Calcium is critical for proper immune cell function, which is very important in transition dairy cows. A key early feature in immune cell activation is calcium signaling, which causes a change in shape of T-cells, cessation of T-cell motility, and movement of the cortical actin cytoskeleton toward the T-cell – antigen presenting cell surface, possibly enhancing the interaction between the T-cell and antigen presenting cell (Gallo et al., 2006). Calcium also regulates cell polarity, which is required for directional cell killing, and it is also involved in the migration of leukocytes to chemokines in the area of inflammation (Gallo et al., 2006). Sustained calcium influx, which is dependent on extracellular calcium entering the cell, activates transcription. These changes in gene expression are necessary for cellular responses such as development and maturation of the lymphocyte and immune activation in the periphery (Gallo et al., 2006). It is well documented that cows are immune suppressed during the transition period (Clemens et al., 1979; Wyle and Kent, 1977; Franklin et al., 1991), and also that as many as 50% of the cows may be at least subclinically hypocalcemic (Horst et al., 2003). The research of Kimura et al. (2006) confirms that during hypocalcemia, intracellular calcium is below optimum levels in peripheral mononuclear cells resulting in a blunted immune cell activation signal.

Since maintaining proper calcium balance is crucial for normal function of many body systems as previously discussed, dietary requirements for different stages of production provide important guidelines for nutritionists to follow. According to Visek et al. (1953), 0.0154g absorbed calcium/kg body weight is necessary for maintenance in a non-lactating cow, which equates to approximately 30g/day (DeGaris and Lean, 2008). For gestation beyond day 190, House and Bell (1993) described an equation to predict

absorbed calcium requirements of the fetus and uterus by day as:

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

where t = day of gestation. For lactating cows, maintenance requirement increases to 0.031g absorbed calcium/kg body weight (Martz et al., 1990). For colostrum production, cows will need approximately 2.1 g absorbed calcium/kg colostrum (NRC, 2001). During lactation, milk production requires 1.22 g absorbed calcium/kg milk produced for Holstein cows (NRC, 2001), and this can exceed a total of 50 g/day lost to milk alone. The amount of calcium required per kg milk varies slightly depending on protein concentration in milk (NRC, 2001). For this reason, higher component breeds require greater amounts of calcium/kg milk produced. Jerseys need 1.45g/kg milk produced, and other breeds require 1.37g/kg milk.

Given the difference between the calcium requirement for dry cow maintenance plus gravid uterus and fetus, and lactating cow maintenance plus milk production, there is potential for a major shortcoming in calcium and development of hypocalcemia. Hypocalcemia is a metabolic disorder in which calcium homeostatic mechanisms fail to maintain normal blood calcium concentrations at the onset of lactation (Goff and Horst, 1997a). The entire extracellular pool of calcium in a cow is around 8-9g, and most cows will secrete 20-30g of calcium into milk each day (Goff, 2000) and may exceed 50g per day (DeGaris and Lean, 2008). In fact, approximately 50% of fresh cows are suspected to be at least subclinically hypocalcemic (Horst et al., 2003), with a total blood calcium level below 2mmol/L or 8mg/dL (Goff, 2008). Approximately 5% of cows develop clinical hypocalcemia (total blood calcium below 1.38 mmol/L or 5.5mg/dL), however, treatment with only 8-12 g calcium intravenously normally corrects the problem (Goff,

2008).

Milk fever, also referred to as parturient paresis, occurs when calcium concentrations in the blood become so low that they can no longer support normal nerve and muscle function (Goff and Horst, 1997b). In these cases, the plasma calcium concentration is normally below 4mg/100ml (Goff and Horst, 1997b) and the cow is unable to rise. If blood calcium loss is greater than 50%, a hypocalcemia crisis is likely to occur (DeGaris and Lean, 2008). In a normocalcemic cow, parathyroid hormone concentrations are sufficient to cause release of calcium from bone, stimulate intestinal absorption via vitamin D3, and stimulate renal reabsorption of calcium (Horst et al., 1994). However, in a hypocalcemic cow, these mechanisms fail to generate sufficient supplies of calcium to maintain normocalcemia and milk fever can result. A schematic representation from Horst et al. (1994) of calcium metabolism in the periparturient cow is provided in figure 1.7.

Milk fever may often predispose the periparturient cow to many other diseases. Cows diagnosed with milk fever have higher cortisol concentrations than those without milk fever (Horst and Jorgensen, 1982), which is associated with immune suppression. Furthermore, Goff and Horst (1997b) have speculated that milk fever results in a loss of muscle tone in the teat sphincter, which may lead to mastitis, especially when considering the immunosuppressive effects of cortisol during this time period.

Cows with parturient paresis also exhibit a greater decrease in dry matter intake than cows without milk fever (Goff and Horst, 1997a), which worsens the negative energy balance that cows are already experiencing. Goff and Horst (1997b) also speculated that the decline in intake also results in a less full rumen which floats above

the floor of the abdomen and reduced depth of the rumen mat. This allows more VFA into the abomasum, reducing contractility (Breukink, 1991). Madison and Troutt (1988) showed that when serum total calcium concentrations were below 1.2 mmol/L, all mechanical and electrical activity in the abomasum was absent. This is only relevant for very hypocalcemic cows, as these authors indicate that few cows will reach serum total calcium concentrations as low as 1.2 mmol/L. All of these variables lead to an increased chance of developing a displaced abomasum (Goff and Horst, 1997b).

Prevention of milk fever has been the focus of much research over the last several decades because of the direct and indirect effects it can have on cow performance. The first method of milk fever prevention was to decrease calcium concentration in the diets of dry cows so parathyroid hormone production would be increased to meet the calcium requirements of the cow and developing fetus, and the homeostatic system would already be working when the cow calved, resulting in quicker mobilization of large amounts of calcium for lactation (Boda and Cole, 1954). However, later work involving dietary cation-anion difference offered an alternative solution to producers who may feed forage sources that are higher in calcium, such as legumes. It is difficult for these producers to obtain low enough calcium content to sufficiently upregulate PTH production. Block (1984) found that significantly fewer cows developed milk fever when fed diets containing greater concentrations of anions (Cl^- and S^- in particular) and less Na. This method of milk fever prevention has been supported by other researchers (Oetzel et al., 1988; Goff et al., 1991; Moore et al., 2000).

Normally, blood calcium is maintained within a range of 8.5-10mg/dL, which is done by rapid replacement of calcium into the bloodstream when it is incorporated into

the milk (Goff, 2000). Parathyroid hormone (PTH) is the main hormone responsible for increasing blood calcium and is released in response to low blood ionized calcium (DeGaris and Lean, 2008). PTH is responsible for the conversion of 25-hydroxycholecalciferol to its active form, 1,25 dihydroxy-vitamin D3 ($1,25(OH)_2D3$) in the kidney, renal reabsorption of calcium, and for osteoclastic resorption of calcium from bone (Goff, 2008). Active vitamin D is responsible for enhanced calcium absorption from the intestines (Horst, 1986). PTH receptors are located on osteoblasts, which stimulate osteoclasts to resorb bone by releasing enzymes onto the surface causing a local reduction of pH at the surface of bone, which may help for mineral dissolution (Block, 1994). Normally, lactational osteoporosis can result in a loss of 9-13% of bone calcium within the first month after calving, but it is reversible once the cow obtains a positive calcium balance (Ellenberger et al., 1932). Unfortunately, calcium mobilization from bones is slower in older than younger cows due to fewer active osteoblasts and fewer receptors (Horst et al., 1994). Vitamin D stimulated intestinal absorption of calcium is also less efficient in older cows (Horst et al., 1990). Couple these factors with higher milk production than a young cow, and it becomes obvious why mature cows are at higher risk for developing milk fever than first lactation animals.

Several things influence effectiveness of calcium homeostatic mechanisms. One major focus for research has been blood acid-base balance. Acid-base balance can be manipulated by adjusting the dietary cation-anion difference (DCAD) in the feed. Dietary cation-anion difference is defined by Ender et al. (1971) as:

milliequivalents of $(Na^+ + K^+) - (Cl^- + SO4^{=}) / kg$ of dry matter.

The way DCAD affects blood acid-base balance was described by Stewart (1983) in the Strong Ion Difference Theory. Two principles drive this theory:

1.) The number of moles of positively charged molecules in any solution must equal the number of moles of negatively charged molecules.

2.) The product of the concentration of hydrogen and hydroxyl ions is always equal to the dissociation constant of water (1×10^{-14}).

Both of these equations must be satisfied simultaneously. Therefore, if negatively charged molecules (anions) are added to the feed in excess of positively charged molecules (cations) there will be a surplus of anions in the blood and the DCAD will be negative. This will be balanced by a proportional change in hydrogen and hydroxyl ion concentration, leading to a decrease in hydroxyl ions (OH^-) and an increase in hydrogen ions (H^+). Since pH is dependent on the concentration of H^+, this means pH of the blood will decrease. This is very important for calcium homeostasis during the periparturient period because at a normal blood pH, parathyroid hormone binds effectively to its receptor, signaling the cascade of events to increase blood calcium (Goff, 2008). However, if pH is higher than normal, there is a conformational change in the PTH receptor so that it does not bind as efficiently, blocking the signal to increase blood calcium (Goff, 2008). Therefore, by feeding anions in the diet, decreasing the DCAD, and decreasing blood pH, the risk of developing milk fever and subclinical hypocalcemia is reduced (Block, 1984; Oetzel et al., 1988; Beede, 1992).

Magnesium also plays an important role in calcium metabolism. It affects the release of PTH during hypocalcemia (Littledike et al., 1983) and also affects tissue sensitivity to PTH (Rude, 1998). Under normal conditions, PTH binds to its receptor in bone or kidney

and normally activates adenylate cyclase, which causes production of cAMP, a second messenger. In other tissues, it binds to its receptor and activates phospholipase C to metabolize phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-tri-phosphate (Goff, 2008), which also act as second messengers. Both adenylate cyclase and phospholipase C have magnesium ion binding sites, which must be filled for optimum function (Rude, 1998). The effect of providing magnesium in excess of requirements offered no advantage in calcium metabolism for periparturient cows on a low DCAD diet (Wang and Beede, 1992). Therefore magnesium deficiency is of greater concern than excess magnesium because it would inhibit the ability of PTH to increase blood calcium.

Phosphorus is another important macromineral that may have altered metabolism during the periparturient period, and it also plays an important role in calcium regulation. Under the influence of hypocalcemia stimulated PTH secretion, phosphorus is released from bone, which increases blood phosphorus along with calcium (Horst, 1986). Furthermore, active phosphorus absorption takes place in the small intestine via 1,25 $(OH)_2$ vitamin D3, which is upregulated by PTH (Horst, 1986). However, PTH also rapidly increases phosphorus excretion in the saliva and urine, therefore, a brief decline in blood phosphorus may be seen before the other mechanisms increase blood phosphorus to normal levels (Goff, 2000). Phosphorus affects calcium homeostasis because when present at high levels, it inhibits activity of the renal enzyme 25-hydroxyvitamin D 1a-hydroxylase (Tanaka and DeLuca, 1973), which is responsible for conversion of 25 hydroxyvitamin D3 to its active form, 1,25 dihydroxyvitamin D3. Kichura et al. (1982) fed diets containing either 10 g or 82 g phosphorus to Jersey cows.

They found that the cows fed the high phosphorus diet had lower plasma 1,25 $(OH)_2$ vitamin D3 prior to parturition and had more hypocalcemia than cows fed low phosphorus diets. This was in accordance with the previous work of Barton (1978). According to Jorgensen (1974), risk of developing milk fever is highest when dietary phosphorus exceeds 90 g per day, and little negative effect is seen when dietary phosphorus is below 50 g per day. According to NRC (2001) recommendations, cows normally need 35 g phosphorus per day or less.

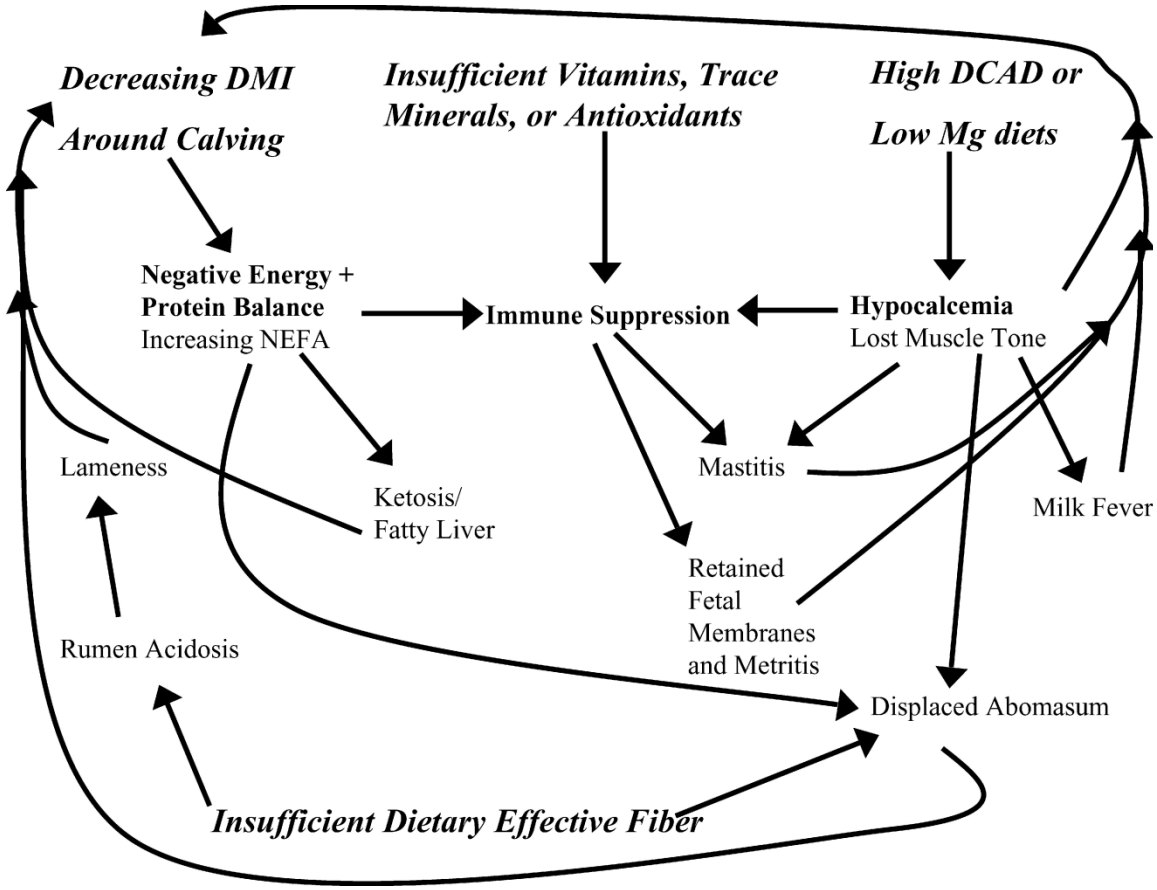
Overall Conclusions

Negative energy balance during early lactation is unavoidable, but managing the transition cow to prevent severe negative energy balance and its harmful ramifications is important. It is known that extensive lipid mobilization may lead to disorders such as ketosis, fatty liver disease (Grummer, 1993), retained placenta, displaced abomasum (LeBlanc et al., 2005), and decreased milk production (Carson, 2008) and fertility (Duffield et al., 2007). Previous research has shown that subclinical hypocalcemia appears to have an association with increased NEFA levels (Horst et al., 2003; Spain et al., 2004), indicating a more severe negative energy balance for hypocalcemic cows. A review of the literature has revealed that there may be a link between calcium and energy metabolism. Intracellular calcium is important for adipocyte regulation of lipolysis (Zemel, 2004) and hepatocellular metabolic signaling for energy metabolism (Hajnoczky et al., 1995). However, if subclinical hypocalcemia truly does effect lipid metabolism, it is unknown if it is significant enough to also elicit differences in liver function, milk production, disease occurrence, or fertility.

Experimental Objectives

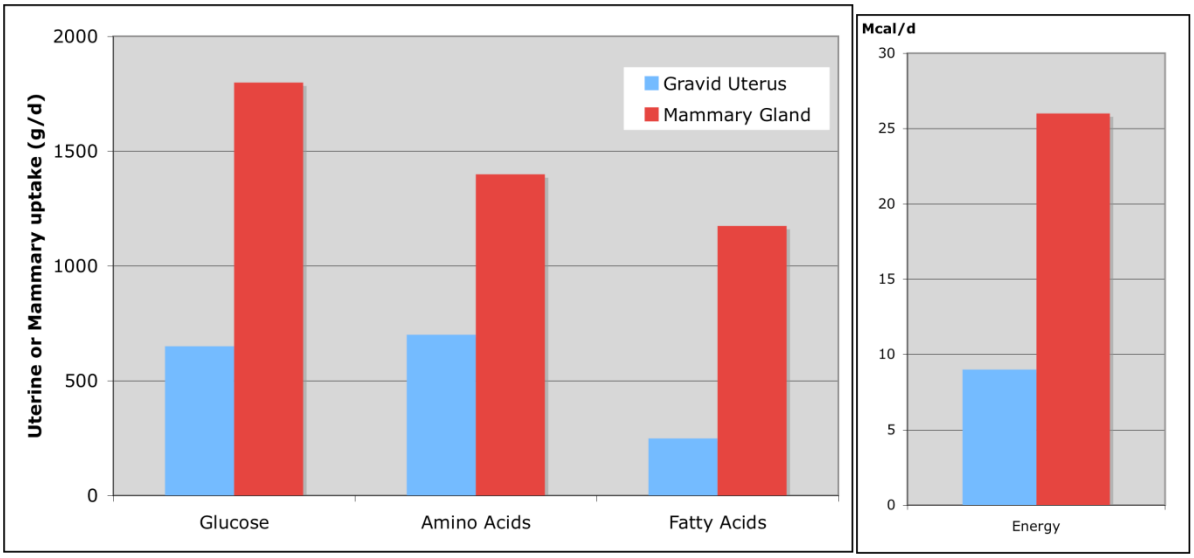
The objective of this thesis was to investigate the effects of subclinical hypocalcemia at calving on plasma biochemical parameters, lipid mobilization, histologic and pathologic changes of the hepatobiliary system, common postpartum diseases, milk production and fertility in periparturient dairy cows.

Figure 1.1. Dietary factors contributing to common postpartum diseases in dairy cows.[1]



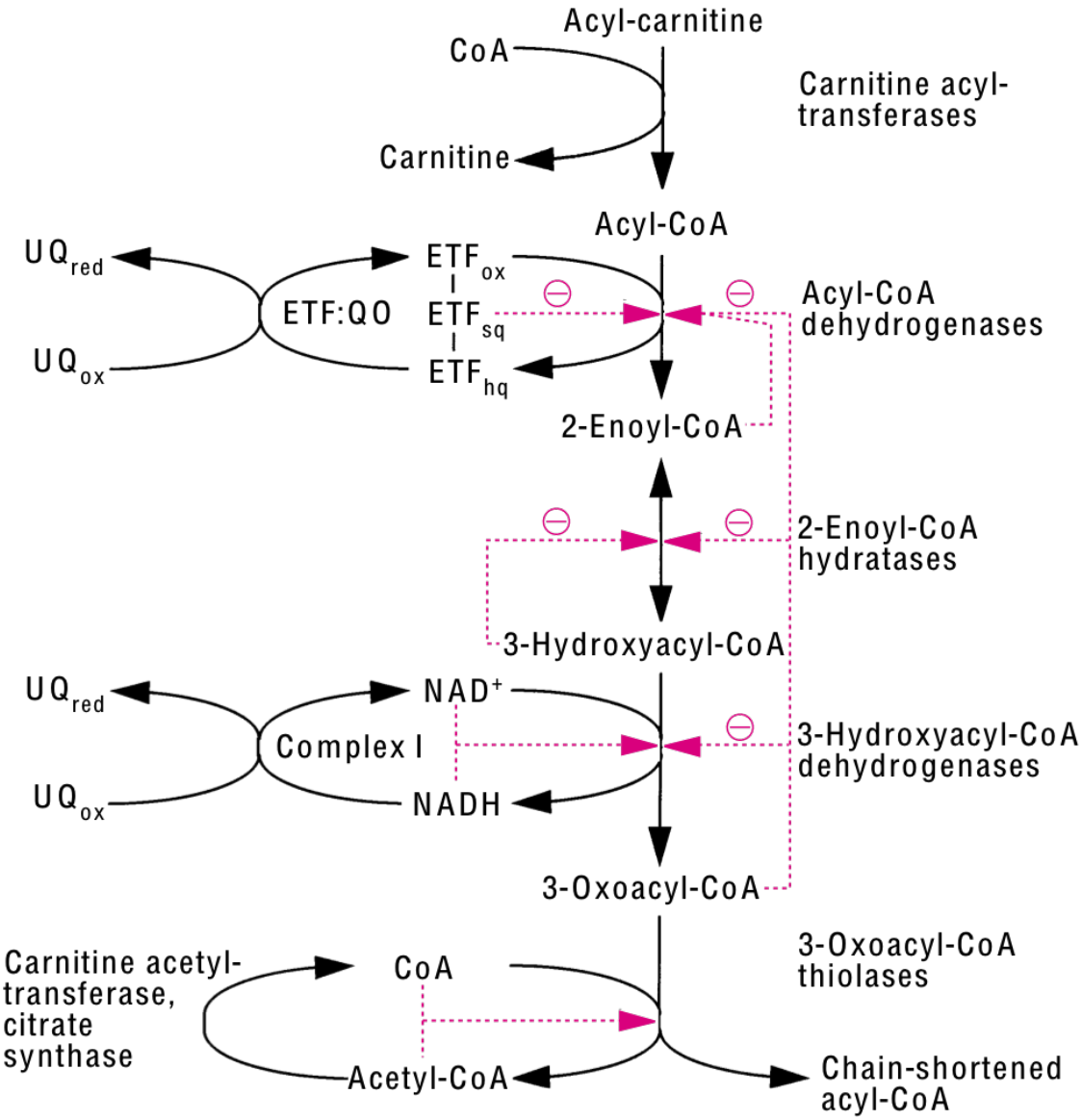
[1]Figure adapted from Goff (2006).

Figure 1.2. Estimated values of uptake for specific nutrients by gravid uterus at day 250 of gestation and for mammary gland at 4 days in milk for Holstein cows.[1]



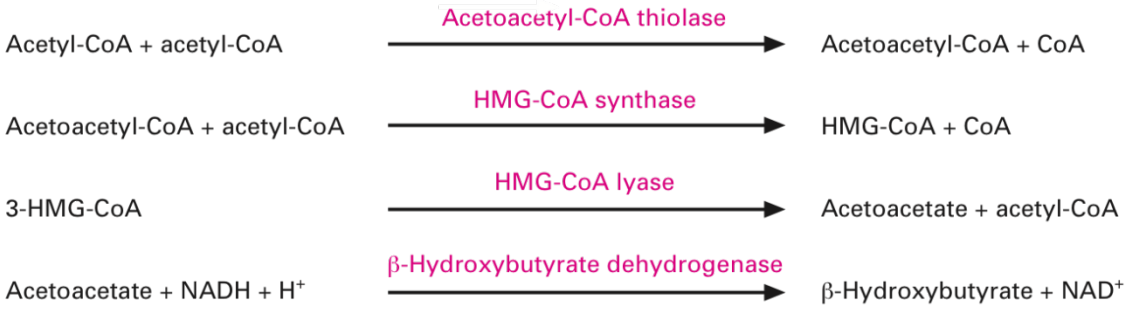
[1]Figure adapted from Bell (1995).

Figure 1.3. Mitochondrial β-oxidation showing the sites of intra-mitochondrial control in red. Abbreviations: UQred, reduced ubiquinone; Uqox, oxidized ubiquinone; ETFox, oxidized ETF; ETFsq, ETF semiquinone; ETFhq, reduced ETF; Complex I, NADH: ubiquinone oxidoreductase[1].



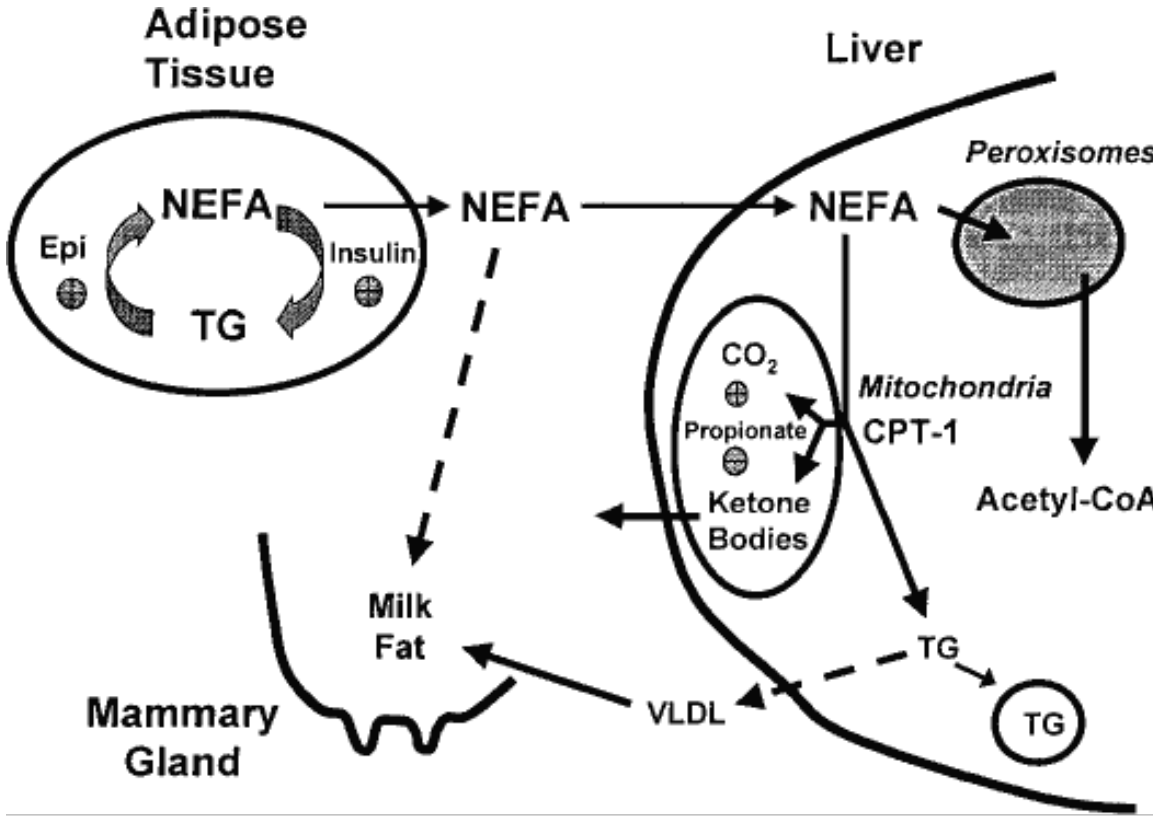
[1]Figure adapted from Eaton et al. (1996).

Figure 1.4. HMG Co-A pathway[1].



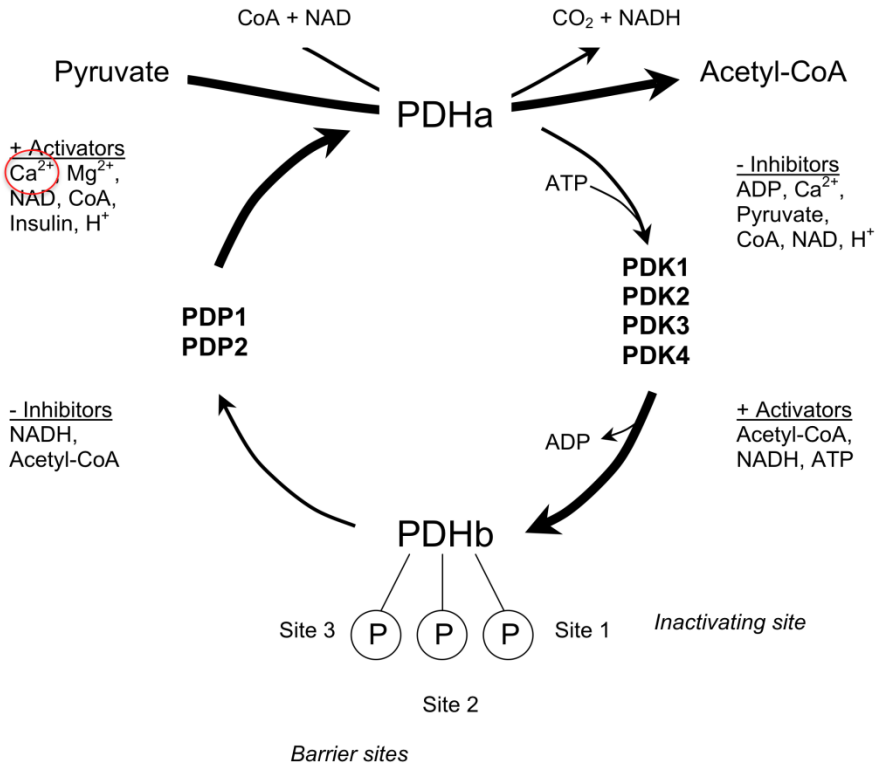
[1]Figure adapted from Hegardt (1999).

Figure 1.5. Representation of relationships among lipid metabolism in adipose tissue, liver, and mammary gland in dairy cows. Plus signs (+) indicate stimulatory effects, minus signs (−) indicate inhibitory effects. Dashed lines indicate processes that occur at low rates or only during certain physiological states. Abbreviations: epi = epinephrine, TG = triglyceride, VLDL = very-low-density lipoproteins, CPT-1 = carnitine palmitoyltransferase 1.[1]



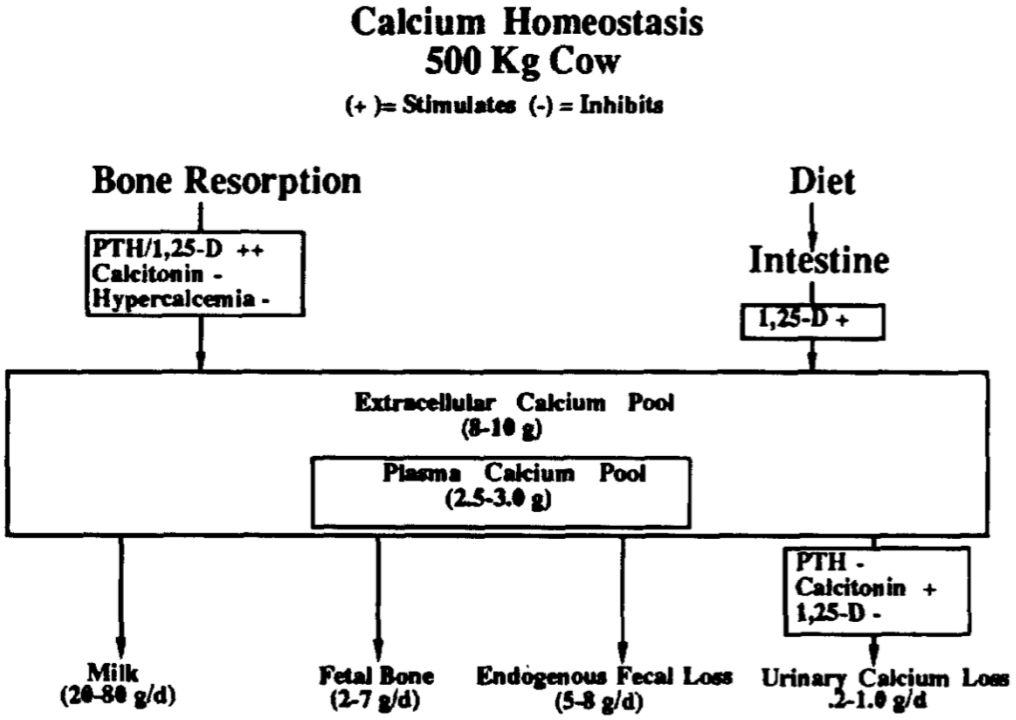
[1] Figure adapted from Drackley (1999).

Figure 1.6. Activation of pyruvate dehydrogenase complex by phosphorylation and dephosphorylation.[1]



[1] Figure adapted from Peters and LeBlanc (2004).

Figure 1.7. Schematic representation of calcium homeostasis in the periparturient dairy cow. PTH = parathyroid hormone; 1,25-D = 1, 25 dihydroxyvitamin D[1].



[1] Figure adapted from Horst et al. (1994).

Table 1.1. Mean incidence and range of common transition cow disorders.[1]

Disorder	Mean (%)		Standard Deviation[2]	Median[2]	Range (%)	
Milk Fever	7.2[1]	4.2[2]	3.4	3.4	0 – 44.1[1]	0 – 13.5[2]
Displaced abomasum	3.3[1]	4.1[2]	3.5	3.1	0 – 14[1]	0 – 15[2]
Ketosis	3.7[1]	2.9[2]	4.0	1.0	0 – 20[1]	0 – 19.3[2]
Retained placenta	9.0[1]	9.1[2]	6.0	7.2	0 – 22.6[1]	0 – 26.3[2]
Metritis	12.8[1]	-	-	-	0 – 66[1]	-
Mastitis	-	21.8[2]	19.0	17.2	-	0 – 92[2]

[1]Table adapted from Jordan and Fourdraine (1993).
[2]Table adapted from McLaren et al. (2006).

Chapter 2

Influence of subclinical hypocalcemia on plasma biochemical parameters, lipid mobilization, liver lipid infiltration, and common postpartum diseases in dairy cows

Introduction

Hypocalcemia is a metabolic disorder in which calcium homeostatic mechanisms fail to maintain normal blood calcium concentrations at the onset of lactation (Goff and Horst, 1997a). While incidence of clinical milk fever in the United States is approximately 5% (McLaren et al., 2006), it is reported that as many as 50% of periparturient dairy cows may suffer from subclinical hypocalcemia (Horst et al., 2003), with total blood calcium being between 1.38 and 2.0 mmol/L (5.5 and 8.0 mg/dL) (Goff, 2008). Prevention of milk fever and its consequences have been the focus of much research in the past, however the effects of subclinical hypocalcemia have received less attention.

It has also been well documented that cows suffer from negative energy balance during the transition period due to decreased dry matter intake and increased energy demands due to lactation (Bell, 1995; Grummer, 1995; Goff, 2006). The hallmark of the dairy cow's negative energy balance is mobilizing fat from body stores as non-esterified fatty acids (NEFA) to utilize for energy. Monitoring NEFA during the prepartum period provides the most utility for predicting future events, as it has been significantly associated with increased risk of developing displaced abomasum (LeBlanc et al., 2005),

retained placenta (LeBlanc et al., 2004), and culling (Duffield et al., 2009). Postpartum monitoring of ß-hydroxybutyrate (BHBA) is often more reflective of pathologic negative energy balance and has been associated with increased risk for developing displaced abomasum (LeBlanc et al., 2005), decreased probability of pregnancy after the first service (Walsh et al., 2007), decreased milk production (Duffield et al., 2009), and increased risk for culling (Seifi et al., 2010). Periparturient lipid-related disorders associated with elevated NEFA and BHBA levels include ketosis and fatty liver disease (Grummer, 1993).

Often when periparturient dairy cows are afflicted by one disease, the risk of experiencing others is increased because many of them are influenced by common factors. However, most research to date has focused on individual diseasaes, and less has provided much information on the interrelation between multiple diseases. As a result few common treatment or management options have been discussed to concurrently prevent or reduce the severity of multiple disease complexes. Some research that has investigated the relationship between multiple diseases has shown that cows experiencing milk fever were nearly 9 times more likely to exhibit signs of ketosis when compared to cows not developing milk fever (Curtis et al., 1983). Hypocalcemia has also been associated with increased risk of developing several other common postpartum diseases such as mastitis, retained placenta, metritis, and displaced abomasum (Curtis et al., 1983; Goff and Horst 1997b; Mulligan et al., 2006; Goff, 2008). However, this association has been studied in cows with clinical milk fever and has not included cows affected by subclinical hypocalcemia. Therefore, more research focusing on subclinical hypocalcemia and its relationship to clinical disease occurrence is warranted.

Previous work has associated subclinical hypocalcemia with increased NEFA mobilization (Horst et al., 2003; Spain et al., 2004), but has not further investigated the relationship between the two disorders.

Based on previous research with milk fever, our alternative hypothesis was that subclinically hypocalcemic cows would have increased plasma NEFA concentrations, and as a result, increased lipid deposition in the liver, elevated biochemical parameters associated with cholestasis, hepatocellular damage, and impaired liver function, increased occurrence of postpartum diseases, decreased milk production, and decreased fertility.

Materials and Methods

Study design

One hundred multiparous Holstein dairy cattle were studied over a two year period. First lactation cattle were not included in this study. All procedures were approved by the University of Missouri Animal Care and Use Committee. Cows were grouped at calving based on whole blood ionized calcium concentrations into two groups 1) hypocalcemic cows (n = 51; [iCa] <1.0 mmol/L), and 2) normocalcemic cows (n = 49; [iCa] ≥ 1.0 mmol/L). Cows were housed at the University of Missouri Foremost Dairy Research Center (Columbia, MO), and were fed a balanced dry cow ration free of anionic salts from the day of dry off until the day of calving. All cows were also fed the same balanced lactation diet during the postpartum portion of the study.

Sample and data collection

Blood samples were collected via coccygeal veinipuncture from all cows into 2 heparinized tubes (Vacutainer®, Becton Dickinson Vacutainer Systems, Franklin Lakes,

NJ) at 14 days from expected calving date (-14), the day of calving (0), and on days 3, 7, 14, 21, and 35 postpartum between 1400 and 1600 hours for measurement of whole blood ionized calcium concentration, pH, packed cell volume, plasma biochemical parameters, and plasma NEFA concentration. Milk samples were collected during the afternoon milking on days 3, 7, 14, 21, and 35 postpartum using an in-line sampler (Westfalia-Surge Inc., Naperville, IL) into vials containing a 2-Bromo-2-nitropropane-1, 3-diol preservative tablet (Broad Spectrum Microtabs II, D & F Control Systems, Inc., Dublin, California, USA) for determination milk protein, fat, solids-not-fat and somatic cell count (Mid-South Dairy Records, Springfield, MO).

To assess the extent of fat mobilization into the liver, liver biopsies were obtained from a subset of 24 cattle in year 1and 22 cattle in year 2 on the day of calving and on days 7 and 35 postpartum. The first 10 cows to calve each year in each group had liver biopsies taken, however, some extra cows were sampled to get biopsies from cows that calved closer together so that they should fit into a block. Liver tissue was obtained via percutaneous biopsy using a large bore biopsy needle similar to that described by Hughes (1962). Briefly, a 5 cm x 5 cm area over the 10^{th} or 11^{th} intercostal space intersecting a diagonal line between the point of the olecranon and the tuber coxae on the right side of the cow was clipped of hair and aseptically prepared. The subcutaneous tissue and intercostal muscle was anesthetized by local infiltration of 10 ml of 2% lidocaine. A #22 scalpel blade was used to make an incision in the skin and the biopsy needle was guided through the skin, subcutaneous tissues, intercostal muscle, peritoneum, and liver capsule to obtain a core of liver tissue. Liver tissue samples were fixed in 10% neutral buffered formalin and stored until analyzed.

The occurrence of postpartum diseases including ketosis, displaced abomasum, retained placenta, metritis, and clinical mastitis were recorded from farm herd health records using definitions contained in the farm's standard operating procedures. These diseases were recorded as present or not present. Ketosis was diagnosed based upon a color change on a urine dip stick (Ketostix, Bayer, Leverkusen, Germany) equivalent to 15 umol/L. Ketones were measured on days 0-14 in milk as part of the farm's fresh cow monitoring program. Displaced abomasum was diagnosed when surgical correction was performed by a veterinarian. Retained placenta was diagnosed when the fetal membranes failed to be completely expelled from the birth canal within 12 hours of parturition. Metritis was defined as a cow with a fever (rectal temperature >103^0F) and a discolored, foul-smelling uterine discharge (Sheldon et al., 2006) diagnosed by farm personnel during daily fresh cow checks on days 0-14 in milk. Per farm protocol, clinical mastitis cases were recorded when a cow was treated intramammary antibiotics. The farm protocol dictates any cow with abnormalities in the milk secretion such as clots, strings, or serum-colored milk, or with an udder that is swollen and/or painful be treated with intramammary antibiotics at first occurrence.

Body condition scoring was done by farm personnel using a 1 to 5 scale with 0.25 unit increments as described by Edmonson et al. (1989) and Ferguson et al. (1994). Body condition scores were recorded at calving, at 14 days in milk, and at first breeding. Body condition change from calving to 14 days in milk was calculated by subtracting the body condition score at calving from the body condition score at 14 days in milk. Body condition change from day 14 to first breeding was calculated by subtracting body condition score at 14 days in milk from body condition score at first breeding. Body

condition change from calving from first breeding was calculated by subtracting body condition score at calving from body condition score at first breeding.

Peak test day milk yield was obtained from herd DHIA records. Estimated 305 day ME 4% fat corrected milk yield was based on the test day milk weight closest to 60 days in milk obtained from herd DHIA records and using the equation 4% FCM = (0.4 * kg ME milk) + (15 * kg ME milk fat) (NRC, 2001). The milk production parameters were only assessed for cows that were still in the herd at the test day around 60 days in milk (n = 92).

Reproductive parameters measured included services per conception, days open, and cyclicity at 50 – 60 days postpartum. Services per conception and days open were obtained from herd DHIA records. The first reproductive tract assessment postpartum was ovarian ultrasound, which was performed per rectum at roughly 50 – 60 days postpartum by a board certified theriogenologist. Cows were not considered to be cycling unless there was evidence of a corpus luteum on one or both ovaries (Peter et al., 2009).

Laboratory methods

Within 30 minutes of sample collection, whole blood ionized calcium concentration was determined using a portable blood chemistry analyzer (VetStat, IDEXX Laboratories, Inc., Westbrook, ME) and blood pH was determined by use of a portable pH meter (Acorn pH 5 meter, Oakton Instruments, Vernon Hills, IL, USA). Packed cell volume was determined by centrifugation (Autocrit II, Becton, Dickinson and

Company, Parsippany, NJ, USA) and visual determination using a micro-hematocrit tube reader (Critocap, Stafford Mfg. Co. Inc., Brooklyn, NY, USA).

Plasma was separated from blood cells by centrifugation for 15 minutes at 2,100 x g at 4^{o}C (RC3B Plus centrifuge, Sorvall Instruments, Newton, CT). Samples for plasma biochemistry analysis were refrigerated overnight and were taken to the University of Missouri Veterinary Medical Diagnostic Laboratory for determination of plasma biochemical parameters using an automated biochemistry analyzer (Olympus AU400, Olympus Corporation, Tokyo, Japan). Biochemical parameters of interest included total calcium, phosphorus, glucose, total bilirubin, direct bilirubin, gamma glutamyl transferase, and aspartate aminotransferase.

Remaining plasma samples were frozen (-20^{o}C) and used for measurement of plasma NEFA concentration at the end of each study year. A NEFA C kit (Wako Chemicals USA, Inc., Richmond, VA) was used for measurement of NEFA concentration according to the procedures of Johnson and Peters (1993).

Formalin-fixed liver biopsy specimens were, longitudinally bisected and paraffin embedded for staining. Sections of tissue were stained with oil red O to detect lipid. Lipid content of 6 randomly selected areas of each specimen were assessed to determine the ratio of oil red O stained to total tissue surface area as described by Sevinc et al. (2003) and Semecan and Sevinc (2005). This was done by taking digital images and counting the number of pixels that were stained red for lipid and dividing by the total number of pixels that were blue for tissue.

Data analysis

Cows were blocked based on parity and calving date for analysis. Parity 2 cows could be blocked with 2nd parity or 3rd parity cows, and 3rd parity cows could be blocked with any parity. Cows greater than 3rd parity could be blocked with any 3rd parity or greater cow. Blocking based on calving date consisted of grouping cows of appropriate parity and within no more than 24 days of calving from each other. This amount of time allowed for including the maximum number of cows into blocks, but still kept those cows exposed to similar seasonal conditions together.

Univariate analysis of variance was performed for repeated measures using PROC MIXED in SAS, which performs an F-test and α was set at 0.05. The model used was: $Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + (\alpha\gamma)_{ik} + (\alpha\beta)_{ij}$ where Y = plasma/blood variables, milk components, BCS change, services per conception, days open, or liver fat percentage; α = calcium status at calving; i = 1,2; ß = block; j = 1,…, 67; γ = day relative to calving; k = -14, 0, 3, 7, 14, 21, 35; αγ = interaction between calcium status and day relative to calving; and αß = interaction between calcium status and block (error term). For variables that did meet the assumption of equality of variance, but failed to meet the normal distribution assumption (NEFA, total bilirubin, GGT, and percent lipid infiltration in hepatocytes), a rank transformation was performed based on the work of Conover and Iman (1981) and then analyses were performed as detailed above.

To further analyze ionized calcium on the day of calving and liver lipid infiltration, the mean was calculated and cows above or below one standard deviation from the mean were compared by analysis of variance in the same manner as described above. This should eliminate cows with similar values directly adjacent to the mean.

This method incorporated 6 hypocalcemic cows compared to 9 normocalcemic cows, and also 7 high lipid cows compared to 9 low lipid cows.

By using PROC CORR in SAS, significant correlations between any variables were found and the variables with the highest r-value were chosen for further analysis with stepwise regression (Table 2.1). Stepwise regression was performed using PROC REG in SAS to compare NEFA to percent lipid in hepatocytes and indirect bilirubin and also to compare ionized calcium concentration and total plasma calcium concentration on day 0 to NEFA, liver lipid infiltration, and indirect bilirubin. The maximum model used was $Y = ß_0 + ß_1x_1 + ß_2x_2 + ß_3x_3 + \ldots + ß_7x_7$. In this model Y = percent lipid in hepatocytes on day 0, 7, or 35 or indirect bilirubin on day -14, 0, 3, 7, 14, 21, or 35, x_1 = NEFA day -14, x_2 = NEFA day 0, x_3 = NEFA day 3, x_4 = NEFA day 7, x_5 = NEFA day 14, x_6 = NEFA day 21, x_7 = NEFA day 35. When comparing ionized and total plasma calcium to the previously described variables, the maximum model was $Y = ß_0 + ß_1x_1 + ß_2x_2 + ß_3x_3 + \ldots + ß_{11}x_{11}$. In this model, Y = ionized or total plasma calcium concentration on day 0, x_1 = NEFA day -14, x_2 = NEFA day 0, x_3 = NEFA day 3, x_4 = NEFA day 7, x_5 = NEFA day 14, x_6 = NEFA day 21, x_7 = NEFA day 35, x_8 = liver lipid day 0, x_9 = liver lipid day 7, x_{10} = liver lipid day 35, and x_{11} = indirect bilirubin day 0. Inclusion criteria for each model were set at P-value 0.10 for entry into the model, and P-value of 0.05 to stay in the model.

Binomial parameters such as postpartum diseases and cyclicity were analyzed using PROC GLIMMIX in SAS. The model used was $Y_{ijk} = \mu + \alpha_i + ß_j + (\alpha ß)_{ij}$ where Y = ketosis, retained placenta, metritis, mastitis, displaced abomasum, or cyclicity; α =

calcium status at calving; i = 1,2; ß = block; j = 1,…, 67; and αß = interaction between calcium status and block (error term).

Results

During the first three months of the study, dry cows were inadvertently fed a diet containing a vitamin and mineral premix balanced for lactating cows, with a higher calcium concentration than the dry cow vitamin and mineral premix (Table 2.2). Any variation caused by this mistake was accounted for because cows were blocked by calving date, putting those cows fed the same diet together in a block. A total of 67 blocks were used for data analysis, 33 of which were complete with two cows per block, and 34 of which were incomplete with one cow per block.

The actual time from first sampling (-14) to calving was 15.7 ± 3.8 days for hypocalcemic cows and 15.2 ± 3.1 days for normocalcemic cows (P = 0.24). . At 14 days from expected calving date there were no differences between groups for any plasma biochemical parameters (Table 2.3).

As expected, on the day of calving, whole blood ionized calcium concentrations were lower for hypocalcemic cows (mean parity ± SD; 3.5 ± 1.5) than normocalcemic cows (mean parity 2.8 ± 1.4) (Figure 2.1). Similarly, plasma total calcium concentrations were lower for hypocalcemic cows than normocalcemic cows (Figure 2.2).

Hypocalcemic cows also had significantly lower plasma phosphorus concentrations on days 0, 7, 14, and 21 compared to normocalcemic cows (Figure 2.3).

Hypocalcemic cows had significantly higher mean NEFA concentrations on days 0 and 21 compared to normocalcemic cows (Figure 2.4).

More normocalcemic cows had liver biopsies sampled by chance because as cows were calving farther apart, it was more difficult to obtain biopsies from cows in different groups that would fit in the same block. Additionally, three hypocalcemic cows initially had liver biopsies taken but were excluded from this portion of the study. One was diagnosed with clinical milk fever, so treatment with intravenous 23% calcium gluconate altered the cow's natural calcium balance. The other two animals did not have liver tissue in the tissue sample collected based on histologic examination.

Hypocalcemic cows had more lipid in the hepatocytes on day 35 than normocalcemic cows (Figure 2.5).

There were no detectable differences in total bilirubin (Figure 2.6), direct bilirubin, indirect bilirubin (Figure 2.7), GGT (Figure 2.8), or AST (Figure 2.9) between hypocalcemic and normocalcemic cows.

For 15 of the 100 cows enrolled in the study, no milk protein, fat, or solids-not-fat were analyzed from the laboratory.

Milk protein concentration was lower on days 21 and 35 for hypocalcemic cows compared to normocalcemic cows (Figure 2.10). There were no differences in milk fat percentage, linear somatic cell score (Figure 2.11), somatic cell count, peak test day milk yield or 305 day ME 4% fat-corrected milk yield (Figure 2.12).

The occurrence of ketosis, displaced abomasum, retained fetal membranes, metritis and mastitis were compared between groups, with no significant differences being found for any of the studied diseases (Table 2.4).

Reproductive parameters studied included cyclicity at 50-60 days postpartum, services per conception, and days open. There were no differences in the

proportions of cows cycling by 50-60 days postpartum, services per conception, or days open between hypocalcemic and normocalcemic cows (Table 2.5).

Timed artificial insemination is performed at the farm the current study was conducted on, so days to first service was not different between groups as hypocalcemic cows had 72 ± 8.4 days to first service and normocalcemic cows had 71.3 ± 9.4 days. This is important to note because this means cows were at the same point in lactation during the first breeding body condition scoring.

Simple linear regression revealed that percent lipid in the hepatocytes and blood glucose concentration were significantly negatively correlated ($P = 0.005$; $R^2 = 0.05$).

There was a significant positive correlation between liver lipid infiltration and AST activity ($P<0.0001$; $R^2 = 0.17$) and between NEFA and AST ($P < 0.0001$; $R^2 = 0.07$).

GGT activity was also significantly correlated with NEFA ($P = 0.02$; $R^2 = 0.008$), however, this is a very weak association based on the R^2 value.

Stepwise regression was performed to compare blood ionized calcium concentration on the day of calving to NEFA, liver lipid content, and plasma indirect bilirubin concentration (Table 2.6). This revealed that the only variables significantly associated with day 0 ionized calcium concentration were NEFA concentration 14 days prepartum ($P = 0.03$) and on the day of calving ($P = 0.02$). However these variables only accounted for 5% and 6% respectively of the total variation in blood ionized calcium concentration on the day of calving. None of these variables were significantly associated with plasma total calcium concentration (Table 2.7).

Stepwise regression was performed to compare NEFA concentrations for the days measured to the total histological lipid content. On the day of calving, hepatocyte lipid

infiltration was significantly associated with NEFA concentrations on days -14 ($P = 0.007$), 7 ($P = 0.03$), and 21 ($P = 0.0008$), and the overall R-squared value of the model fitting all three variables was 0.48. On day 7 postpartum, hepatocyte lipid infiltration was significantly associated with NEFA concentration on day 3 ($P = 0.002$; $R^2 = 0.24$). Hepatocyte lipid infiltration on day 35 postpartum was significantly associated with NEFA concentration on day 14 prepartum ($P < 0.0001$; $R^2 = 0.54$) (Table 2.8).

It was also found with stepwise regression that NEFA for 14 days prepartum ($P = 0.03$) and the day of calving ($P < 0.0001$) were significantly related to the unconjugated bilirubin concentration on the day of calving, and the overall R-squared for the model was 0.67 (Table 2.9).

Cows were also grouped into two groups, one above and one below one standard deviation from mean iCa on day 0. Hypocalcemic cows had significantly lower iCa on day 0 compared to normocalcemic cows ($P < 0.0001$) (Figure 2.13). The hypocalcemic cows had significantly higher BCS on day 0 (3.08 ± 0.12) than normocalcemic cows (2.54 ± 0.12; $P = 0.05$) however, there were no differences in body condition changes. Mean NEFA concentrations did not significantly differ between groups (Figure 2.14). Despite this, hypocalcemic cows had significantly more liver lipid infiltration on days 0, 7, and 35 compared to normocalcemic cows on days 0 ($P = 0.01$), 7 ($P = 0.02$), and 35 ($P = 0.04$) (Figure 2.15).

Data from liver biopsied cows were separated into two groups, one greater than and one less than one standard deviation from the mean of all liver biopsies on day 7 postpartum in order to further characterize the clinical significance of extensive hepatic lipid infiltration. There were no differences in ionized calcium concentration between

groups (Figure 2.16). Cows with greater liver lipid lost more body condition in the first two weeks postpartum (-0.65 ± 0.05 BCS vs. -0.27 ± 0.04 BCS; $P = 0.01$) and also from calving until first breeding (-0.96 ± 0.07 BCS vs. -0.29 ± 0.04 BCS; $P = 0.02$) than cows with lower liver lipid content (Table 2.10). While it may be difficult to discern a difference between a loss of 0.65 and 0.27 body condition scores in the first two weeks postpartum, a difference between approximately one body condition score loss and approximately one-fourth of a body condition score loss is more appreciable. This was somewhat reflected in NEFA values between groups as high liver lipid cows had significantly greater NEFA on day 7 (1245.01 ± 184.08 uEq/L vs. 617 ± 134.74 uEq/L; $P = 0.04$) compared to cows with lower liver lipid content (Figure 2.17). The elevated NEFA concentrations of high liver lipid cows contributed to significantly higher liver lipid infiltration on day 7 and 35 compared to lower liver lipid cows on days 7 ($P = 0.003$) and 35 ($P = 0.02$), as would be expected (Figure 2.18). This greater deposition of lipid in the liver may have resulted in some pathologic insult because blood AST activity was significantly higher on days 7 and 14 in high lipid compared to low liver lipid cows (Figure 2.19). High liver lipid content cows also had a 100% occurrence (7/7) of ketosis compared to lower liver lipid content cows which had 33% occurrence (3/9; $P = 0.002$). There were also 6/8 low lipid cows cycling and 2/6 high lipid cows cycling (P=0.11) at 50-60 days postpartum. Even though there was possibly a minor degree of hepatic insult and there was greater ketosis occurrence, there were no differences in milk production between groups.

Discussion

Dairy cows experience subclinical hypocalcemia when whole blood total calcium is less than 2.0 mmol/L or 8.0 mg/dL (Goff, 2008). Since approximately half of total

calcium is ionized (Ballantine and Herbein, 1991), 1.0 mmol/L was chosen as the cut point to differentiate subclinical hypocalcemia from normocalcemia. There are possible disadvantages to differentiating hypocalcemic cows from normocalcemic cows. Biologically speaking, cows just above and just below the cut point may be no different from each other. For this reason, regression analysis was also done with the data.

In our study 51% of cows experienced subclinical hypocalcemia, which is supportive of the observations made by Horst et al. (2003), who stated that on average, approximately 50% of mature dairy cows will experience subclinical hypocalcemia at calving. In the current study, hypocalcemic cows recovered to the same blood ionized calcium level as normocalcemic cows by day 3 postpartum, which is described in the literature (Goff and Horst, 1997a; Moore et al., 2000; Goff et al., 2002).

For the first three months of the study, the diet was incorrectly balanced, which may have influenced the results, as there were more hypocalcemic than normocalcemic cows during that time period. Nonetheless, blocking the cows should have minimized any influence this mistake would have had on the results.

There were 34 incomplete blocks consisting of one cow per block because cows either did not match up due to parity or calving dates too far apart. Of the 34 cows, 18 were hypocalcemic and 16 were normocalcemic, so this should not have introduced a great deal of bias based on the number of hypocalcemic versus normocalcemic cows.

At baseline (14 days from expected calving) cows were metabolically similar based on the plasma biochemical profile results not being significantly different between groups. Because no measurements were made between baseline and calving it was not possible to determine whether significant differences occurred between these baseline

measurements and calving. Measurements within a week of parturition would have been valuable because they would have allowed for observation of any possible differences between groups.

The lower phosphorus on the day of calving could reflect an increase in parathyroid hormone because hypocalcemic cows were in greater need of increasing blood calcium than normocalcemic cows. Parathyroid hormone will result in phosphorus release from bone along with calcium, however salivary and renal excretion of phosphorus results in a net loss of phosphorus (Goff, 2000). This does not explain the differences that occurred at one, two, and three weeks postpartum, however, because blood calcium was no different between groups by that point in time. Nevertheless, none of these values were out of the normal reference interval for periparturient dairy cows: 3.22 – 8.45 mg/dL (Quiroz-Rocha et al., 2009).

Hypocalcemic cows had a maximum NEFA concentration on the day of calving, and normocalcemic cows at three days postpartum, which was consistent with the findings of Grummer (1993) and Ingvartsen and Andersen (2000). The previous studies stated that plasma non-esterified fatty acid concentration typically begins to increase several weeks prepartum, with a rapid increase to a peak concentration around the time of calving and then a gradual decline as feed intake increases. NEFA levels have been used as an estimate of energy balance (Kunz et al., 1985). Based on the data presented herein, hypocalcemic cows appeared to experience a more severe negative energy balance on the day of calving and at 21 days postpartum. The association between subclinical hypocalcemia at calving and NEFA found in the present study is similar to that found in previous reports (Horst et al., 2003; Spain et al., 2004). This relationship may be explained by findings in humans,

where adipocytes stimulated with calcium-sensing receptor agonists had decreased basal lipolysis (Cifuentes and Rojas, 2008) suggesting that lipolysis may increase with calcium depletion. Additionally, calcium is an important second messenger for energy metabolism in hepatocytes. It has been shown that increases in cytosolic calcium concentration lead to parallel increases in the concentration of calcium in mitochondria, and that this is important for the stimulation of mitochondrial oxidative metabolism (Hajnoczky et al., 1995). Calcium flux into the mitochondria phosphorylates pyruvate dehydrogenase to its active form (Denton et al., 1972), which converts pyruvate to Acetyl CoA to enter the TCA cycle. This also causes an increase in NADH that is responsible for donating electrons during oxidative phosphorylation (Robb-Gaspers et al., 1998). A schematic representation for this process can be seen in figure 2.20 (Peters and LeBlanc, 2004). Theoretically, hypocalcemia could influence hepatocellular calcium concentration. If this is true, pyruvate dehydrogenase may not be activated as efficiently with insufficient intracellular calcium. As a result, carbohydrate metabolism via the TCA cycle would be decreased, resulting in a more severe negative energy balance than a cow would see without hypocalcemia.

Aslan et al. (1988) related the amount of liver lipid infiltration to the concurrent severity of fatty liver disease as lipid infiltration of 0 – 10% being mild, 10 – 20% moderate and >20% severe. This was based on clinical signs such as anorexia, decreased milk production, and progressive debilitation, as well as hematologic signs such as decreased albumin and cholesterol (Sevinc et al., 2003). Based on these values, both groups were experiencing mild to moderate fatty liver disease on the day of calving, which is consistent with the work of Djokovic et al. (2007) who noted that in apparently healthy cows on the

day of calving, the liver fat content is approximately 8%. On day 7 postpartum, the hypocalcemic cows appear to have severe disease, and the normocalcemic cows moderate to severe. By 35 days postpartum, the hypocalcemic cows were still in the moderate to severe range, but the normocalcemic cows were in the mild range. The difference at 35 days postpartum may indicate that hypocalcemic cows truly had greater peak lipid infiltration in the hepatocytes, which commonly occurs between two and three weeks postpartum (Van den Top et al., 1996; Rukkwamsuk et al., 1999). If this was true, hypocalcemic cows would have had more lipid to clear from the liver. Alternatively, this could mean that normocalcemic cows cleared the lipid faster than hypocalcemic cows, and did not necessarily have more lipid infiltration earlier in lactation.

The difference in milk protein concentration on day 21 postpartum could possibly be a function of increased milk yield by hypocalcemic cows and a dilution of the protein. Alternatively, if the normocalcemic cows had increased their dry matter intake more than hypocalcemic cows, they could have been incorporating more protein into milk at that point in time.

The fact that there was no difference in milk yield between groups could indicate that the greater NEFA mobilization and greater hepatic lipid infiltration were not significant enough to affect production. Alternatively, this may indicate that genetic potential of the hypocalcemic cows was not being reached. Hypocalcemic cows may be attempting to produce more milk than normocalcemic cows, and as a result, have lower blood calcium and higher NEFA. Curtis et al., (1984) showed that cows with higher production potential were at greater risk of developing milk fever. As seen in an experiment by McNamara and Hillers (1986), higher genetic merit cows mobilize more fat

in early lactation, as did our hypocalcemic cows. However, our production measure, 305 day ME 4% FCM is likely not sensitive enough to detect differences in production during early lactation. Ostergaard and Larsen (2000) also noted that blood calcium at calving did not affect fat-corrected milk yield.

The postpartum diseases of interest in this study were chosen because they have commonly been associated with hypocalcemia in the literature (Curtis et al., 1983; Goff and Horst 1997b; Mulligan et al., 2006; Goff, 2008). Hypocalcemia may result in reduced smooth muscle function, which may predispose to displaced abomasum due to abomasal atony and gas accumulation (Madison and Troutt, 1988; Doll et al., 2009). Massey et al. (1993) reported that subclinical hypocalcemia was a significant risk factor for development of displaced abomasum. More recently, Seifi et al. (2011) showed that cows with total calcium $\leq$ 2.3 mmol/L at any time within the first week postpartum were five times more likely to have a displaced abomasum than cows above this threshold. However, our results indicate that subclinical hypocalcemia does not appear to increase the occurrence of displaced abomasum, granted the current study had relatively few cows compared to a major retrospective study such as Geishauser et al. (1997) and LeBlanc et al. (2005). Similarly, Geishauser et al. (1997) and LeBlanc et al. (2005) found that blood calcium levels were not significantly associated with risk of developing displaced abomasum in 256 cows and 1044 cows, respectively.

Hypocalcemia has also been shown to cause immunosuppression by depleting intracellular calcium stores in peripheral blood mononuclear cells (Kimura et al., 2006). It may also decrease smooth muscle contractions, thereby affecting the teat sphincter and uterus, possibly increasing the risk of mastitis, metritis, and retained placenta (Goff and

Horst, 1997b). However, our results indicate that there is no detectable difference in the occurrence of any of these diseases due to subclinical hypocalcemia. This is in contrast to Curtis et al. (1983) who stated that cows with clinical milk fever were three times more likely to develop retained placentas and eight times more likely to develop mastitis, however it is important to note that this was milk fever and not subclinical hypocalcemia. During the current study, retained placenta was defined as a placenta that was not expelled within 12 hours after parturition. Although authors define retained placenta as either retention for greater than 12 or 24 hours (Kay, 1978; Lin et al., 1989; Kelton et al., 1998), 95% of cows expelling the placenta within 24 hours had already expelled it at 12 hours (Van Werven et al., 1992). The definition of mastitis in this study does have its weaknesses as the farm employees may not catch every case of clinical mastitis, or alternatively may fail to record the case even if treated. However, it was not feasible to have a person present at every milking for the sole purpose of mastitis detection, so this was the most solid definition usable in this situation. Regardless of the accuracy of mastitis detection, the two groups were subjected to the same error and the milkers did not know which cows were in which group. Therefore, this should have not had any significant impact on interpreting the results.

Roche (2006) stated that clinical hypocalcemia is a significant risk factor for decreased conception rates. Maizon et al. (2004) found that fertility was decreased in cows with dystocia, retained placenta, and metritis, all of which are possibly related to hypocalcemia. Since the major risk factors listed were some of the diseases considered in this study, it seems logical that differences in reproductive performance may not have occurred because there were no differences in disease occurrence between groups.

Djokovic et al. (2007) stated that the correlation coefficient between liver fat percentage and blood glucose concentration was -0.69, indicating a moderate negative association. This implied that more severe lipid infiltration would possibly impair the liver's gluconeogenic capacity, as was suggested by Rukkwamsuk et al. (1999b). The association between liver lipid content and blood glucose in the current study was virtually nonexistent compared to observations made by Djokovic et al. (2007).

As the amount of lipid deposition increases in the liver, more hepatocelluar damage can occur (Van den Top et al., 1996), which may explain the association between liver lipid and AST in the current study. Since increased NEFA results in increased liver lipid deposition, AST can be indirectly increased by elevated NEFA levels. However, AST is also found in other cells including cardiac and skeletal muscle, so the health of these cells may explain another significant amount of variation in AST activity as NEFA and liver lipid only accounted for little variation in the current study, 7% and 17%, respectively.

Increased NEFA may also indirectly cause increased GGT activity because as NEFA increases, hepatocellular lipid deposition increases, which may lead to an obstructive cholestasis (Cebra et al., 1997).

The significant associations between NEFA and liver lipid content observed in the present study can be explained by the fact that as NEFA concentrations increase, the ability of the liver to ß-oxidize and export triglycerides may become overwhelmed and triglycerides may be stored directly in hepatocytes (Hayirli, 2006). However, it was also noted in the present study that the lipid infiltration on the day of calving was associated with NEFA on day 21 postpartum. This relationship may exist because if there is greater lipid infiltration at day 0, the liver's function may be compromised to a point that worsens

the negative energy balance, requiring greater NEFA mobilization in an attempt to maintain milk production, which results in further decreases in liver function. On two of the three days liver biopsies were taken, there was a significant association with the prepartum NEFA concentration. This was similar to the trend noted by Bertics et al. (1992) who noted that prepartum NEFA concentrations were related to postpartum liver lipid infiltration.

Based on the results of stepwise regression in the current study, it can be seen that NEFA concentrations explain a significant amount of the variation in liver lipid content. Since hypocalcemic cows had greater NEFA concentrations than normocalcemic cows, this relationship may explain why hypocalcemic cows also had greater liver lipid infiltration on day 35 compared to normocalcemic cows. Alternatively, there may be a difference in liver lipid metabolism resulting in decreased lipid clearance by hypocalcemic cows or increased lipid clearance by normocalcemic cows.

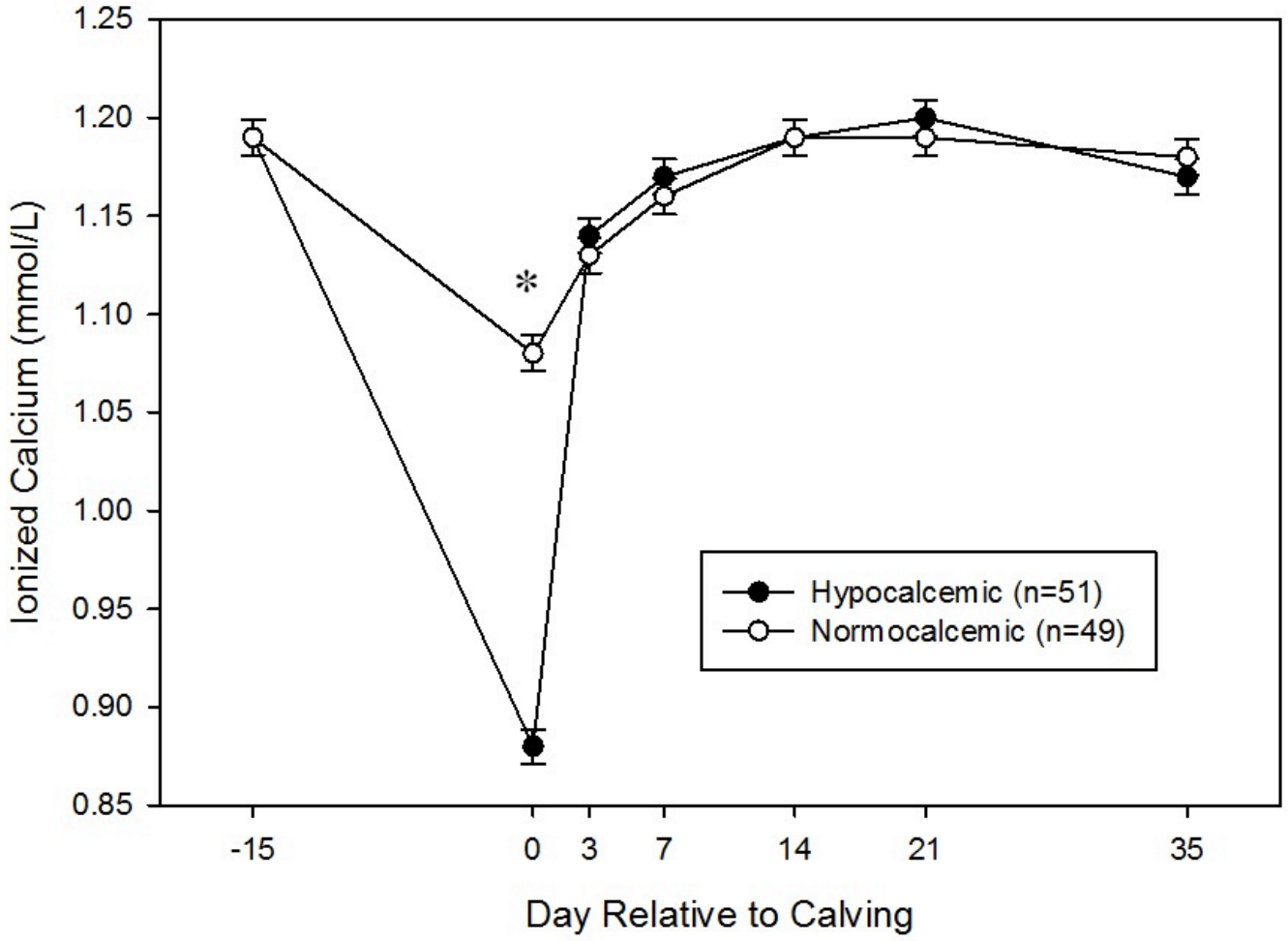
When lipolysis results in increased NEFA circulation in the blood, the NEFA compete with bilirubin for uptake into hepatocytes to be metabolized (Reid et al., 1977). This may explain the strong association noted between the two parameters via stepwise regression in the current study.

Since there is no major difference in plasma NEFA concentration between groups separated based on one standard deviation above and below mean ionized calcium concentration, but there was a difference in liver lipid content, there may be a difference in liver lipid metabolism depending on calcium status. However, since there was already more lipid in the hepatocytes on the day of calving, a difference in NEFA mobilization may

have occurred between baseline sampling and calving, leading to the difference in liver lipid deposition.

These data indicate that subclinical hypocalcemia may lead to increased NEFA concentrations on days 0 and 21 postpartum and increased lipid deposition in the liver on day 35 postpartum, but based on our measures, this results in no detectable differences in hepatocellular or biliary damage or function. Additionally, subclinical hypocalcemia does not appear to alter milk production at peak production or through an entire lactation, alter fertility, or result in any difference in disease occurrence for ketosis, displaced abomasum, mastitis, metritis, or retained placenta.

Figure 2.1 Whole blood ionized calcium concentration vs. day relative to calving by group (P = 0.0003;* P < 0.0001).



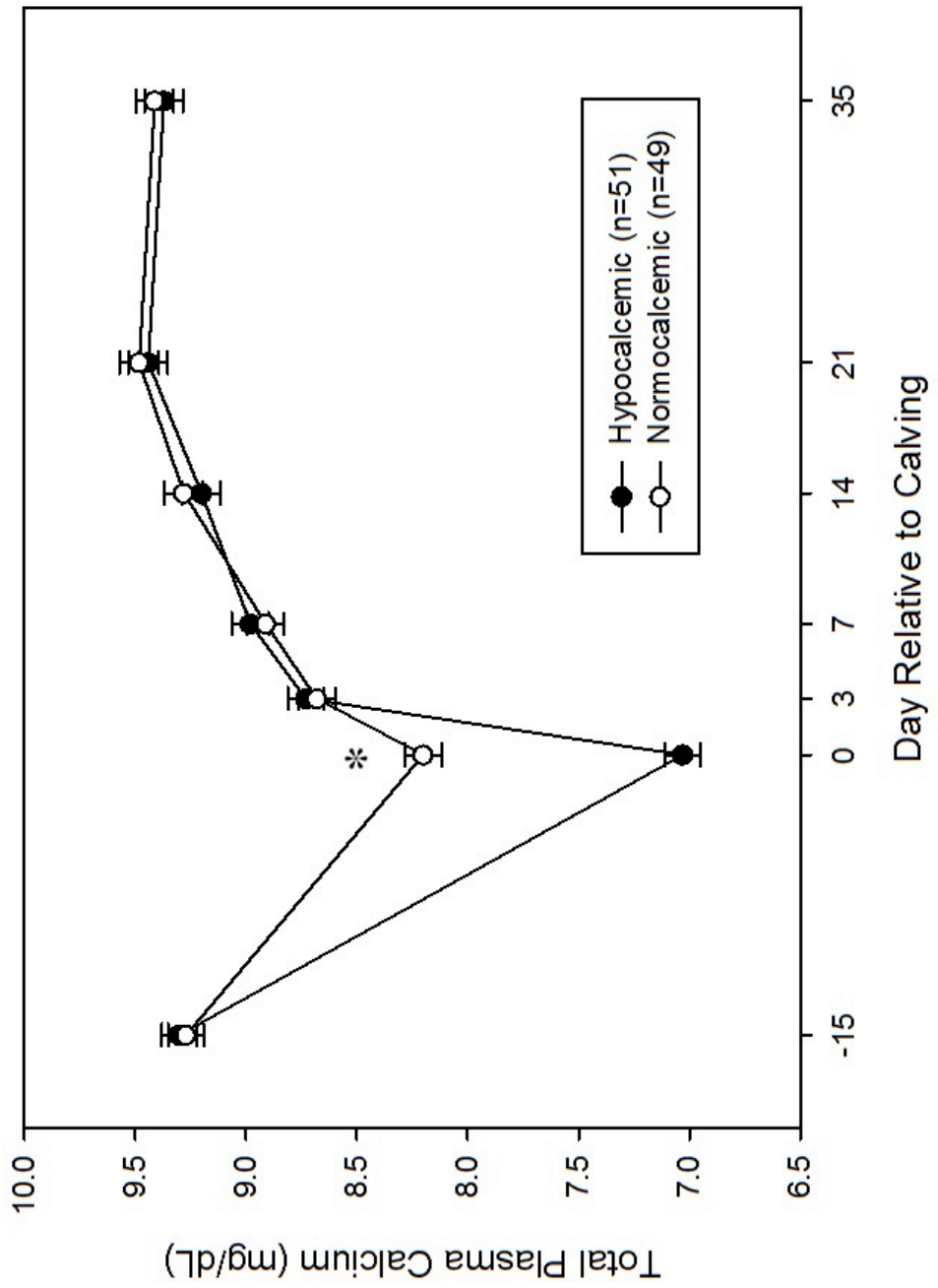


Figure 2.2 Plasma total calcium concentration vs. day relative to calving by group ($P = 0.05$; *$P < 0.0001$).

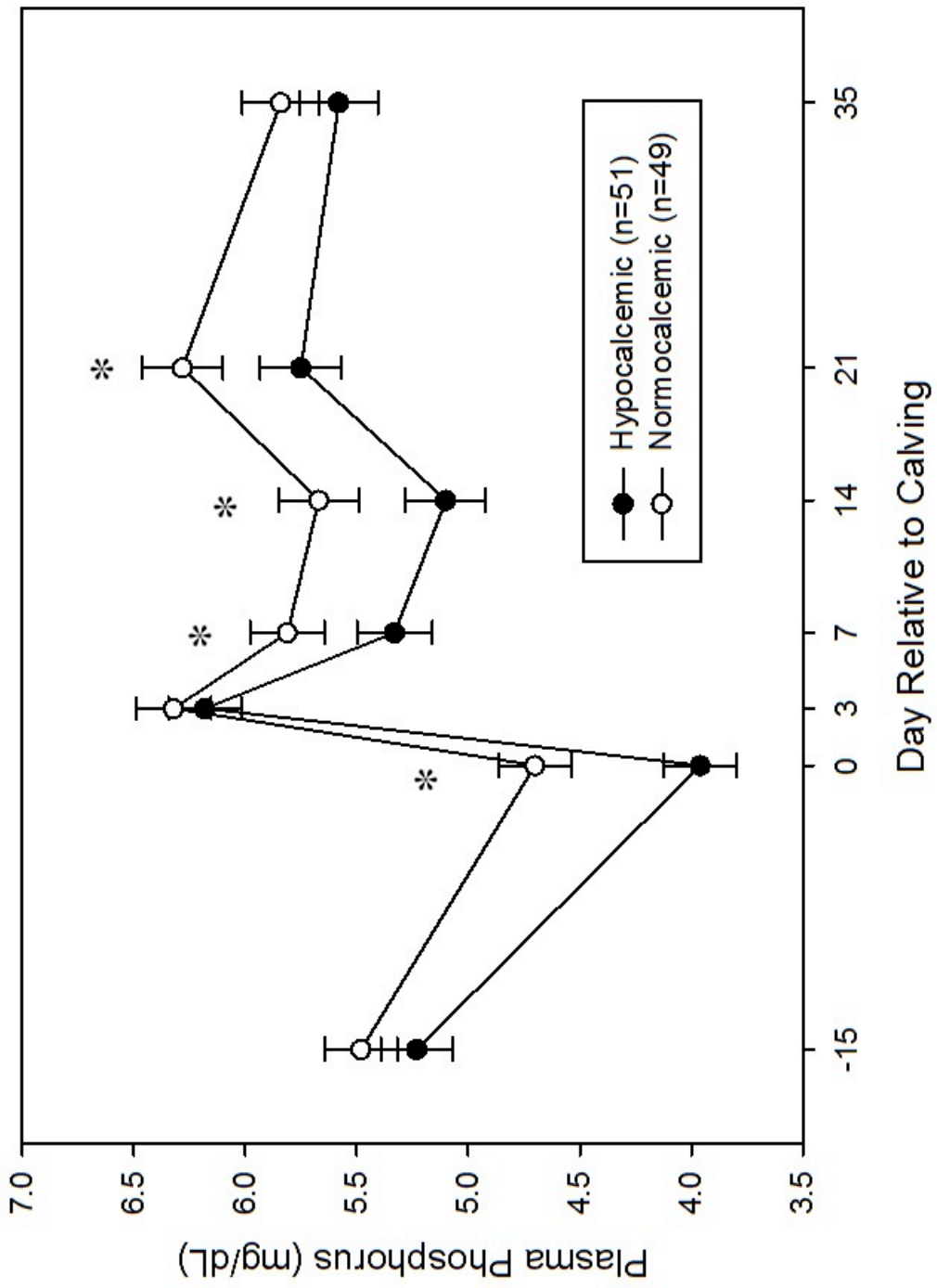


Figure 2.3 Plasma phosphorus concentration vs. day relative to calving by group ($P = 0.009$; * $P \leq 0.05$).

Figure 2.4 Plasma NEFA concentration vs. day relative to calving by group (P = 0.08; * P ≤ 0.02; $^{+}$ P = 0.12).

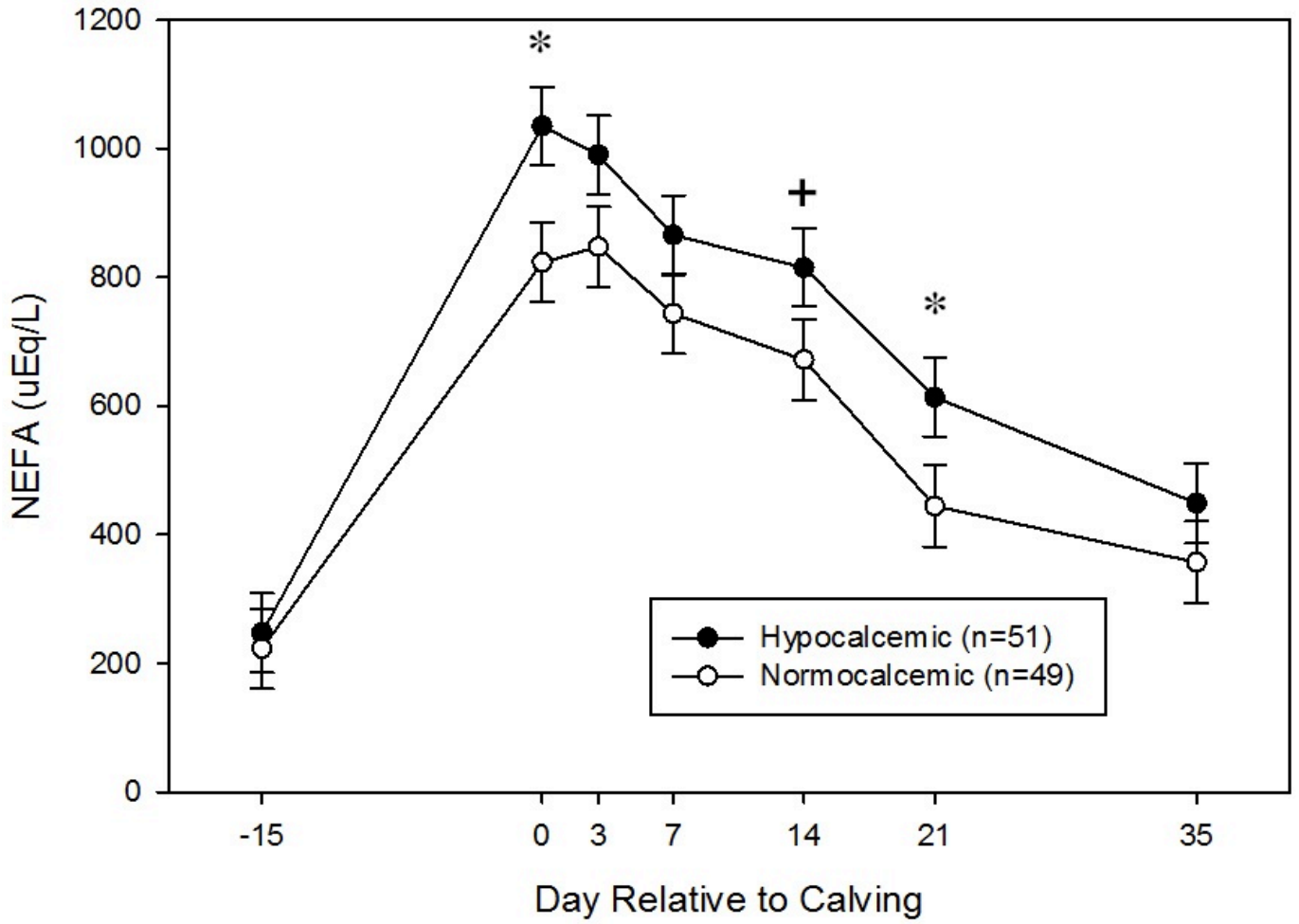
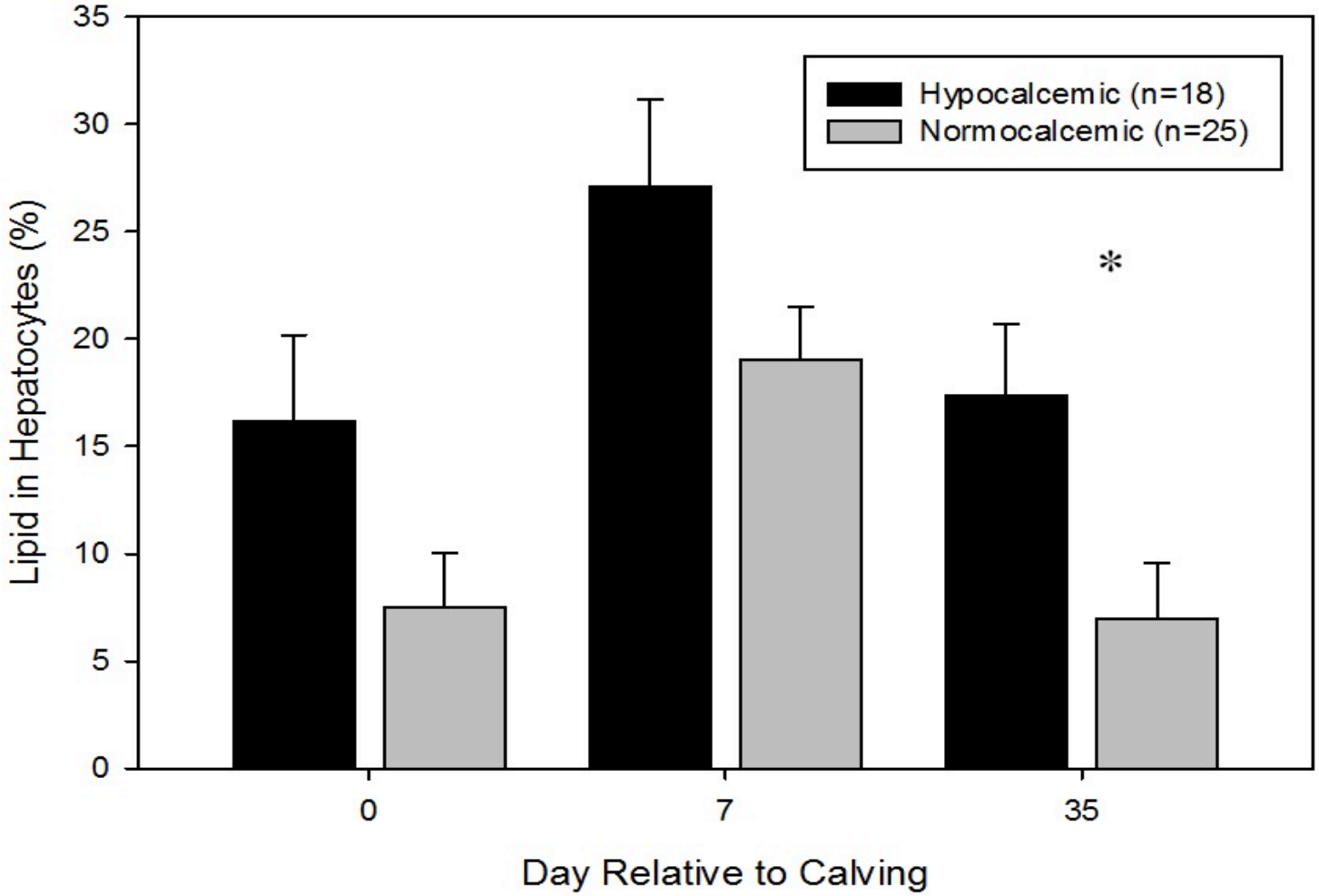


Figure 2.5 Total lipid concentration determined by histopathology vs. day relative to calving by group (P = 0.07; *P = 0.0008).



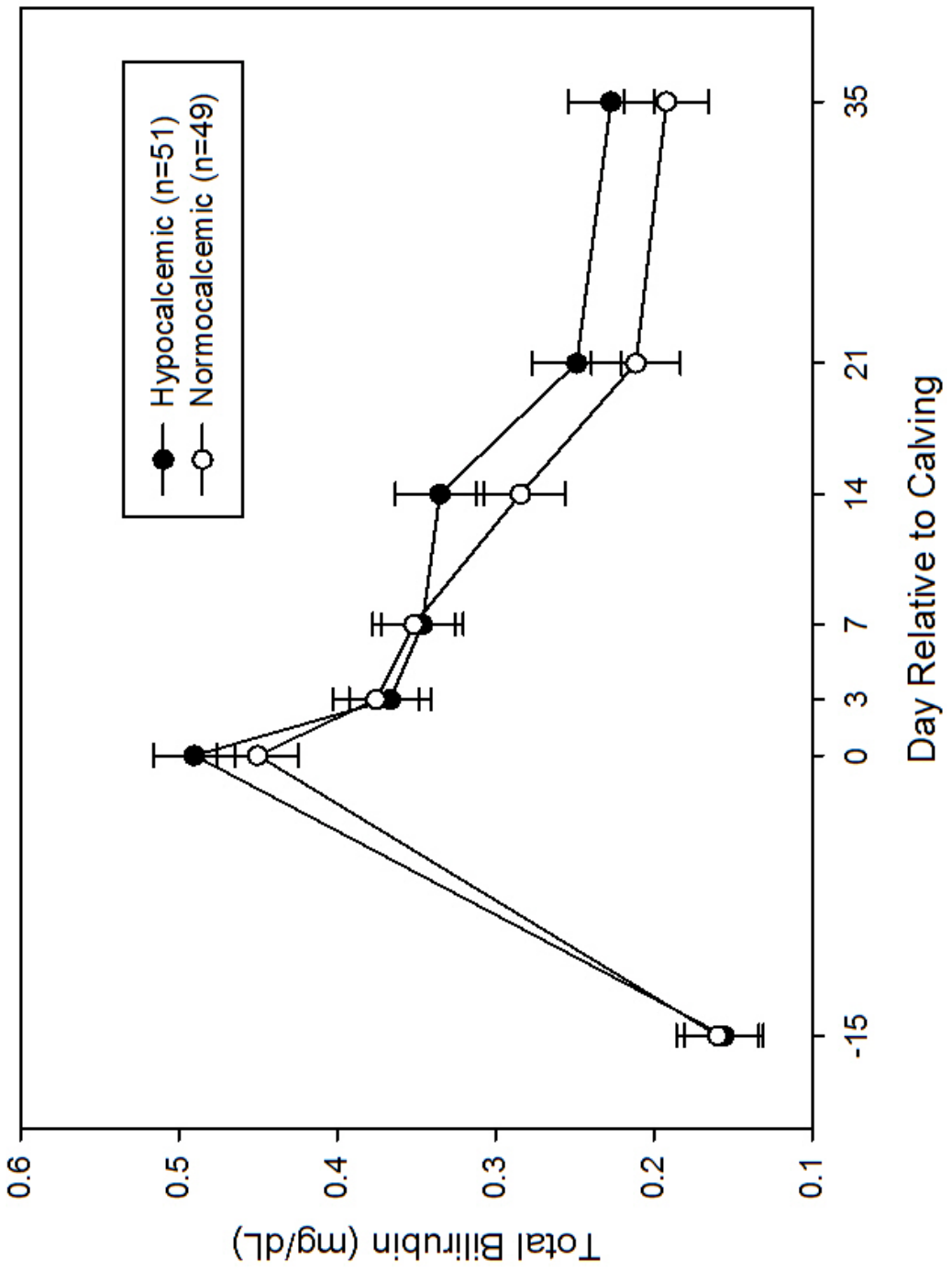


Figure 2.6 Plasma total bilirubin concentration vs. day relative to calving by group (P = 0.51).

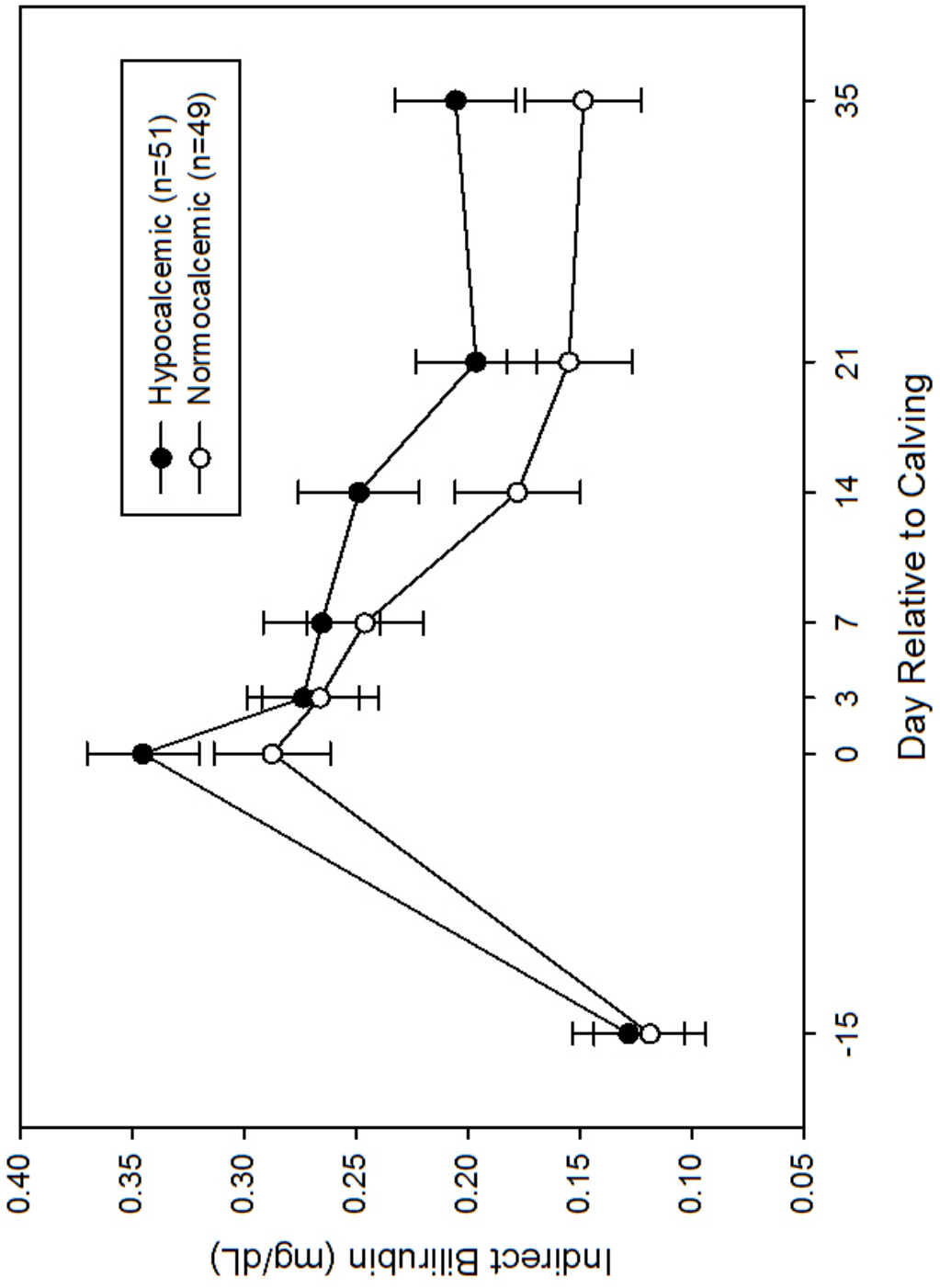


Figure 2.7 Plasma indirect bilirubin concentration vs. day relative to calving by group (P = 0.42).

Figure 2.8 Plasma gamma glutamyl transferase activity vs. day relative to calving by group (P = 0.95).

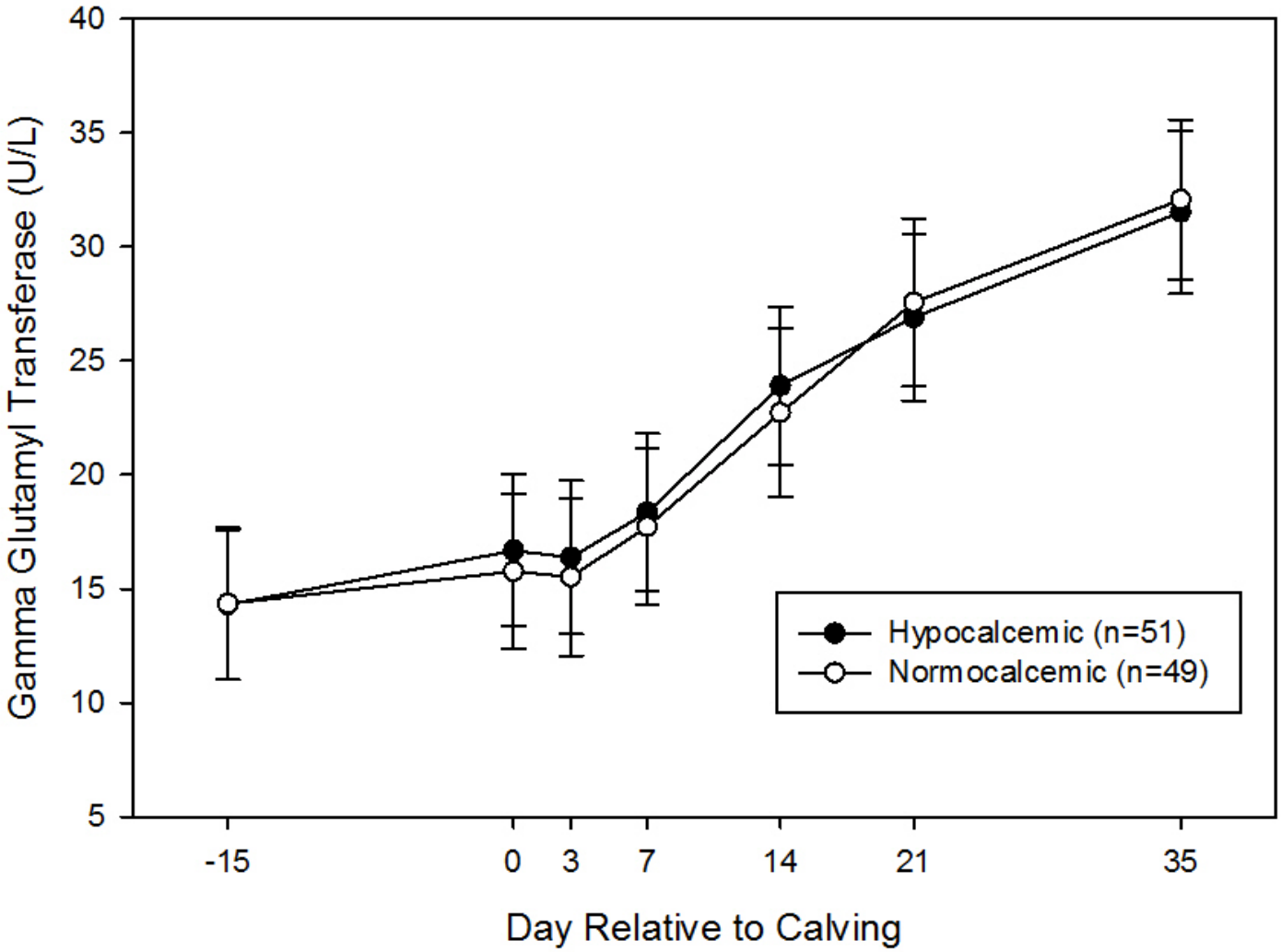
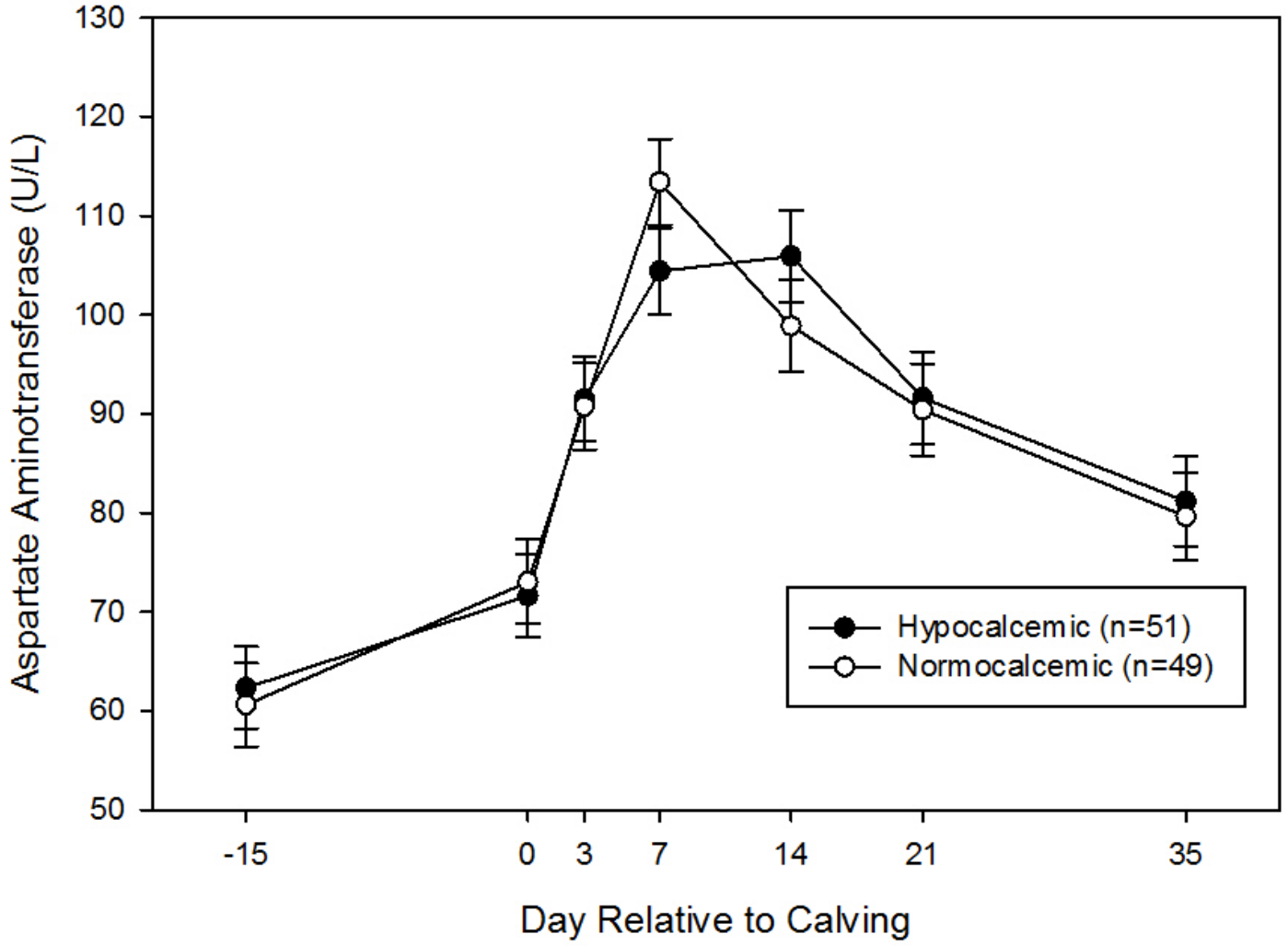


Figure 2.9 Plasma aspartate aminotransferase activity vs. day relative to calving by group (P = 0.95).



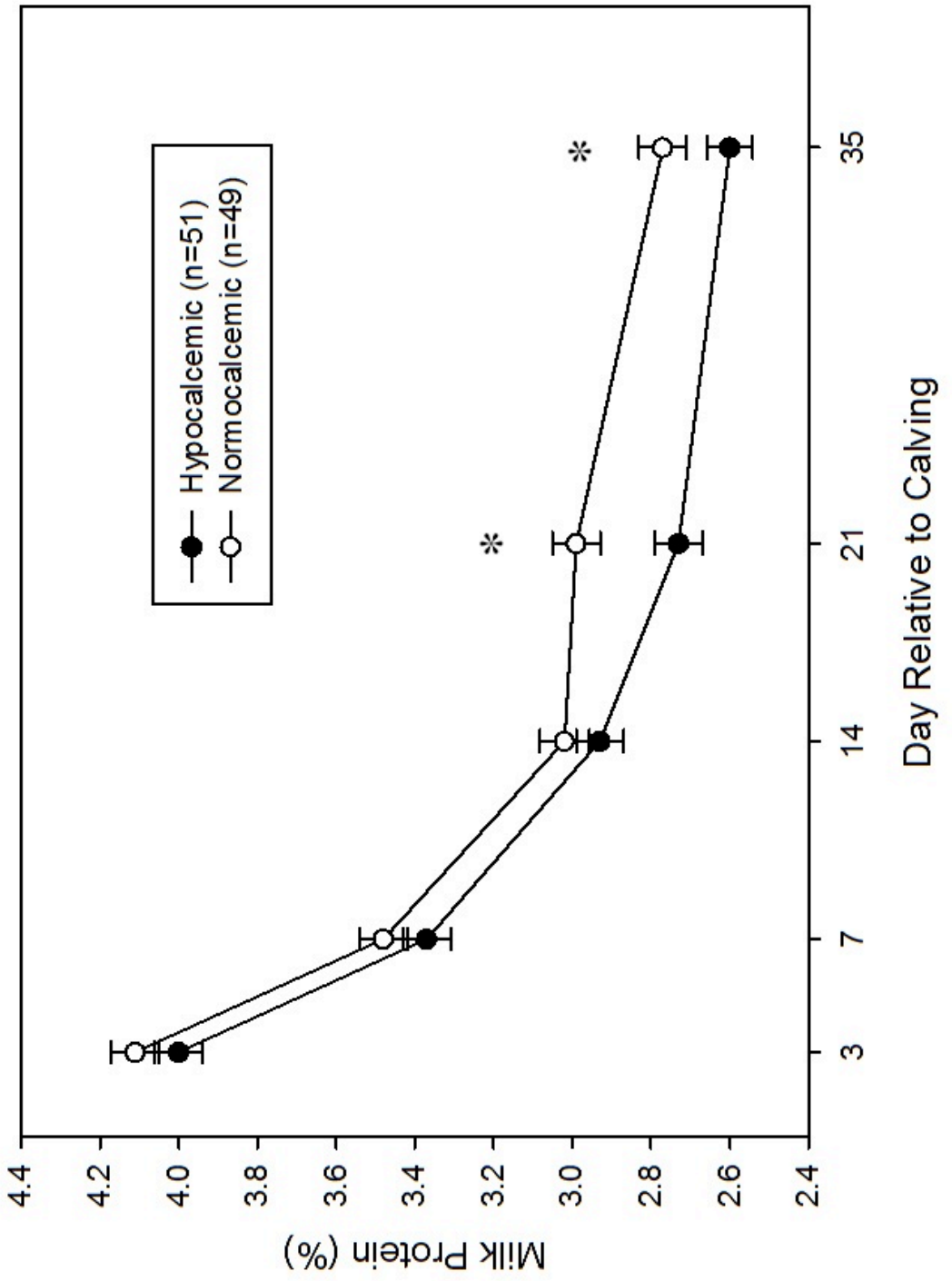


Figure 2.10 Milk protein concentration vs. day relative to calving by group ($P = 0.02$; *$P < 0.05$).

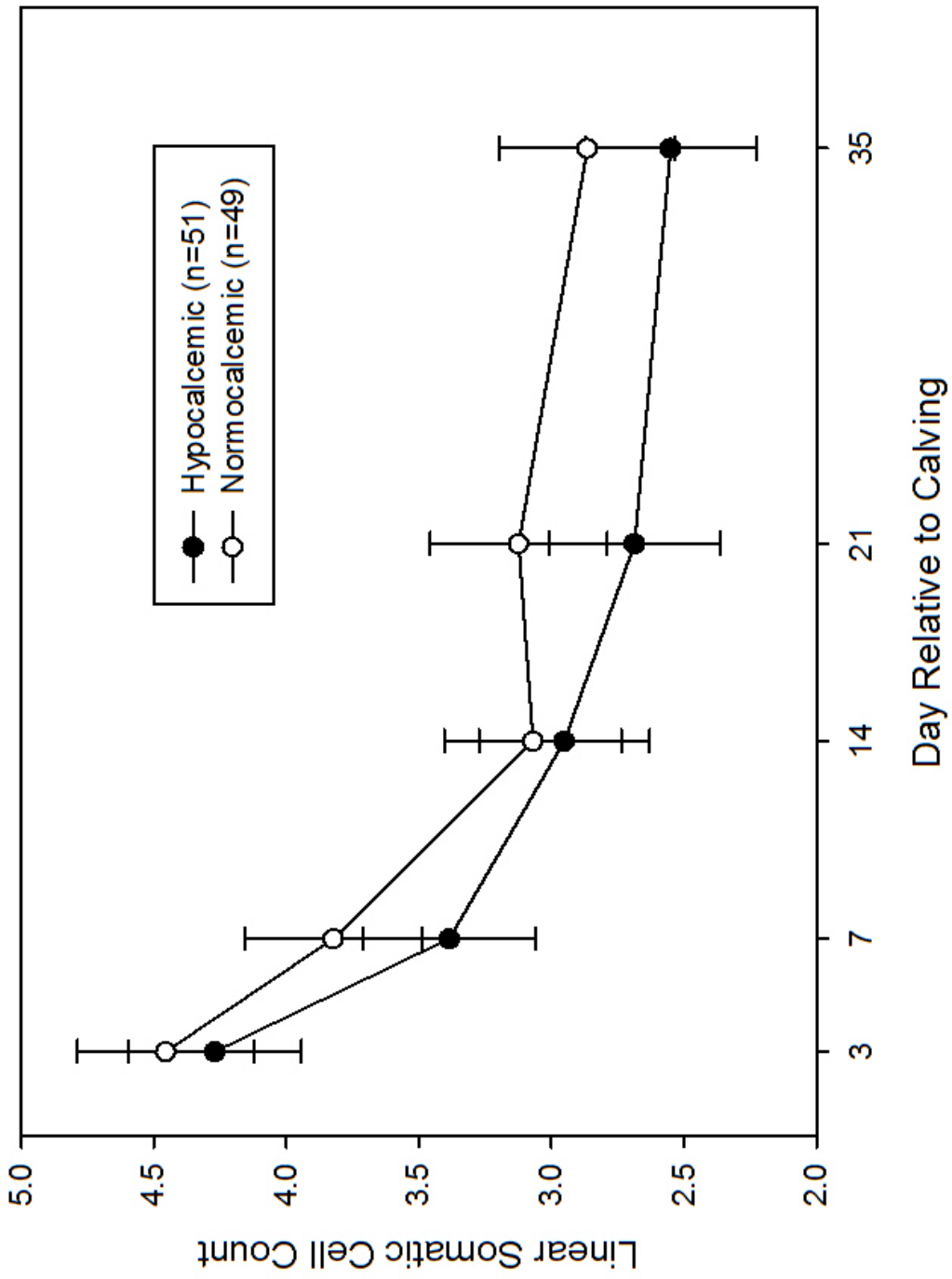


Figure 2.11 Milk linear somatic cell count vs. day relative to calving by group (P = 0.48).

Figure 2.12 Peak test day milk yield (P = 0.80) and 305 day ME 4% fat corrected milk yield (P = 0.85) by group.

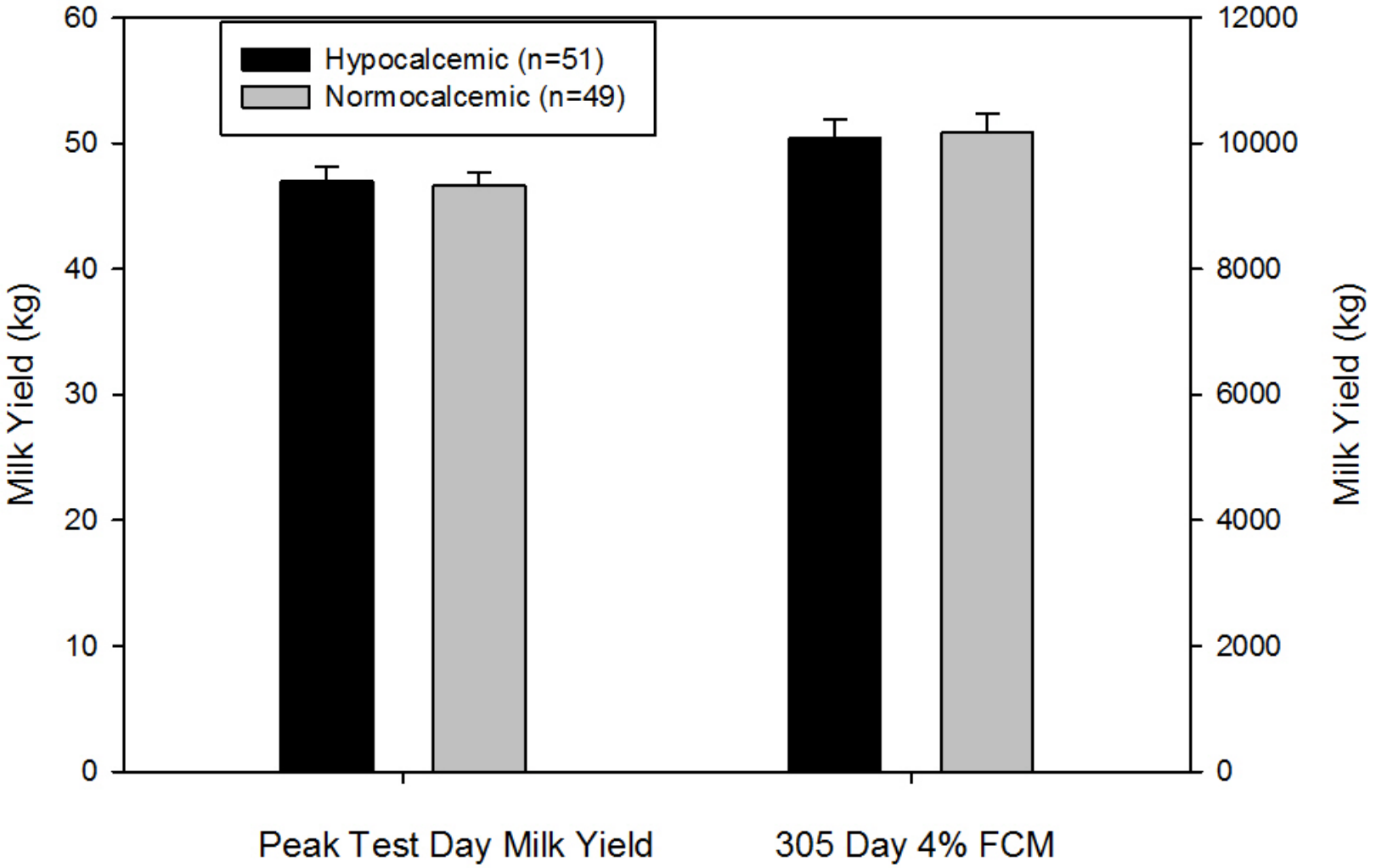


Figure 2.13 Mean ionized calcium concentration vs. time for cows outside one standard deviation from mean ionized calcium. (*$P < 0.0001$).

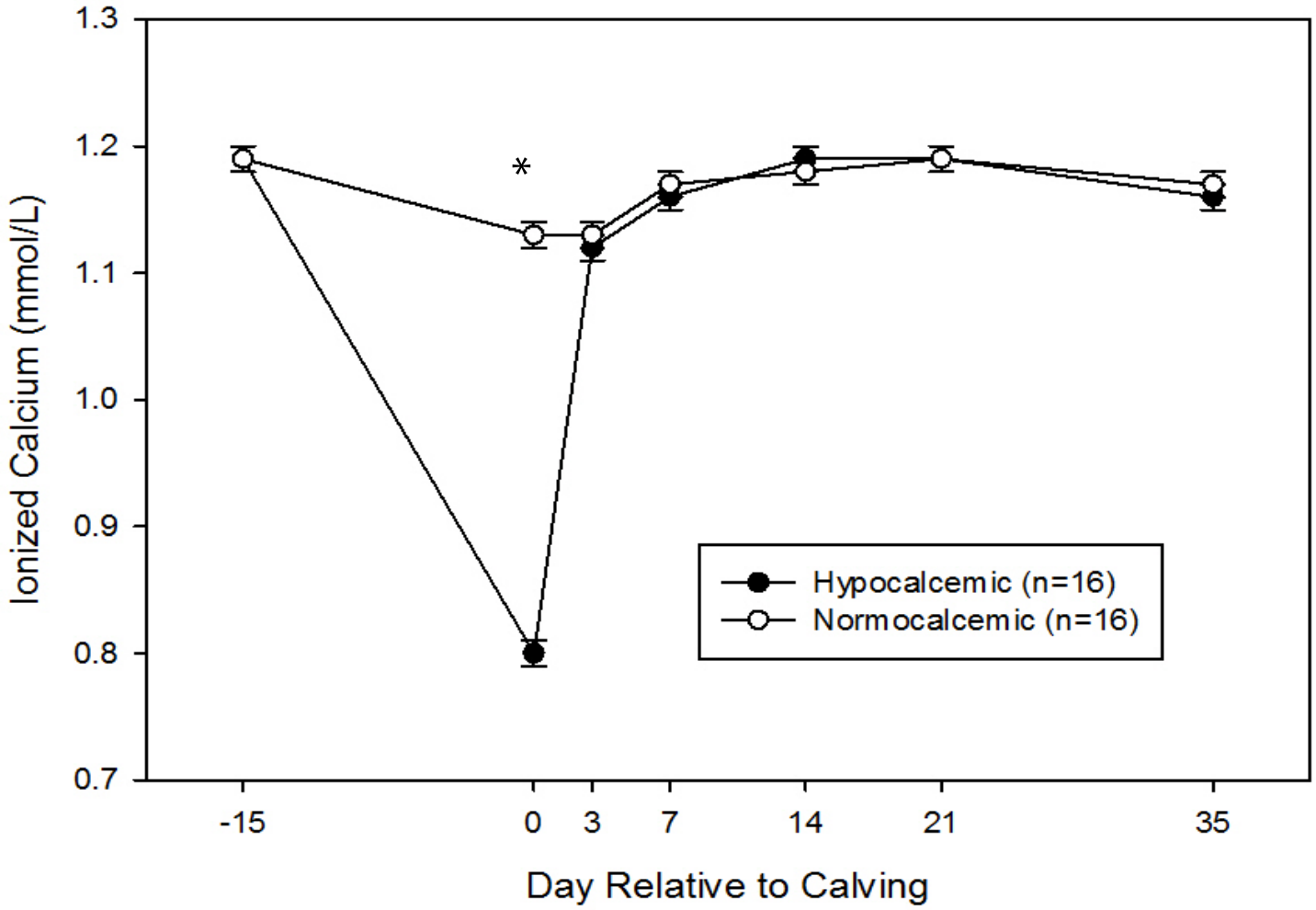


Figure 2.14 Mean NEFA concentration vs. time for cows outside one standard deviation from mean ionized calcium. ($^{+}P = 0.11$).

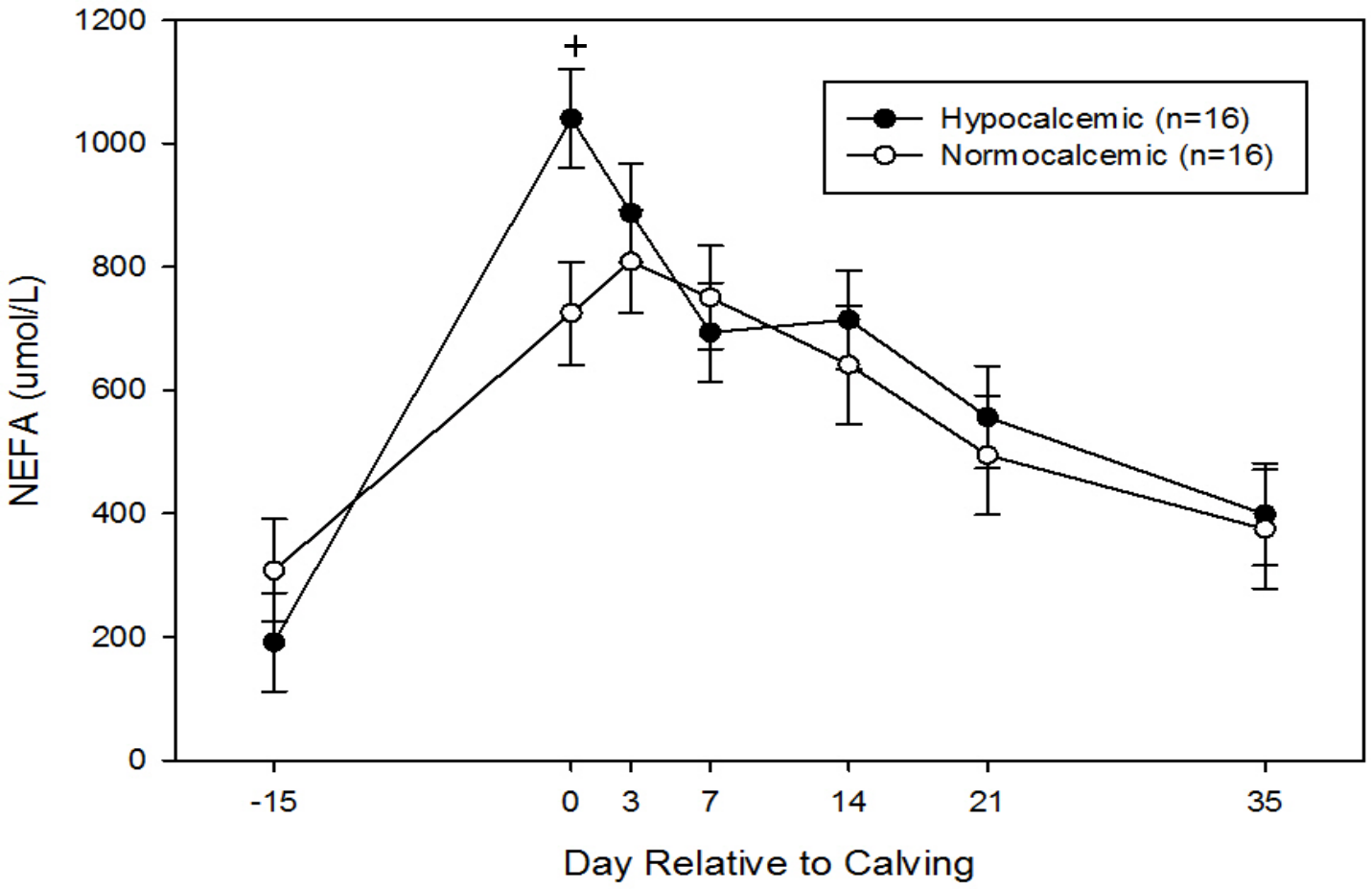
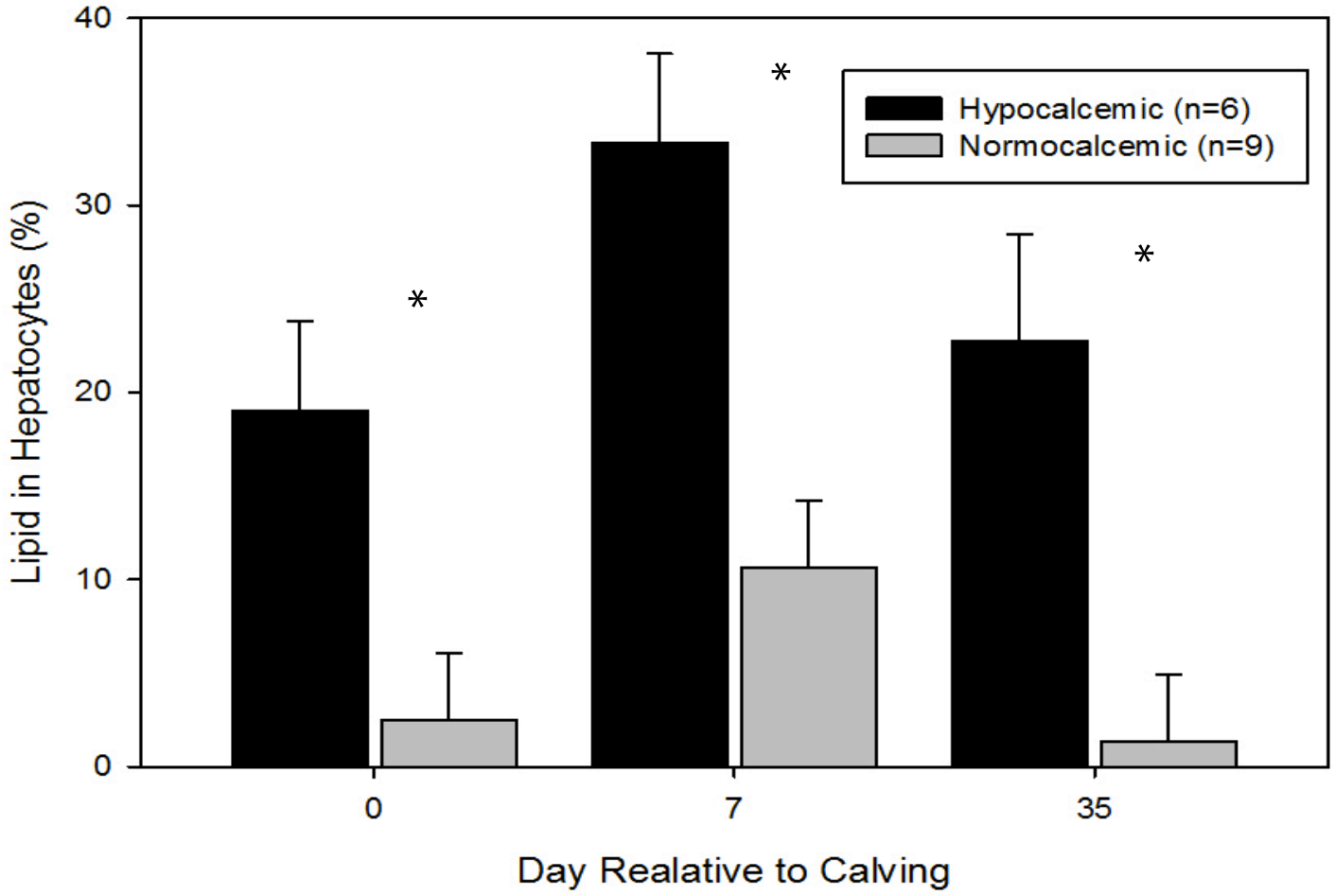


Figure 2.15 Mean hepatocellular lipid infiltration vs. time for cows outside one standard deviation from mean ionized calcium. ($^{*}P < 0.05$).



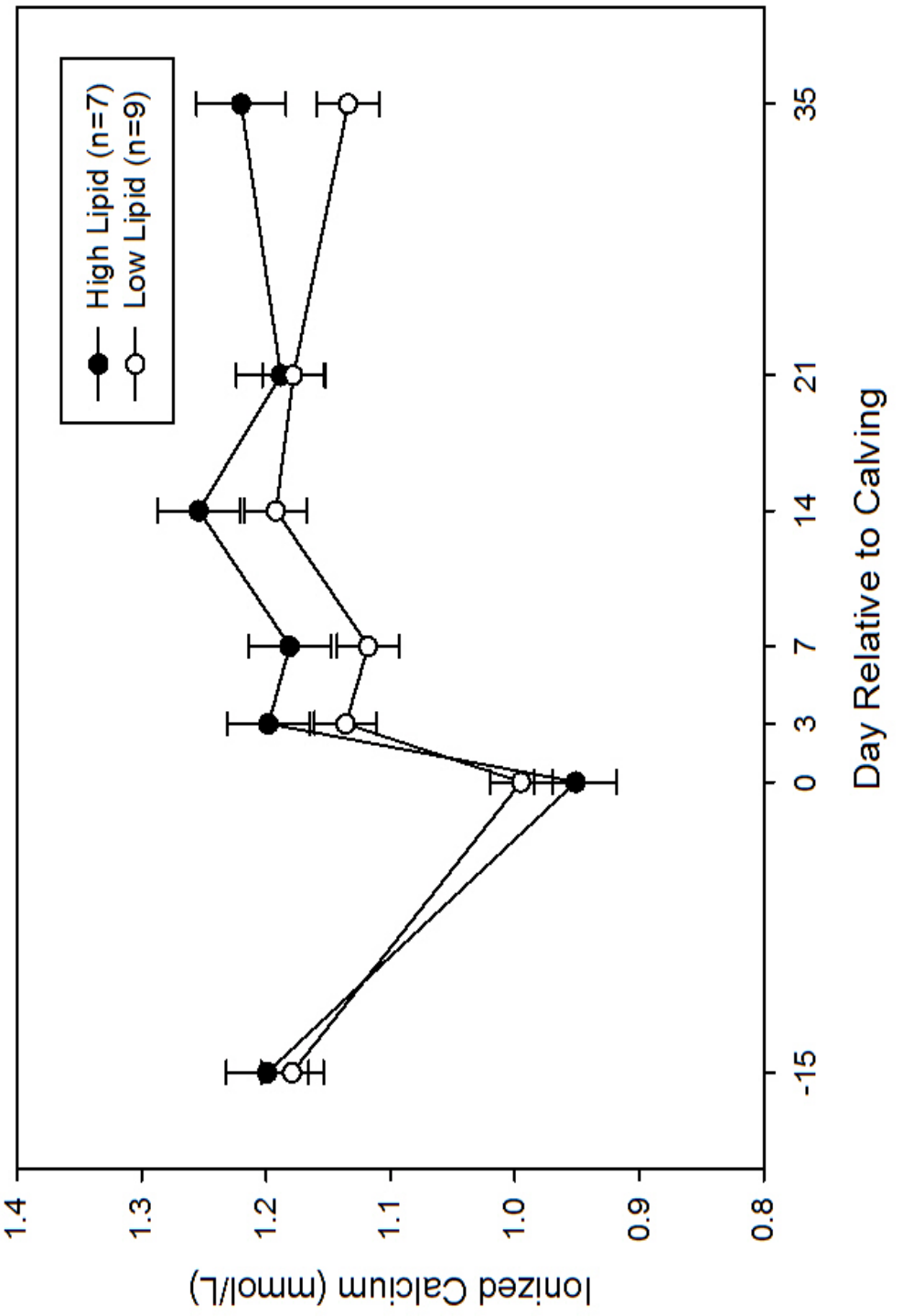


Figure 2.16 Whole blood ionized calcium vs. time for cows outside one standard deviation from mean liver lipid on day 7 ($P = 0.27$).

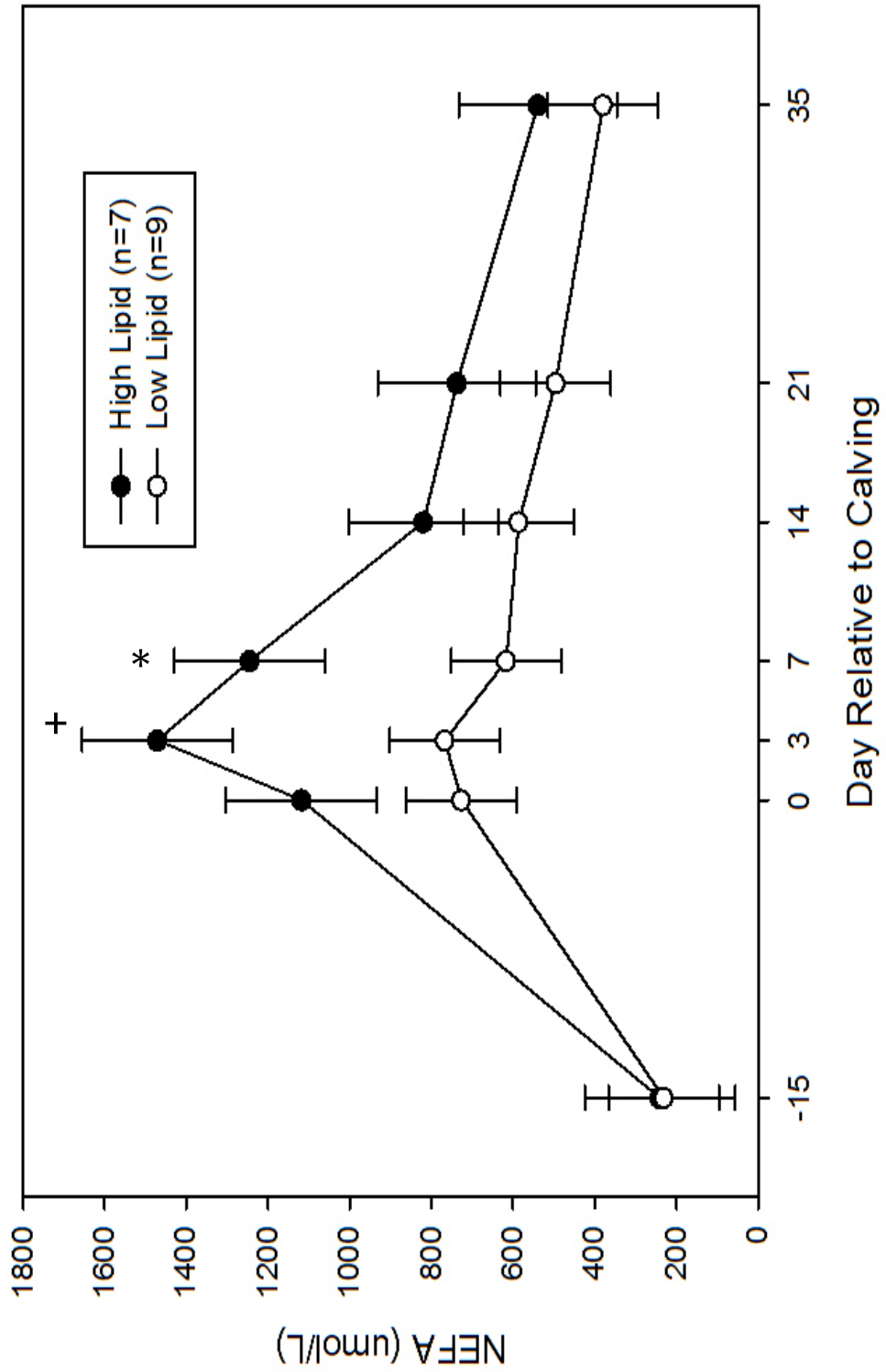


Figure 2.17 Mean NEFA concentration vs. time for cows outside one standard deviation from mean liver lipid on day 7 ($^{+}P = 0.08$; $^{*}P = 0.04$).

Figure 2.18 Percent lipid in hepatocytes vs. time for cows outside one standard deviation from mean liver lipid on day 7 ($^{+}P = 0.10$; $*P < 0.05$).

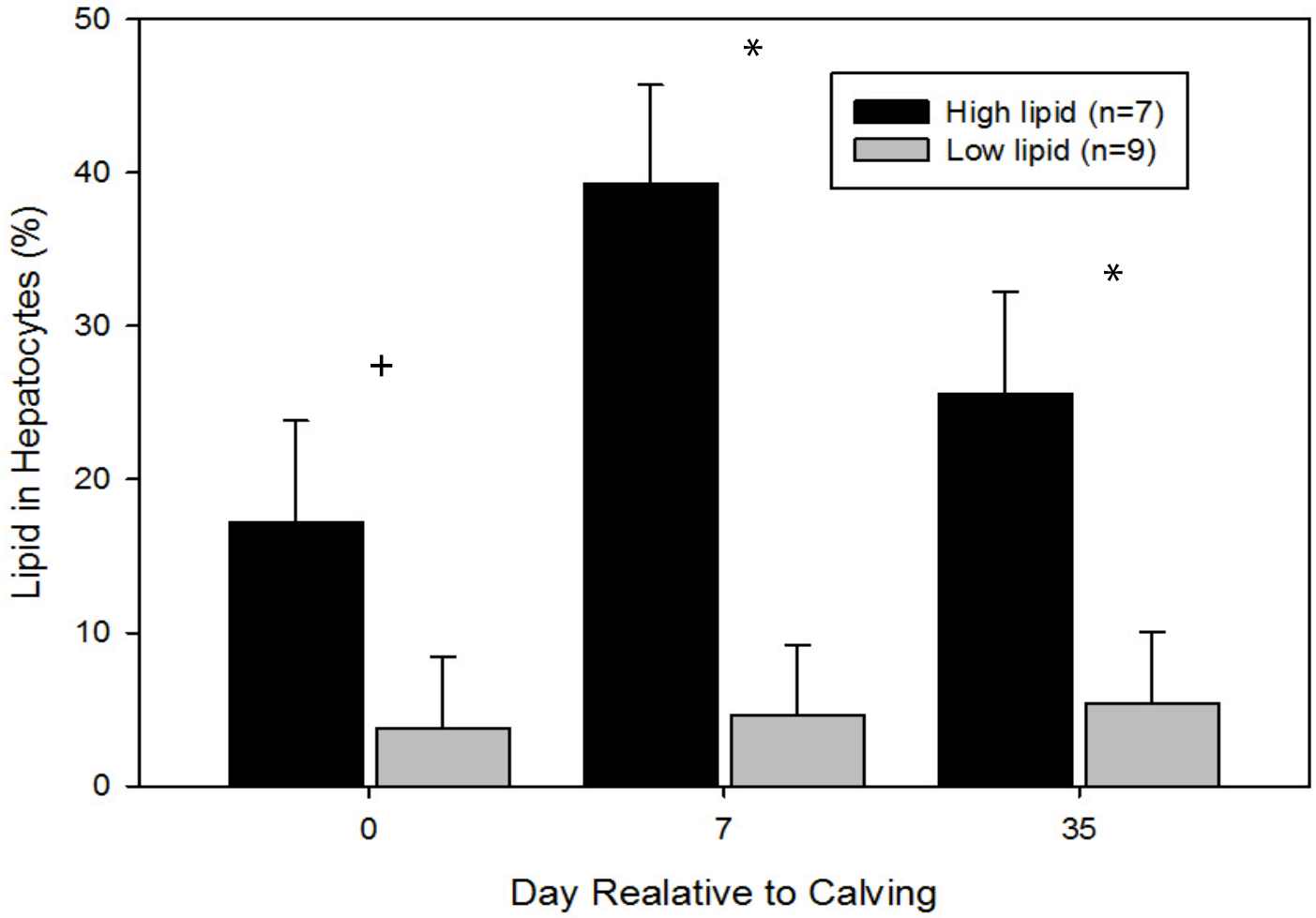


Figure 2.19 Mean plasma AST activity vs. time for cows outside one standard deviation from mean liver lipid on day 7 (*$P < 0.05$).

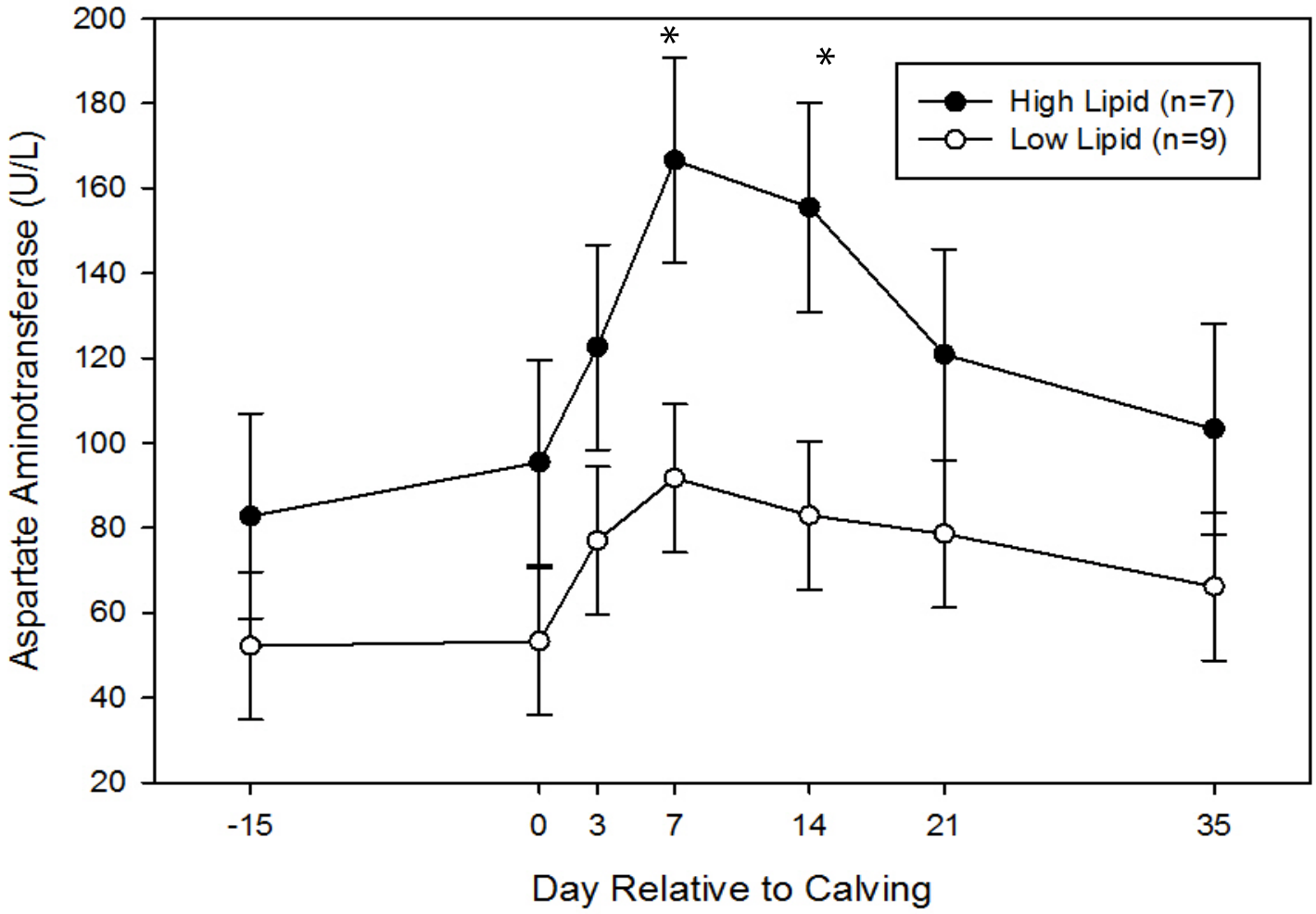


Figure 2.20 Activation of pyruvate dehydrogenase complex by phosphorylation and dephosphorylation.

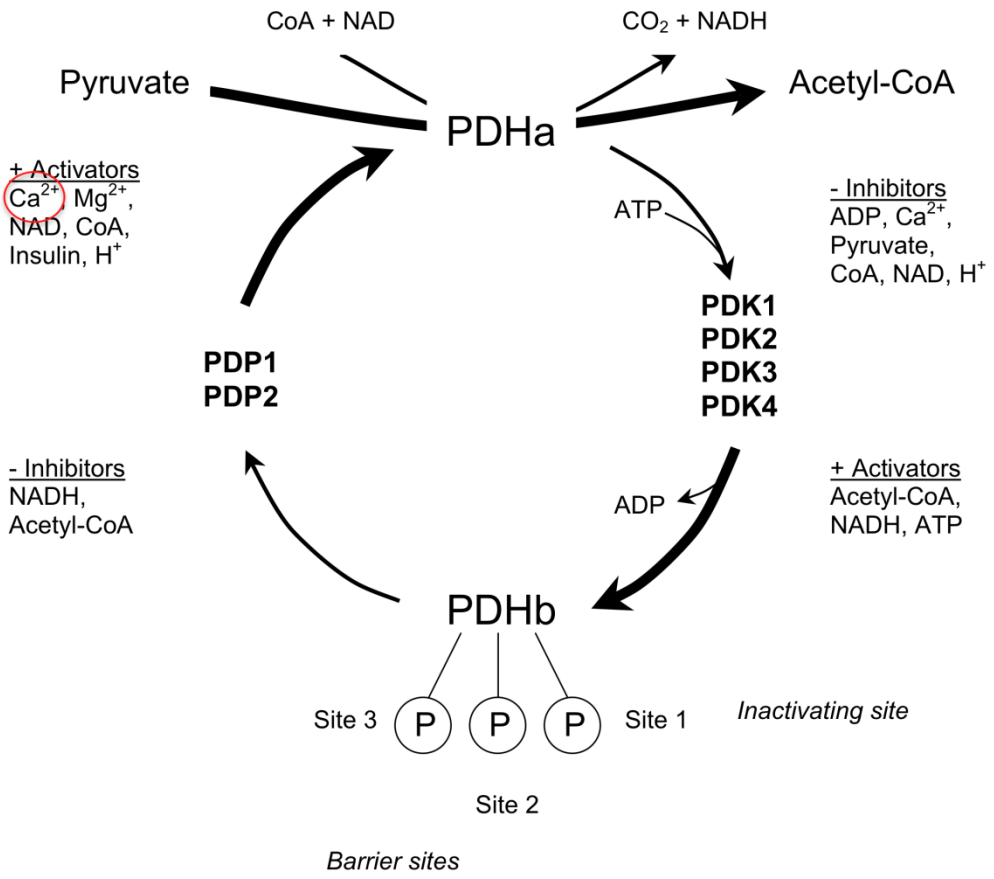


Table 2.1 Significant correlations between variables.

Correlation	P-value	r-value
Liver lipid to NEFA	0.001	0.39
Liver lipid to glucose	0.03	-0.27
NEFA to iCa	<0.0001	-0.25
NEFA to total Ca	<0.0001	-0.31
NEFA to indirect bilirubin	<0.0001	0.61
NEFA to Anion Gap	<0.0001	0.24
NEFA to Total CO_2	<0.0001	-0.17

Table 2.2 Composition and chemical analysis of dry cow rations.

	Diet containing dry cow premix[1]	Diet containing lactating cow premix[1]
Ingredient	% Dry Matter	
Grass hay	20.9	20.9
Corn silage	33.9	33.9
Ground corn	10.3	10.3
Soybean meal 48%	7.5	7.5
Soy hulls	26.8	26.8
Salt	0.2	0.2
Vitamin ADE premix[2]	0.1	0.1
Vitamin E premix[2]	0.3	0.3
Trace minerals	0.04	0.04
Chemical analysis		
Dry matter	50.2	53.3
Crude protein	14.89	-
ADF	32.74	-
NDF	49.32	-
Calcium	0.68	0.89
Phosphorus	0.31	0.35
Magnesium	0.20	0.22
Potassium	1.68	1.41
Sulfur	0.14	0.16
Sodium	0.10	0.16
Chloride	0.32	0.47
DCAD[3]	+33.67 mEq/ 100g	+25.37 mEq/ 100g

[1] Diet fed from dry off (approximately 60 days before expected calving date) until parturition

[2] Diet balanced to contain 1260 IU/lb vitamin A, 504 IU/lb vitamin D, and 61 IU/lb vitamin E

[3] No anionic salts were added to either diet

Table 2.3 Prepartum blood and plasma biochemical parameters of interest.

Parameter	Group		P-value
	Normocalcemic	Hypocalcemic	
Ionized calcium (mmol/L)	1.19 ± 0.01	1.19 ± 0.01	0.98
Total calcium (mg/dL)	9.27 ± 0.08	9.3 ± 0.08	0.79
NEFA (umol/L)	223.07 ± 62.22	247.51 ± 61.45	0.78
Total bilirubin (mg/dL)	0.17 ± 0.04	0.12 ± 0.04	0.40
GGT (U/L)	67.2 ± 5.58	56.46 ± 5.85	0.17
AST (U/L)	13.25 ± 4.2	14.51 ± 4.4	0.83

Table 2.4 Proportion of cows with each clinical disease by group.

Calcium Status	Ketosis (Small – Moderate) $P = 0.80$	Ketosis (Large – Extreme) $P = 0.80$	Mastitis $P = 0.95$	Metritis $P = 0.44$	Retained Placenta $P = 0.28$	Displaced Abomasum $P = 0.96$
Hypocalcemic	0.14 (7/51)	0.35 (18/51)	0.10 (5/51)	0.06 (3/51)	0.10 (5/51)	0.06 (3/51)
Normocalcemic	0.18 (9/49)	0.29 (14/49)	0.10 (5/49)	0.10 (5/49)	0.04 (2/49)	0.06 (3/49)

Table 2.5 Reproductive parameters by group.

Calcium Status	Cyclicity $P = 0.36$	Services per conception $P = 0.72$	Days Open $P = 0.67$
Hypocalcemic	57.4%	2.56 ± 0.25	127.6 ± 9.5
Normocalcemic	62.8%	2.43 ± 0.25	122 ± 9.5

Table 2.6 Stepwise regression of day 0 blood ionized calcium concentration on NEFA, liver lipid content, and indirect bilirubin.

iCa day	Variable/ day	Partial R^2	Model R^2	P-value
0	NEFA/ -15	0.05	0.11	0.03
0	NEFA/ 0	0.06		0.02
0	NEFA/ 3	-	-	0.83
0	NEAFA/ 7	-	-	0.90
0	NEFA/ 14	-	-	0.45
0	NEFA/ 21	-	-	0.12
0	NEFA/ 35	-	-	0.63
0	Indirect Bilirubin/ 0	-	-	0.70
0	Liver lipid/ 0	-	-	0.55
0	Liver lipid/ 7	-	-	0.23
0	Liver lipid/ 35	-	-	0.19

Table 2.7 Stepwise regression of day 0 total plasma calcium concentration on NEFA, liver lipid content, and indirect bilirubin.

iCa day	Variable/ day	Partial R^2	Model R^2	P-value
0	NEFA/ -15	-	-	0.61
0	NEFA/ 0	-	-	0.07
0	NEFA/ 3	-	-	0.50
0	NEAFA/ 7	-	-	0.72
0	NEFA/ 14	-	-	0.52
0	NEFA/ 21	-	-	0.10
0	NEFA/ 35	-	-	0.53
0	Indirect Bilirubin/ 0	-	-	0.48
0	Liver lipid/ 0	-	-	0.72
0	Liver lipid/ 7	-	-	0.49
0	Liver lipid/ 35	-	-	0.35

Table 2.8 Stepwise regression of NEFA and liver lipid infiltration.

Liver biopsy day	NEFA day	P-value	Partial R-squared	Model R-squared
0	-15	0.007	0.27	0.48
	7	0.03	0.14	
	21	0.0008	0.07	
	0	0.73	-	-
	3	0.19	-	-
	14	0.34	-	-
	35	0.22	-	-
7	3	0.002	0.24	0.24
	-15	0.57	-	-
	0	0.38	-	-
	7	0.41	-	-
	14	0.88	-	-
	21	0.43	-	-
	35	0.93	-	-
35	-15	<0.0001	0.54	0.54
	0	0.39	-	-
	3	0.30	-	-
	7	0.33	-	-
	14	0.65	-	-
	21	0.67	-	-
	35	0.18	-	-

Table 2.9 Stepwise regression of indirect bililrubin concentration on NEFA.

Indirect Bilirubin day	NEFA day	P-value	Partial R-squared	Model R-squared
0	-15	0.03	0.02	0.67
	0	<0.0001	0.65	
	3	0.08	-	-
	7	0.21	-	-
	14	0.14	-	-
	21	0.73	-	-
	35	0.42	-	-

Table 2.10 Body condition parameters of cows outside one standard deviation from mean liver lipid on day 7.

Group	BCS at calving	BCS loss first 2 weeks postpartum*	BCS loss from calving to 1st breeding*
High Lipid	3.0 ± 0.1	-0.65 ± 0.05	-0.96 ± 0.07
Low Lipid	2.75 ± 0.1	-0.27 ± 0.04	-0.29 ± 0.04

* $P < 0.02$

Addendum: Cows removed from study reported by group and cause of removal.			
ID	Group	Lactation Number	Reason
824	hypocalcemic	4	Clinical milk fever
2064*	hypocalcemic	3	No liver tissue biopsied
697*	hypocalcemic	6	No liver tissue biopsied

Totals:
3 Hypocalcemic

*Only excluded from liver biopsy portion of data analysis.

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