Management of Peripartum Dairy Cows for Metabolic Health and Immune Function

By

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ABSTRACT

MANAGEMENT OF PERIPARTUM DAIRY COWS FOR METABOLIC HEALTH AND IMMUNE FUNCTION

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This thesis was conducted to investigate interactions among metabolic and immune system health and management of dairy cows in the peripartum period. Several management interventions recommended for the benefit of peripartum cows were explored to improve their evidence base. A randomized controlled trial evaluated the effect of a prophylactic calcium supplementation product on blood calcium concentrations, the incidence of clinical disease and culling, milk production and the probability of pregnancy at first insemination. Secondly, a study was conducted to evaluate if administration of a calcium supplement product at calving altered the immune function parameters neutrophil oxidative burst or phagocytosis capacity. Finally, a randomized controlled trial was performed to test whether reducing social stress by providing non-competitive access to feeding and lying modifies metabolic health and immune function, and if differences in metabolic health and immune function could be explained using a measure of group social status.

Treatment with prophylactic subcutaneous calcium increased blood calcium levels at 24 hours after treatment and reduced the proportion of cows treated with calcium for stage 1 clinical hypocalcemia, but had no effect on the risk of other disease or culling, milk production or reproductive performance. Supplemental calcium given to low parity parturient cows did not
alter oxidative burst or phagocytosis capacity of neutrophils. The calcium product may prevent visible signs of hypocalcemia in some cows but emphasize advisors must prioritize hypocalcemia prevention throughout the peripartum period.

A non-competitive housing strategy modestly improved albumin and calcium concentrations during the peripartum period and improved the oxidative burst function among cows with higher social status compared to low social status cows. On average, crowding in a small, stable group through the close-up dry period without crowding or social instability in the postpartum period did not negatively affect the metabolic health and immune function of cows. As only high-rank (dominant) cows extracted an advantage from lower density housing, future research should focus on alternative strategies that better support low-rank (subordinate) cows in group housing.
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Chapter 1

LITERATURE REVIEW

The peripartum period, which comprises the three weeks before and after parturition in dairy cattle, is characterized by marked physiological, metabolic and endocrine changes as the cow transitions from the metabolically demanding states of pregnancy to lactation (Roche et al., 2013; Grummer et al., 1995). In addition to enduring the parturient phase itself, the transition cow concurrently experiences the challenge of re-establishing calcium homeostasis, an acute diet change requiring rumen adaptation, and modifications in glucose, fatty acid and mineral metabolism with concurrent negative energy and protein balance (Goff and Horst, 1997; Overton and Waldron, 2004). After parturition, dry matter intake cannot meet the demands of milk production, necessitating adaptation on the part of the cow. Concomitant depressed immune capacity throughout this period poses an additional risk for peripartum diseases (Mallard et al., 1998). Despite transition cow biology and management remaining a core focus of nutrition and physiology research for more than 25 years, the peripartum period continues to be problematic on many dairies, with between 30 and 50% of dairy cows affected by at least one infectious or metabolic disease after calving (LeBlanc, 2006) and the highest proportion of culled cows leaving the herd in the first 60 days (Godden et al., 2003). These rates impair the welfare of dairy cows and the economic profitability of dairy farms. Greater recognition of the inter-relationships between fat and energy metabolism, immune function and inflammation in other species, particularly humans and mice, has emphasized that all these components are central to the health of peripartum cows (LeBlanc, 2012).
Metabolic Adaptations during the Peripartum Period

Homeorhesis, the orchestrated control in metabolism of body tissues necessary to support a physiological state (Bauman and Currie, 1980), is most exceptional in postpartum dairy cows. The dramatic change in nutrient demands that characterizes the onset of lactation in the dairy cow requires a coordinated metabolic response and a tremendous shift in nutrient partitioning. Within a few days after calving, the requirements of the mammary gland alone drastically exceed the maintenance requirements of late term pregnancy, with approximately three times the glucose requirement, double the amino acid requirement and five times the fatty acid requirement before calving (Bell, 1995). The ability of key metabolic pathways to rapidly adapt after calving is fundamental to a successful transition period.

Glucose Metabolism

The demand for glucose after calving is primarily driven by need in the mammary gland for energy and the synthesis of lactose, resulting in low blood glucose concentrations and associated low blood insulin concentrations (Lucy, 2008). The primary homeorhetic mechanisms employed to meet this demand are an increase in hepatic gluconeogenesis from volatile fatty acids, principally propionate and decreased glucose usage by insulin dependent peripheral tissues. Increased propionate supply is associated with increased capacity of the liver to use propionate for gluconeogenesis during periods of negative energy balance, representing up to two thirds of the glucogenic precursors (Drackley et al, 2001). Propionate supply for glucose synthesis is limited by dry matter intake; therefore additional pathways must also be employed (Drackley and Caroso, 2014). Supplementary contributors to the total hepatic gluconeogenesis include lactate (from the rumen, skeletal muscles and uterus), glycerol (from triglyceride lipolysis in adipose
tissue) and amino acids (from intestines and skeletal muscles; De Koster and Opsomer, 2013; Drackley et al., 2001).

**Lipid Metabolism**

The period of negative energy balance during which feed consumption does not meet the demands of milk production necessitates the mobilization of body fat resources. High circulating growth hormone and low insulin concentrations trigger adipose tissue to mobilize stored triacylglycerols which are released into the bloodstream in the form of non-esterified fatty acids (NEFA) (Drackley and Caroso, 2014). Increased NEFA is used as an energy source, allowing the cow to conserve glucose for the mammary gland. Non-esterified fatty acids can be used for synthesis of mammary triacylglycerol (Roche et al, 2013) or metabolized to be used as an energy source by non-mammary tissues, Circulating NEFA also initiate feedback to regulate lipolysis (Sordillo et al., 2009). When successful, this homeorhetic mechanism maintains a supply of glucose for milk synthesis, and NEFA is metabolized to meet energy needs without excessive accumulation in the blood (Herdt, 2000). When lipid mobilization is excessive or begins prematurely, the capacity of hepatocytes to export triglycerides synthesized from re-esterified NEFA is overwhelmed, resulting in increased blood NEFA concentrations and accumulation of triglyceride in the liver (Sordillo and Raphael, 2013). Among high producing dairy cows, it is likely that some triglyceride will accumulate in the liver during early lactation (Overton and Waldron, 2004), however excessive accumulation (above 5% liver triacylglycerol based on wet weight; Bobe et al., 2004) results in fatty liver as well as reduced ability for gluconeogenesis, limiting glucose available for milk production (Sordillo and Raphael, 2013). Additionally, uptake of NEFA by the liver causes an increase in ketone body production, an intermediate product of fatty acid oxidation. When released in the blood, ketones can be used as an energy substrate by
some tissues, however during excessive fat mobilization, ketone production exceeds use and ketone concentrations are elevated in the blood (Drackley et al, 2001). Concentrations of NEFA greater than 0.29 mEq/L prepartum or the ketone body beta-hydroxybutyrate (BHB) greater than 10mg/dL postpartum are associated with clinical disease including retained placenta, metritis, clinical ketosis and displaced abomasum (Ospina et al., 2010).

Calcium Metabolism

The onset of lactation brings about a huge demand for calcium for colostrum and milk synthesis, resulting in a substantial drop in blood calcium levels around the time of calving (Horst et al., 1986). Typically, blood calcium reaches a nadir 12 to 24 hours after calving with recovery by 48 to 72 hours (Goff, 1999). Exceedingly low blood calcium levels cannot support nerve and muscle function and if left untreated would result in recumbency and death (Oetzel, 2011). Among those that recover from clinical hypocalcemia (milk fever), there is increased risk of dystocia, uterine prolapse, retained placenta, metritis, mastitis and displaced abomasum (Curtis et al., 1983; Markusfeld, 1987). More recent research has identified that subclinical hypocalcemia is highly prevalent among periparturient cows with its own consequences including increased risk of ketosis (Horst et al, 1997), displaced abomasum (Chapinal et al., 2011a), milk production losses (Chapinal et al, 2012) and increased culling risk in early lactation (Roberts et al., 2012). Cows classified as high risk for metritis (a cow with one or more calving disorders such as dystocia, twins, stillbirth and retained placenta) that were able to maintain serum calcium concentrations above 2.15 mmol/L had one-half and one-third the incidence of metritis and puerperal metritis, respectively, when compared to low metritis risk cows that were below this cut-point in the first 3 days in milk (DIM; Martinez et al., 2012). The major homeostatic mechanisms to replace extracellular calcium lost to milk and maintain normocalcemia involve withdrawing calcium
from bone and increasing the absorption efficiency of dietary calcium (Goff, 2008). During the critical first few days of lactation, homeostasis is restored by release of parathyroid hormone (PTH) which reduces urinary calcium losses, stimulates bone calcium resorption and increases 1,25-dihydroxyvitamin D synthesis to enhance active intestinal transport of dietary calcium (Goff and Horst, 1993). Hypocalcemia prevention strategies initiate homeostatic control mechanisms ahead of calving and prime the response for the decrease in blood Ca following calving (Thilsing and Hansen, 2009).

**Metabolism and Inflammation**

**Insulin Resistance**

The hormone insulin is primarily responsible for increasing glucose transport into cells. Insulin resistance can result from either decreased insulin secretion in response to glucose or a diminished response of tissues to insulin (Kahn et al., 1978; Sano et al., 1991). A physiologic period of insulin resistance characterizes the end of gestation and early lactation such that glucose is directed to the pregnant uterus or the mammary gland where glucose uptake is independent of insulin (Lucy, 2008; De Koster and Opsomer, 2013). In order to sufficiently supply glucose to the fetus near the end of gestation, an insulin-independent process, there is a reduction in the number of glucose transporters in insulin-sensitive tissues including liver, muscle and adipose tissue and therefore diminished glucose utilization by peripheral tissues (Thorens, 1996). Insulin-sensitive tissues can be more flexible for their source of energy, using other fuels like NEFA or BHBA instead of glucose (Heitmann et al., 1987). In the postpartum period, insulin resistance is more severe due to the increased energy demands of lactogenesis
(Bell and Bauman, 1997). Low circulating insulin will induce glucose sparing for the mammary gland (Bossaert et al., 2008) while also contributing to lipolysis (Spicer and Echternkamp, 1995).

Negative energy balance (NEB) and insulin resistance are interconnected. High circulating growth hormone (GH) and NEFA that characterize NEB antagonize insulin action and exacerbate insulin resistance (Lucy, 2008; Kerestes et al., 2009). Insulin resistance can be induced experimentally through intravenous infusion of tallow (triacylglycerol; TG) resulting in hyperlipidemia characterized by high TG and NEFA concentrations (Pires et al., 2007). The authors of this study concluded hyperlipidemia-induced IR with excessive elevation of NEFA may disrupt adipose tissue function resulting in a cycle in which adipocytes become more insulin resistant and plasma NEFA concentrations are further increased leading to energy-related metabolic disorders. Studies in humans have demonstrated that many mediators of inflammation (e.g. TNFα, IL-1β) can block intracellular signalling of insulin receptor substrate proteins, exacerbating IR (Hotamisligil, 2006). In people, adipose tissue has been shown to contribute pro-inflammatory signals as well as NEFA, which can activate TLR4 (a receptor for lipopolysaccharide from gram negative bacteria), activating nuclear factor Kβ and causing secretion of tumour necrosis factor alpha (TNFα) and interleukin 1 (IL1) that upregulate inflammation and increase IR. The peripartum period in dairy cattle is characterized by similar mediators and an understanding of similar phenomena is only beginning to emerge, but the metabolically active nature of adipose tissue suggests similar events likely occur.

Interactions between Metabolism and the Inflammatory Response

Adipose tissue is able to synthesize and secrete many biologically active substances, including pro-inflammatory cytokines such as TNFα and IL-6 that are involved in regulating inflammatory
responses (Tilg and Moschen, 2008; LeBlanc et al., 2014; Sordillo and Mavangira, 2014). Elevated serum TNFα concentrations were observed in cows with moderate to severe fatty liver disease (Ohtsuka et al., 2001). Adipose macrophages are capable of producing TNFα and IL-1B, the same mediators immune cells produce in response to pathogen detection (Lumeng and Saltiel, 2011) and important mediators in the endometrial immune response after calving.

However, even cows without microbial infection or other signs of infectious disease can display a systemic inflammatory response around calving (Bertoni et al., 2008). The effects of these pro-inflammatory cytokines on metabolism are only beginning to be understood. Late lactation cows intravenously infused with TNFα developed insulin resistance and elevated liver triglyceride concentrations (Bradford et al., 2009). A similar infusion in the first week after calving was associated with an increase in haptoglobin and several eicosanoids (markers of inflammation), decreased feed intake and tended to impair health status but did not alter glucose or lipid metabolism (Yuan et al., 2013). Milk production was reduced in response to decreased feed intake, suggesting that cows prioritized avoiding further negative energy balance over production.

Oxylipids, a class of lipid mediators derived from the oxygenation of polyunsaturated fatty acids (Raphael et al., 2014), along with cytokines can alter the initiation, magnitude and duration of the inflammatory response, including acute phase protein release by the liver, and alter body temperature, heart rate and dry matter intake (Lumeng and Saltiel, 2011). Intense lipid mobilization and changes in plasma NEFA during the peripartum period has been shown to change the phospholipid content of immune cells associated with altered leukocyte functions (Contreras et al., 2010) and modify the profile of oxylipids produced (Sordillo and Mavangira, 2014). Circulating NEFA concentrations may be a factor impacting the inflammatory response of
transition cows based on evidence from an in-vitro study that found NEFA concentrations in the range of those typically found in transition cows enhance the pro-inflammatory phenotype of endothelial cells (Contreras et al., 2012). Clearly, more research is needed to better understand the consequences of these inter-relationships.

**Peripartum Immune Function**

Through the last decade, an understanding has developed that metabolic and immune pathways are linked, particularly during times of altered nutrient metabolism, resulting in increased disease risk through the peripartum period (Sordillo and Mavangira, 2014). There is a formidable body of evidence that peripartum cows experience decreases in immune function and nonspecific host defense mechanisms during the peripartum period (Guidry, 1976, Kehrli, 1989a, 1989b; Gilbert, 1993, Mallard, 1998, Kim, 2005). Reduced immune function extends to multiple cell types and their functions, increasing the risk of disease (Detilleux et al., 1995; Mallard et al., 1998) and affecting all peripartum cows to varying degrees (Sordillo and Aitken, 2009).

**Innate Immunity**

Innate immunity is the non-specific defence against microorganisms, the first line of defense against injury or infection and is characterized by rapid onset. The innate system includes the physical barriers of skin and mucosa, antimicrobial peptides, the complement system and cells such as macrophages and neutrophils that detect and respond to microbes (Turner et al., 2012). Cells of the innate system rapidly identify cells as damaged, infected or non-self by means of specific pattern recognition receptors on host cells to bind molecules called pathogen-associated molecular patterns (PAMP) which are conserved among a diverse group of proteins (Turner et al., 2012; Wellnitz and Bruckmaier, 2012). The rapid action of the innate system is particularly
important to the transition dairy cow when a timely response is critical, whereas the adaptive immune system can take significantly longer to activate (Mallard et al., 1998). Cells of the innate immune system are principally responsible for quick action in the mammary gland (Wellnitz and Bruckmaier, 2012) and uterus (Sheldon et al., 2009) after parturition. A rapid immune cell response to the uterus is necessary to prevent metritis or endometritis (Sheldon et al., 2009). Gilbert et al. (2007) observed a greater proportion of neutrophils in the uterus on the day of calving to be associated with lower bacterial load and lower risk of subclinical endometritis 6 weeks after calving. Similarly, reduced innate immune defenses in the mammary gland around parturition increase the risk of mastitis during the peripartum period (Burvenich et al., 2003; Sordillo, 2005).

Although all components of the innate immune system have reduced function during the periparturient period, most noteworthy are neutrophils which are the primary cells recruited to both the uterus and mammary gland in response to bacterial recognition (Cai et al., 1994; Detilleux et al., 1995; Wellnitz and Bruckmaier, 2012). Briefly, resident macrophages release inflammatory mediators (e.g. IL-1β) when they come into contact with bacterial pathogens, resulting in endothelial surface changes that recruit neutrophils to the site of infection. Circulating neutrophils must slow their movement and adhere to the endothelium in order to exit the vasculature to migrate to the affected tissue. This is accomplished through several recognized steps including tethering, rolling, adhesion, crawling and transmigration either between endothelial cells or directly through them (Kolackowska and Kubes, 2013). Once at the site of infection, neutrophils phagocytose the pathogen and use reactive oxygen species or antibacterial proteins to eliminate them (Cai et al., 1994; Kolackowska and Kubes, 2013).
Diminished migration and functional capacity of neutrophils in the periparturient period has been demonstrated in many studies (Kehrli et al., 1989b; Cai et al., 1994; Kim et al., 2005). Typically, the number of circulating neutrophils increases on the day of calving but is reduced through the postpartum period due to a large exodus of neutrophils to the uterus and mammary gland (Guidry et al., 1976). Recovery to pre-calving levels is usually reached by four weeks postpartum (Cai et al., 1994; Kim et al., 2005). Although the absolute number of circulating neutrophils remains unchanged until calving, neutrophil oxidative burst function is already compromised in the prepartum period, declining from 2 weeks before calving and reaching its nadir about one week after calving (Kehrli et al., 1989; Detilleux et al., 1995). Phagocytosis function does not decline until after calving (Kehrli et al., 1989, Kim et al., 2005) or not at all (Sander et al., 2011). A few populations have been identified as experiencing a greater reduction in neutrophil function including high producing cows (Kehrli et al., 1989, Goff and Horst, 1997), older cows (Gilbert et al., 1993), and cows that develop retained placenta (Kimura et al., 2002) or uterine disease (Cai et al., 1994; Kim et al., 2005; Hammon et al., 2006).

Adaptive Immunity

The adaptive immune response is similarly altered among peripartum cows. Adaptive immunity is an antigen-specific response with enduring memory. Once an antigen is recognized, lymphocytes (T and B cells) and antibodies are produced that are able to recognize the antigen in the future with improved ability to rapidly eliminate the invader. The proportions of B cells and T cells in circulation are influenced by parturition, with B cell populations either remaining unchanged or briefly decreasing after calving and T cell populations significantly reduced, which may be associated with increased trafficking to the mammary gland (Mallard et al., 1998; Van Kampen et al., 1999). Kehrli et al. (1989a) reported postpartum impairment of lymphocyte
function in blood and milk. There has been less peripartum change demonstrated in serum antibody levels, but serum concentrations of immunoglobulin G (IgG) have been shown to be reduced around calving, leading to less adaptive protection (Kehrli et al., 1989; Detilleux et al., 1994; Detilleux et al., 1995). Furthermore, cell-mediated immunity is reduced during the peripartum period, associated with the diminished number of circulating T cells, which is reduced due to the effects of peripartum changes in circulating corticosteroids, progesterone and estrogen (Van Kampen and Mallard, 1997; Mallard et al., 1998).

**Postpartum Uterine Involution, Inflammation and Disease**

Uterine involution is a complex process accompanied by gross and molecular changes that occur over several weeks postpartum. The discharge of the fetus, membranes and fluid associated with calving initiates uterine involution in the cow (Sheldon et al., 2008). Normal uterine involution is characterized by contraction and shrinkage as the uterus and cervix rapidly reduce in size over 3 to 6 weeks (Gier and Marion, 1968), which can be attributed to glandular and muscle atrophy as well as sloughing of caruncles (Sheldon et al., 2008; Chapwanya et al., 2009). Sloughed caruncles along with remaining fetal fluids and umbilical blood make up lochia which is normally passed for 2 to 3 weeks after calving (Gier and Marion, 1968; Lewis, 1997).

The majority of cows (up to 90%) have bacterial contamination in the uterus after calving (Sheldon et al., 2002; Sheldon and Dobson, 2004). At the time of parturition, an open cervix allows bacteria from the environment or vagina to enter the uterus (Noakes et al., 1991). Uterine bacterial contamination activates the local innate immune response to resolve inflammation and clear infection; however the reasons for which some cows successfully clear infection while others develop uterine disease are likely multifactorial, but are not entirely clear.
Uterine inflammation is considered normal and necessary as part of uterine involution due to the typical bacterial contamination and significant endometrium repair required (LeBlanc, 2014). Inflammation is a critical part of pathogen defense and activates immune responses; however, derangements in the inflammatory response may be responsible for greater uterine disease risk. Initiation and resolution of inflammation must be appropriately balanced because an excessive inflammatory response can result in damage to host tissues (Aitken et al., 2011). The normal, local uterine innate immune response is characterized by elevated expression of genes encoding toll-like receptors (TLR4), inflammatory mediators (nuclear factor kappa B1, IL-1A, IL-6, IL-8, IL-12A) and effector molecules (acute phase proteins, antimicrobial peptides; Chapwanya et al., 2009). Overly robust production of pro-inflammatory mediators may be a key factor in cows that develop purulent vaginal discharge (PVD) or subclinical endometritis. Herath et al. (2009) demonstrated that cows with persistent endometritis that were infertile had a greater pro-inflammatory response to bacterial infection during the week after calving, specifically higher ratios of mRNA for the pro-inflammatory cytokines IL-1A and IL-1B to the anti-inflammatory cytokine IL-10. Galvão et al. (2011) suggested an alternative hypothesis after observing TNFα and IL-1β gene expression were decreased in uterine tissue the week after calving among cows diagnosed with endometritis at 42 days. The authors hypothesized that cows that develop endometritis might have compromised ability to up-regulate gene expression of pro-inflammatory cytokines, which could result in poor chemotaxis and activation of neutrophils and monocytes.

Disordered inflammation may alternatively consist of a hyporesponsive state in which migration of immune factors is delayed. When activation of neutrophils is compromised, bacterial clearance from the uterus is impaired, predisposing cows to developing uterine disease. Greater
recruitment of neutrophils to the uterus on the day of calving is associated with lower prevalence of positive bacterial culture (Gilbert et al., 2007). Kimura et al. (2002) demonstrated that neutrophils isolated from blood of cows with retained placenta had significantly lower neutrophil function before calving as measured by chemotaxis toward cotyledon preparations and myeloperoxidase activity. For cows with metritis or subclinical endometritis, neutrophil myeloperoxidase activity has been demonstrated to decrease sharply prior to parturition (Hammon et al., 2006; Mateus et al., 2002). It remains unclear if neutrophil phagocytosis ability is compromised in animals with uterine disease. Kim et al. (2005) reported diminished phagocytosis capacity among cows with endometritis but others have not found this association (Hoedmaker, 1992; Cai et al., 1994).

**Interactions among Endocrine Profiles, Metabolic Health and Peripartum Neutrophil Function**

At the time of parturition, dairy cows experience a dramatic increase in several steroid hormones that alter neutrophil function at a molecular level (Burton et al, 2005). These changes are well recognized, however hormonal changes are short lived so do not fully explain reduced neutrophil function throughout the transition period (Roche et al., 2013). Glucocorticoids, which are well recognized to alter the inflammatory response by suppressing antibody and cytokine production (Khansari et al., 1990), are increased in plasma at calving (Goff and Horst, 1997). Greater adrenal secretion of cortisol at calving has been associated with down-regulation of glucocorticoid receptors and adhesion molecules (L-selectin and CD18) in bovine mononuclear leukocytes (Burton et al., 1995; Preisler et al., 2000a, b). These molecules are necessary for successful activation and migration of leukocytes to the sites of tissue injury. The synthetic glucocorticoid dexamethasone has been demonstrated to decrease the number, distribution and function of leukocytes in bovine blood (Burton and Erskine, 2003). Burton et al. (2005)
postulated that glucocorticoids are critical in host defense during stress, responsible for enhancing production of neutrophils, prolonging their survival in blood by decreasing the expression of genes and membrane proteins associated with apoptosis, and prioritizing extracellular matrix remodeling of the reproductive tract and placenta to ensure the fetus is born alive in the event the tissues become damaged, but at the expense of reduced antibacterial defense in the mother. Elevated cortisol above typical levels at parturition may have negative consequences for risk of disease. Galvão et al. (2010) found greater concentrations of cortisol on the day of calving among cows that developed metritis compared to those without metritis.

Other hormone changes including progesterone and estradiol concentrations have been reported to have direct and indirect effects on immune cells (Sordillo and Mavangira, 2014). Progesterone levels are high throughout pregnancy and important in preventing fetal rejection (Weinberg, 1987; Bonizzi et al., 2003). Close to calving, progesterone concentrations fall and remain low for several weeks postpartum (Goff and Horst, 1997). Increased serum progesterone has been associated with reduced phagocytic activity by neutrophils (Roth et al., 1983) and reduced oxidative burst activity of neutrophils has been observed when cells were incubated in vitro with concentrations of progesterone above 6.56 ug/ml (Chaveiro and Moreira da Silva, 2010).

Estrogen receptor β has been demonstrated to be present in neutrophils suggesting the opportunity for direct effects on neutrophil function (Lamote et al., 2006a). An in vitro model by Lamote et al. (2004) demonstrated a significant decrease in the number of viable neutrophils after 17β-estradiol treatment. Among late gestation cows, Lamote et al. (2006b) demonstrated 17β-estradiol decreases expression of CD47, an integrin-associated protein important for neutrophil migration through collagen. Similar to cortisol, serum estradiol concentrations peak
just before calving and quickly decrease (Radcliff et al., 2003) suggesting its effects are only one component of reduced immune function. However Galvão et al. (2010) measured higher circulating estradiol at calving in cows that developed metritis compared to those unaffected by uterine disease, so it is likely an important facet of peripartum immune dysfunction.

*Associations between Peripartum Neutrophil Function and Energy Status*

Peripartum immune function and metabolic health are intricately linked. Two studies that compared energy balance and immune parameters around calving between mastectomised cows and those with functioning mammary glands observed lymphocyte and neutrophil function were reduced for longer among lactating cows (Kimura et al., 1999, 2002). From this work, one can conclude that it is not only the dramatic hormone profile changes around calving that influence peripartum immune function, but the metabolic demands of early lactation that have an adverse impact on immune cells. Diminished neutrophil function has been associated with negative energy or protein balance, insulin resistance and metabolic disease in the transition period (Ingvartsen and Moyes, 2013). Hammon et al. (2006) observed neutrophil function was significantly reduced in cows with greater peripartum negative energy balance as characterized by elevated blood NEFA and decreased dry matter intake, with changes already detected in the prepartum period. In vitro experiments that exposed neutrophils to levels of NEFA and BHBA consistent with subclinical ketosis in dairy cows resulted in reduced oxidative burst function (Scalia et al., 2006; Hoeben et al., 1997). Furthermore, neutrophils recovered from cows with high liver triacylglyceride (TAG) content (>40mg/g) consistent with fatty liver disease had lower oxidative burst function compared to neutrophils from cows with normal liver TAG content (Zerbe et al., 2000).
Altered neutrophil function may be related to availability of energy resources for neutrophils. Immune cells such as macrophages and neutrophils have high energy requirements in order to support their antimicrobial functions, and glucose is the primary fuel source for such cells (Calder et al., 2007). Glycogen, a storage form for glucose and the main fuel of neutrophils, has been found to be lower in neutrophils during the first three weeks postpartum which may contribute to decreased cell function (Galvão et al., 2010). Bovine monocytes and neutrophils have both insulin and IGF-1 receptors and exposure to these two hormones together has been shown to enhance neutrophil function (Nielsen et al., 2003). Glucose availability decreases dramatically around parturition when glucose is preferentially directed to the mammary gland to meet milk production needs. Peripheral concentrations of insulin and IGF-1 are also low, all of which may contribute to reduced immune function.

**Associations between Peripartum Neutrophil Function and Hypocalcemia**

Intracellular calcium signaling is a key element in immune cell activation, including the activation of neutrophils, by way of an influx of calcium from the extracellular space when antigen receptors are triggered (Vig and Kinet, 2009; Burgos et al., 2011). Reduced neutrophil activation likely contributes to periparturient immune suppression as periparturient cows have lower intracellular calcium stores than non-parturient cows, and blunted calcium flux from the extracellular to intracellular space in response to activation signals (Kimura et al., 2006). Calcium is important for the function of neutrophils. Neutrophils treated in vitro with ethylene diamine tetraacetic acid (EDTA), an extracellular calcium ion chelator, had severely reduced phagocytosis capacity (Ducusin et al., 2001). Furthermore, neutrophils collected from cows with parturient paresis had lower intracellular calcium concentrations and impaired phagocytosis compared to cows without parturient paresis (Ducusin et al., 2003). Martinez et al., (2012)
demonstrated total circulating neutrophil number, neutrophil phagocytosis and neutrophil oxidative burst capacity to be reduced among cows with blood calcium concentrations < 2.15 mmol/L during the first three DIM compared with cows with calcium above 2.15 mmol/L. Blood calcium concentrations below 2.15 mmol/L were associated with greater risk of developing metritis, suggesting that reduced neutrophil capacity increased susceptibility to disease.

Management Strategies for the Peripartum Period

There are many management factors that contribute to periparturient physiological adaptations. The principle of many strategies is to ensure that cows maintain dry matter intake around calving and in the postpartum period. Factors important to intake include avoiding excessive body condition, preventing over-consumption of energy relative to requirements during the dry period, ensuring adequate forage intake after calving, attention to the Dietary Cation Anion Difference (DCAD) of the diet, and minimizing environmental stressors and maximizing cow comfort (Drackley and Cardoso, 2014). A review of peripartum nutrition is beyond the scope of this paper and has been reviewed elsewhere (Overton and Waldron, 2004; Friggens et al., 2004; Beever, 2006; Ingvartsen, 2006; Roche et al., 2013; Drackley and Cardoso, 2014), however nutritional management through the peripartum period is critical for cows to successfully adapt to the physiological stresses encountered during this time. Briefly, the primary objective is to provide a palatable, well-balanced diet that attempts to meet nutritional requirements. Furthermore, the diet must promote good appetite and high dry matter intake around and after calving. A well-formulated diet would ideally minimize the extent of body fat mobilization, provide adequate protein to meet amino acid requirements for maintenance, fetal growth and
milk production, maintain immune function throughout the peripartum period and support mineral balance around calving (Drackley et al., 2005).

Along with diet, managing body condition is critical but will only briefly be reviewed. A large body of evidence exists to assert that cows with excessive body condition are at greater risk during the peripartum period and obesity is a major contributing factor to failure to adapt successfully to negative energy balance (Ospina et al., 2010). Cows with a body condition score ≥ 4.0 on a 5-point scale at calving are at greater risk of parturient health problems including ketosis, DA and fatty liver than cows with a BCS of 3 to 3.5 (Grummer et al., 1993; Ingvartsen, 2006). Although there is some evidence that excessive feeding will be associated with a large calf and dystocia (Maree et al., 1986), the primary concern with over-condition is the rate of postpartum lipid mobilization. Among cows with a large adipose mass, the homeorhetic mechanisms employed after calving result in excessive lipid mobilization and high blood NEFA concentrations that cannot be effectively processed by hepatocytes (Grummer et al., 2004; Friggins et al., 2004). Furthermore, obesity in dairy cows may lead to a state of chronic low-grade inflammation resulting in insulin resistance and metabolic disorders and rendering these cows at higher risk of infectious disease. Given these relationships, it is clearly important to manage cattle to calve at an appropriate body condition score (3 to 3.5) such that they have adequate reserves to endure subsequent negative energy balance without excessive lipid mobilization. There is generally little opportunity to significantly add or reduce body condition during a traditional dry period length of 6 to 8 weeks, requiring cows be dried off at an appropriate BCS, avoid long dry periods and provide appropriate dietary energy levels. In the last decade, feeding a single controlled energy diet (low energy: high fiber offered ad libitum) throughout the far off and close up dry periods has shown significant potential to optimize body
condition score, energy balance and performance of transition cows (Beever, 2006; Janovick and Drackley, 2010).

Two central themes of this thesis are the management of hypocalcemia around calving and the peripartum environment cows experience in the weeks around calving. Recognizing that there are many facets of peripartum cow health and production, only these two will be described in greater detail.

**Control Strategies to Mitigate Hypocalcemia**

Although it is possible to treat the majority of clinical hypocalcemia (milk fever) cases by administering intravenous calcium solutions, affected cows are at greater risk of metabolic and infectious disease (Curtis et al., 1983). Furthermore, subclinical hypocalcemia by its very nature cannot be visually appreciated and is impractical to rapidly measure. This necessitates strategies that target calcium metabolism before circulating concentrations decline to levels associated with increased disease risk or clinical symptoms of milk fever. Although many hypocalcemia control strategies have been explored in the literature, typically four are used which include calcium supplements around the time of calving, feeding acidifying rations using anionic salt supplementation during late pregnancy, feeding low calcium rations during late gestation, or prepartum administration of vitamin D or its metabolites (Thilsing and Hansen, 2002).

**Dietary Strategies**

Dietary hypocalcemia prevention strategies involve priming the cow to more efficiently manage the period of negative mineral balance (Horst et al., 1997). Vitamin D supplements are minimally available in North America and are not a commonly employed strategy. Restricting calcium in the prepartum diet stimulates parathyroid hormone secretion ahead of calving, promoting bone
calcium resorption and the production of 1,25-dihydroxyvitamin D (Oetzel, 2011), essentially activating calcium homeostatic mechanisms earlier to minimize hypocalcemia in the days after parturition. The feedstuffs available for dry cow diets often make formulating a prepartum diet with such a low calcium level (< 20 g/d) impractical, therefore this strategy is minimally employed. Feeding anionic salts is an effective method to mitigate hypocalcemia by bringing the cow into a state of physiologic compensated metabolic acidosis. Acid-base status of the cow dictates the sensitivity of tissues to PTH stimulation, with metabolic alkalosis blunting tissue responsiveness. When anions are added to the diet, parathyroid hormone dependent functions are enhanced, including bone resorption and renal production of 1,25-dihydroxyvitamin D (Goff and Horst, 2003). Although this strategy can significantly reduce the incidence of clinical and subclinical hypocalcemia, the necessity of performing urinary pH monitoring, potentially separating nulliparous and multiparous animals, and reduced palatability leading to decreased dry matter intake (DMI) has limited its employment on some dairies.

**Calcium Supplements**

A wide variety of calcium supplements have been developed that can be given to parturient cows as subcutaneous or oral therapies. An oral calcium bolus tested in 10 subclinically hypocalcemic cows compared to 10 subclinically hypocalcemic controls found serum ionized calcium was significantly higher for the bolus group at 13 hours postpartum with a mean increase of 0.15 mmol/L (Sampson et al., 2009). Oral products composed of calcium chloride cause systemic acification resulting in increased parathyroid hormone sensitivity in target tissues. Subcutaneous calcium injection products provide a slow release of calcium from subcutaneous depots to support the animal. There are few studies documenting the effects of subcutaneous calcium therapies, but administration of 500mL of calcium borogluconate (23%) subcutaneously to 6
Jersey cows was shown to raise blood calcium levels to about 120% above baseline for approximately 6 hours after calving (Goff, 1999).

There is scant evidence of improvements in health subsequent to administration of calcium supplementation products after calving. A controlled trial involving 204 Holstein cows from one herd administered three to four tubes of CaCl$_2$ gel (54 g elemental calcium per tube) prophylactically and found treated cows had reduced incidence risks of MF and DA (Oetzel, 1996). No other calcium supplementation study has found an effect of any product on DA incidence. A trial with oral (calcium chloride and calcium sulfate) boluses involving 927 multiparous cows from 2 herds had no effect on any study outcome including health events, production, first service conception or removal from the herd when all cows were considered together. The study did observe that cows with a previous lactation mature-equivalent milk production above 105% of herd mean that received boluses produced 2.9 kg more milk at first DHI test after calving and supplemented lame cows averaged fewer health events (Oetzel and Miller, 2012). Correspondingly, no differences in parturient disease incidence, reproductive performance or milk production were associated with treatment with CaCl$_2$ gel in cows with retained placenta (Hernandez et al., 1999) or in cows supplemented with anionic salts prepartum (Melendez et al., 2003) although both were small studies that lacked power to detect several of these outcomes.

**Management of the Peripartum Cow Environment**

Awareness of the importance of the non-nutritional components of peripartum management has increased through the last decade. Creating an environment that is low-stress and comfortable in both the pre- and postpartum period is likely as critical as the nutritional strategy employed and
will impact success on individual farms. Dry matter intake has been shown to be strongly associated with the degree of negative energy balance through the peripartum period (Drackley, 2005). The peripartum environment, particularly one that limits DMI and lying time, may have enduring effects on transition cow health. Feeding behaviour has been used to predict metabolic and infectious disease in research herds. At one week before calving, time spent feeding and DMI were able to identify cows at risk for severe metritis after calving (Huzzey et al., 2007). The underlying reason for reduction in feeding behaviour in some cows and not others was not determined but cows later diagnosed with severe metritis engaged in fewer aggressive interactions at the feedbunk and had reduced feeding and drinking times one week before calving. Goldhawk et al. (2009) found that cows that later developed ketosis ate less, spent less time eating and participated in fewer interactions up to two weeks before calving.

There is great variation in the management practices employed during the transition period with many of these practices based on management ease or diet delivery rather than cow preference or expression of natural behaviour. Management of cows in the peripartum period is often dictated by farm size and the nutritional strategy employed (Overton and Waldron, 2004). This period may be accompanied by several regroupings, pen changes, and overstocking (Cook and Nordlund, 2004). There are few behaviour studies using cows in the periparturient phase with much of the research originating from lactating cows. Of those in the transition period, a major limitation of many is that they are conducted on small research dairies with small numbers of cows and using group sizes that would not be practical on some commercial dairies. However, extrapolation of results from lactating cows or small transition groups may still be useful for successfully managing the cow environment.
Competition and Stocking Density

The pen size in transition cow facilities is usually based on an estimate of the proportion of the herd that will be in the transition stage of the lactation cycle, accounting for the targeted duration of time in the group (Cook, 2007). If pen sizes are based on the average flow of cows through the transition facility, the normal ebb and flow of calving over time will not be accounted for and pens may be overcrowded up to half of the time (Cook, 2009). Depending on pen design, crowding may be present at the feedbunk, at stalls or for bedded pack lying space, or both. Several studies have demonstrated that with increasing stocking density, feeding and lying behaviour will be altered (Olofsson, 1999; DeVries et al., 2004; Proudfoot et al., 2009). Moderate overcrowding in lactating pens has been reported to not affect stall access (Krawczel et al., 2008) or milk production if feeding is well managed (Krawczel et al., 2012), however recommendations for transition cow pens are that overcrowding should be avoided (Grant and Albright, 2001; Cook and Nordlund, 2004).

Research in lactating cow groups found that when the amount of feeding space was doubled from 0.5 to 1.0 m per cow in a free-stall barn, there were 57% fewer aggressive interactions while feeding (De Vries et al., 2004). Short term increases in lactating pen stocking densities for headlocks and stalls from 100% (1 cow per freestall and headlock) to 142% found a linear increase in displacements from the feedbunk, reduced lying time for stocking densities of 131 and 142% compared with 100 or 113%, and decreased rumination above 131%, but no differences in terms of fecal cortisol metabolites, milk yield or composition (Krawczel et al., 2012). However, the experience may not be the same for all cows in pens. A field study that housed primiparous and multiparous animals together reported a decrease of 0.7 kg/day in daily
milk yield for first-lactation cows for every 10% increase in stocking density above 80% headlocks (Oetzel et al., 2007).

Few studies have examined the effects of feeding space allowances in transition cows. Proudfoot et al (2009) examined 110 primiparous and multiparous cows in either a competitive (2 cows per feed bin) or non-competitive (1 cow per feed bin) transition environment and found that a competitive feeding environment increased displacements at feed bins regardless of parity. Competition had a tendency to decrease feed intake (-1.7 kg DM/d) of multiparous dry cows in the week before calving, driven by the tendency for smaller intakes per meal. Multiparous cows adapted to increased competition by two weeks postpartum by increasing their rate of feed intake at each visit. Additionally, competition increased the standing time of multiparous cows in the week after calving and decreased their lying time. The feeding bins used in research are not found on commercial dairies. Whether findings would be replicated at a feed rail or with headlocks is unknown. There is evidence that cows will typically only occupy 80% of 24” headlocks after fresh feed delivery which is accepted as peak feeding time (Huzzey et al., 2006; Nordlund et al., 2006). Some recommendations for transition cow pens take this into consideration and propose not stocking beyond 80% of feeding spaces (Nordlund et al., 2006). However, a controlled study with 728 Jersey cows that aimed to stock prepartum pens at 80% cows to headlocks compared to 100% of headlocks through the close-up dry period found no difference in RP, metritis, PVD, NEFA or BHB concentrations, early lactation culling, pregnancy to first or second AI, or milk yield up to 155 days postpartum (Silva et al., 2014).
Grouping Strategies

On many dairies, cows move through a series of pens including one or more prepartum pens, a calving pen and one or more postpartum pens, totalling as many as 5 pen changes during the 6 week period (Cook and Nordlund, 2004). These pen movements are typically management-driven, allowing for precise feeding in each phase and ease of handling. Along with these pen movements, cows may be added to groups weekly or even daily in fresh cow pens resulting in changing social structures. When cattle are moved between groups, a period of increased social interaction follows, including physical (bunting and fighting) and non-physical (threatening and avoidance behaviours) to determine social hierarchy (Kondo and Hurnik, 1990). When lactating cows were introduced into a new pen of 12 animals, regrouped cows spent 15 min less eating in the first hour after morning fresh feed delivery and experienced more displacements from the feedbunk during the first two days after regrouping, which was accompanied by a loss of 3.7 kg milk production on the day after regrouping (von Keyserlingk et al., 2008).

Many guidelines recommend maintaining a stable social group in transition pens to reduce stress (Grant and Albright, 2001; Cook and Nordlund, 2004). Cook (2009) has advocated a series of stable social group bedded pack pens sized to accommodate 140% of the average number of weekly calvings as the preferred prepartum close-up housing method. Such a design assembles a new stable close-up group each week and allows cows to calve in the same pen without necessitating a move to a separate calving pen. Despite this recommendation, there are few grouping studies using transition cows. Lobeck-Lucterhand et al. (2014) monitored 224 primiparous and multiparous close-up Jersey cows and reported fewer displacements from the feedbunk among cows assigned to an all-in-all-out housing system where no cows were added after the group was formed versus a traditional grouping strategy with twice-weekly additions to
maintain a desired stocking density of 100% of stalls and 91.6% of head gates. Whether changes in behaviour result in differences in metabolic and reproductive health is less clear. In the experiment cited above but using a larger sample of 567 Jersey prepartum cows, treatment did not affect the incidence of RP or metritis, NEFA or BHB concentrations, removal from the herd or energy corrected milk yield (Silva et al., 2013a). In a smaller study using 85 Holstein or Holstein-Jersey crossbred cattle, Coonen et al. (2011) demonstrated that cows housed in a socially stable prepartum pen for 2 to 4 weeks before expected calving had similar DMI, NEFA and in milk yield in the first 30 DIM compared with cows housed in pens with twice weekly entries, but sample size was insufficient to detect meaningful differences in these outcomes.

Social Ranking

The lack of a consistent effect of stocking density or regrouping on health or performance may be because not all cows are affected equally, and the experience is worse for some cows. Cows form dominance hierarchies within a group which are strongly associated with age, body size and seniority in the herd (Dickson et al., 1970). The permanence of these social relationships is uncertain with some work suggesting that once formed they tend to last for a long time (Beilharz and Zeeb, 1982) and other work suggesting they might change with weight changes or pen movements (Schein and Fohrman, 1955). Hasegawa et al. (1997) examined the effect of pen moves on dominant, middle-rank and subordinate first lactation cows and found that middle and subordinate heifers produced less milk in the week after regrouping, and subordinate heifers exhibited disrupted lying behaviour as evidenced by more time standing and shorter, more frequent lying bouts.

There may also be interactions between social rank and overstocking cows, as demonstrated by a stall access study conducted by Wierenga and Hopster (1990). Above 125% stocking density,
low rank cows shifted lying time to early evening hours when competition for stalls was less but at 155% stocking density, stall access was overwhelmed in the evening as well and lying time was reduced. A displacement index first established in pigs (Mendl et al., 1992) has been described for cattle (Galindo and Broom, 2000) as an index of success in agonistic interactions when feeding, and assigns cows as exhibiting low, moderate or high success at displacing other cows from the feedbunk. In herds stocked at 100% cows to stalls, low-success cows had lower lying times and greater time standing, with greater time perching in stalls than moderate or high success cows, and had significantly higher lameness incidence by mid lactation than high-success cows (60% vs. 18%; Galindo and Broom, 2000). Proudfoot et al. (2010) examined associations between behaviour around calving and the development of hoof lesions 8 to 12 weeks later and found cows that subsequently developed lesions spent more time standing and perching, ate faster during the two weeks before calving and were more likely to be low or high-ranking cows than cows without lesions. Researchers hypothesized that high ranking cows might be older cows with increased risk for lameness but did not have an adequate sample size to test this hypothesis.

DeVries et al. (2004) used this index to define low rank individuals in a group of lactating cows and found that when feeding space was increased from 0.5 to 1.0 m, feeding activity was increased among all cows, but most evidently for low-rank cows. Huzzey et al. (2012) examined 40 late gestation dry cows and found no differences in daily feeding time, total number of displacements or time to approach the feedbunk after feed delivery between the index levels; however cows in the low success group had a different physiological profile than high success cows, with greater average daily fecal cortisol metabolite concentrations, greater NEFA concentrations and a difference in peak insulin response to a glucose tolerance test.
Linking Immune Function and the Peripartum Environment

Many have theorized that stressors encountered in the transition period greatly contribute to the reduction in peripartum immune function during this time. One theory that would link infectious, environmental, social and nutritional stressors is the release of pro-inflammatory cytokines such as TNFα, IL-1β, and IL-6 and their associated signal-transduction mechanisms (Drackley et al., 2005). The role of inflammation in periparturient disease has previously been described but it is worth reiterating that an unregulated inflammatory response likely links the increased incidence of both metabolic and infectious disease during the peripartum period (Sordillo and Raphael, 2013). Stressors in the forms of pen changes or competition and resulting cytokine release would have the ability to impact peripartum cows in several ways, including decreasing dry matter intake resulting in increased body fat mobilization (Bertoni et al., 2008) or diverting nutrients to support the stress response (Moberg and Mench, 2000). There is some evidence that cytokine release can result in increased cortisol concentrations, resulting in greater reduction of immune function (Kushibiki et al., 2003; Waldron et al., 2003a). The release of hormones associated with the stress response such as glucocorticoids and epinephrine may alter immune function (Khansari et al., 1990) and result in altered IGF-1 (Kushibiki et al., 2003), insulin and glucagon secretion (Waldron et al., 2003a) with metabolic health and milk production consequences. When mid-lactation cows were infused with lipopolysaccharide (LPS), immune activation resulted in decreased serum concentrations of calcium and phosphorus, suggesting that cytokines might be involved in the etiology of hypocalcemia (Waldron et al., 2003b). Additionally, pro-inflammatory cytokines might alter the acute phase response in the liver as they have the ability to decrease synthesis of negative acute phase proteins and increase synthesis of positive acute phase proteins (Ingvartsen and Anderson, 2000; Drackley et al., 2005). All of these cytokine
cascades may interfere with the normal homeorhetic response and metabolic adaptations to lactation, putting cows with greater stress through the peripartum period at greater risk for metabolic and infectious disease.

Despite these hypothesized mechanisms, direct evidence for the role of stressors in increasing periparturient disease is lacking. Silva et al.(2013b) compared an all-in-all-out prepartum pen to twice weekly additions to maintain stocking density of 100% stalls and 92% of head gates using 68 prepartum Jersey cows. Although cows in the twice weekly addition pen had significantly higher cortisol one week prior to calving, weekly entry of new cows into the prepartum pen was insufficient to cause immunosuppression measured by neutrophil phagocytic and oxidative burst activities, expression of L-selectin or CD-18 or concentration of IgG anti-ovalbumin. In view of the overall importance of DMI in determining the extent of negative energy balance in the postpartum period (Drackley, 2005), practically, efforts to minimize stressor-induced limitations in DMI should be emphasized, but more research is necessary to better understand these mechanisms.

Research Objectives

The overall goal of the research described in this thesis is to contribute to the emerging understanding of the interactions between metabolic and immune health and management of dairy cows in the peripartum period.

The first objective was to evaluate the effect of prophylactic administration of a calcium supplement on blood calcium concentrations, the incidence of clinical disease and culling, milk production in early lactation and the probability of pregnancy at first insemination.
The second objective was to evaluate if administration of the same calcium supplement product at time of calving increased neutrophil oxidative burst or phagocytosis capacity.

The final objective was to test whether reducing social stress by providing non-competitive access to feeding and lying modified metabolic health and immune function and if differences in metabolic health and immune function could be explained using a measure of social status in the group.
REFERENCES


Chapter 2

RANDOMIZED CLINICAL TRIAL OF A CALCIUM SUPPLEMENT FOR IMPROVEMENT OF HEALTH IN EARLY LACTATION DAIRY COWS

ABSTRACT

Prophylactic calcium supplementation immediately after calving is a common strategy to prevent clinical and subclinical hypocalcemia in parturient dairy cows. The objective of this study was to evaluate the effect of prophylactic administration of Theracalcium® on blood calcium concentration at 24 and 48 hours after treatment, incidence of clinical disease and culling, milk production in early lactation, and on the probability of pregnancy at first insemination. Cows (n = 984) from 7 farms were blocked by parity and randomly assigned to receive either calcium gluconate (35% w/v) in combination with calcium glucoheptonate (10% w/v; Theracalcium, Vétoquinol Canada Inc., Lavaltrie, Quebec) or a placebo (medication vehicle solution with no calcium) at first contact with each cow after calving and again 12 - 24 hours later when available for lockup. Each dose was 120mL injected subcutaneously over two sites. Total serum calcium concentration (tCa) was measured from coccygeal blood samples before (time 0) and 24 and 48 hours after first treatment in a subsample of cows (n = 127). Beta-hydroxybutyrate (BHB) concentrations were measured from all cows twice between 3 and 16 DIM at weekly visits and cows were evaluated for vaginal discharge once between 28 and 42 DIM. Disease events, production data from the first three milk tests, reproduction and culling data were collected from each herd. For cows that had received 1 injection of calcium before the blood sample at 24 h (n = 95), tCa was significantly higher in the treated cows (P=0.01): mean ± SE 2.03 ± 0.03 versus 1.90 ± 0.03 mmol/L. At 48 h there was no significant difference in tCa between treatment and
control (mean ± SE 2.12 ± 0.02 and 2.10 ± 0.03 mmol/L, respectively). Cows treated with Theracalcium® were significantly less likely to have received supplemental calcium for exhibiting clinical signs of hypocalcemia than control cows (5.0% vs. 8.4%; P=0.02). There was no effect of treatment on retained placenta, metritis, hyperketonemia, prevalence of purulent vaginal discharge, culling from the herd, early lactation production, probability of pregnancy to first AI or time to pregnancy. With this subcutaneous prophylactic calcium treatment regimen, blood calcium levels were temporarily increased at 24 hours after treatment, however, there was no effect of supplemental calcium on the risk of disease or culling, milk production or reproductive performance.

INTRODUCTION

Milk fever (MF) or clinical hypocalcemia is a metabolic disorder that results when homeostatic mechanisms fail to maintain blood calcium levels around calving to the point that visible signs of muscle weakness occur. The lactational incidence risk (LIR) has been reported to range from 3 to 6% of cows of all parities depending on geographical location (DeGaris and Lean, 2009). McLaren et al. (2006) reported a mean milk fever incidence of 4.2% in a sample of 48 Ontario dairy herds. Milk fever is a serious management concern as failure to treat affected cows in a timely manner will result in death in nearly all affected animals (Oetzel, 2011). Cows that recover after intravenous calcium treatment are at increased risk for dystocia, uterine prolapse, retained placenta, metritis, mastitis, ketosis and displaced abomasum (Curtis et al, 1983; Markusfeld, 1987). Calcium (Ca) is critical for muscle and nerve function and reduced blood Ca compromises skeletal muscle strength and gastrointestinal motility which can predispose to reduced dry matter intake, increased incidence of metabolic diseases and decreased milk yield.
(Oetzel, 2013). Milk fever has also been associated with immune suppression by impairing the activity of mononuclear blood cells (Kimura et al., 2006).

Subclinical hypocalcemia (SCH) as a distinct but related disease entity to MF has been recognized more recently and been the focus of much research. The principle of SCH is that there are thresholds of blood Ca concentration below which there are undesirable consequences despite an absence of visible signs. The prevalence of plasma calcium between 1.5 and 2.0 mmol/L within 48 hours postpartum has been reported to range from 25 to 54% depending on parity (Reinhardt et al., 2011). Subclinical hypocalcemia around calving has been associated with increased odds of a displaced abomasum (DA), greater odds of culling, reduced milk yield in early lactation and a difference in early lactation fatty acid metabolism (Chapinal et al. 2011; Roberts et al. 2012; Chamberlin et al. 2013). Martinez et al (2012) observed that cows with a blood calcium concentration less than 8.59 mg/dL (2.15 mmol/L) at any point during the first three days in milk had lower concentrations of neutrophils, impaired neutrophil function and increased incidence of metritis compared to cows that maintained blood Ca above 2.15 mmol/L.

The consequences of milk fever and the inability to rapidly and practically identify SCH have emphasized the importance of prevention. Prior to calving, prevention strategies are principally dietary focused with attention to the dietary cation-anion difference (DCAD) of the pre-calving ration. One method to lower the DCAD includes the supplementation of anions, which increases the mobilization of Ca from bone before calving and reduces the risk of clinical milk fever (Charbonneau et al., 2006). Challenges with this approach include potentially needing a close up dry cow group for mature cows only, the necessity of urinary pH monitoring and reduced palatability leading to decreased dry matter intake (DMI). Other prevention methods including calcium deficient diets that stimulate parathyroid hormone secretion, feeding calcium binders or
feeding vitamin D or its analogues, have been less widely adopted due to a lack of practicality on account of available feedstuffs, cost and availability in North America (Goff, 2008; Oetzel, 2011).

A large number of calcium supplementation products that can be given orally or subcutaneously have been promoted to producers as additional insurance against hypocalcemia. These can be given ahead of expected parturition or immediately after if early milk fever symptoms are present. Administration to all cows or all multiparous cows after calving has been instituted in some herds. Some oral supplements such as calcium chloride (CaCl$_2$) are acidifying in addition to providing rapidly absorbed calcium. Given as a drench, gel or paste, or as a component of a calcium bolus, several studies have shown CaCl$_2$ raises blood calcium levels for 12 hours after administration (Goff and Horst, 2003; Sampson et al, 2009; Blanc et al, 2014). Subcutaneous administration of 500mL of calcium borogluconate (23%) has been shown to raise blood calcium concentrations to about 120% above baseline for approximately 6 hours after calving (Goff, 1999).

Several studies have examined the effects of calcium supplements on disease, production and reproduction of parturient dairy cows. A controlled trial administering CaCl$_2$ gel prophylactically to 204 Holstein cows in one herd found treated cows had higher serum calcium concentrations at days 1 and 2 after calving and reduced incidence rates of MF and DA (Oetzel, 1996). Conversely, Hernandez et al (1999) administered CaCl$_2$ gel to 60 cows of which one third had a RP and did not find a significant effect of treatment on serum calcium, the incidence of metritis or DA, time to first insemination, pregnancy status after first insemination or milk production. However, the small sample size limited ability to detect a difference in many of these outcomes. Melendez et al (2003) examined calcium chloride gel and a second calcium propionate
supplement on 479 cows in a single herd feeding anionic salts and found no treatment effect on milk fever, retained fetal membranes, metritis, ketosis, DA, conception at first service, overall pregnancy rate and services per pregnancy. More recently, a large controlled trial involving 927 multiparous cows from 2 herds evaluated the effect of supplementation with oral Ca boluses after calving and found that among cows with a previous lactation mature-equivalent milk production greater than 105% of herd rank, supplementation was associated with 2.9 kg more milk at first test after calving (Oetzel and Miller, 2012). Cows that were lame in the dry period that received the Ca boluses had a lower score for the sum of health events in the first 30 days after calving (health events included were metritis, ketosis, DA, mastitis, pneumonia and herd removal or death); however, there was no treatment effect on the other diseases, production or reproduction outcomes examined.

There are no large scale trials on the effects of any prophylactic subcutaneous calcium products. Theracalcium®, a combination of calcium gluconate and calcium glucoheptonate, is a calcium supplementation product that can be administered subcutaneously or intravenously. There is currently no peer-reviewed literature available on its efficacy in the treatment of milk fever or subclinical hypocalcemia.

The objective of this study was to evaluate the effect of prophylactic administration of Theracalcium® on the incidence of clinical disease and culling, milk production in early lactation, and on the probability of pregnancy at first insemination. In addition, the effect of administration of Theracalcium® on blood calcium concentrations at 24 and 48 hours after treatment, in cows without clinical hypocalcemia was evaluated.
MATERIALS AND METHODS

Study Population

The study was conducted on seven commercial dairy farms in Ontario from June 2013 to March 2014. Herd size ranged from 40 to 500 lactating cows. Herds were purposively selected on proximity to the University of Guelph and willingness to comply with the calcium supplementation protocols. To be eligible for enrollment study herds had to be subscribed to a milk recording service (Canwest DHI), administer calcium supplementation according to protocol directions to all cows calving in the herd during the study period and refrain from use of other forms of prophylactic calcium supplementation for the duration of the study. All herds fed a total mixed ration (TMR) and prepartum cows did not receive supplemental anionic salts. All herds consented to a study protocol that had been reviewed and approved by the University of Guelph Animal Care Committee.

Study Design and Data Collection

Cows were enrolled in the study by farm personnel within eight hours after calving. A sample size of 1000 cows was planned to detect a difference between 6.5% and 3% for the incidence of DA, 25% and 18% prevalence of subclinical ketosis, 16% and 10% prevalence of purulent vaginal discharge, 42% and 50% cows pregnant by 120 DIM and a 1kg/d milk yield difference with 95% confidence and 80% power. Cows were blocked within each herd as being in first or second parity, or third and greater parity. Within each block, cows were randomly assigned to receive the calcium supplementation product Theracalcium® or a placebo (identically packaged product vehicle without calcium). Farm personnel administered and recorded experimental treatments according to randomized assignment sheets provided and were blinded to the
treatment given. Each treatment was contained in one 250mL bottle, identified with a number. Treatments were randomly allocated and balanced in sets of 10 bottles. Cow ID, parity, calving ease and time of treatment administration were recorded by farm personnel.

Cows in the treated group received calcium gluconate (35% w/v) in combination with calcium glucoheptonate (10% w/v) for a total of 9.46g of calcium (Theracalcium, Vétoquinol Canada Inc., Lavaltrie, Quebec) divided into two doses, at first handling after calving as soon as possible to a maximum of 8 hours after calving, and again 12 to 24 hours later. Each dose was 120mL given over two sites (60mL per site) subcutaneously. Cows in the control group received a similar volume of placebo (medication vehicle solution with no active ingredient) at the time of enrollment and 12 to 24 hours later. During the first ten weeks of the study, a negative control was used because the production of the placebo was delayed. During this time producers enrolled cows as a treatment or negative control according to randomized assignment sheets but were not blinded.

Cows displaying signs of milk fever or injury resulting in inability to rise at time of first examination after calving were excluded from enrollment. If cows enrolled in the study began to show signs of clinical hypocalcemia, farm personnel recorded any supplemental calcium treatments given to the cow.

Prior to receiving treatment or placebo, whole blood samples were collected for calcium analysis on five of the seven farms by the farm personnel. Whole blood was collected from the coccygeal vein using a 20 gauge, 1-inch hypodermic needle (BD Vacutainer Precision Glide, Becton, Dickinson and Co., Franklin Lakes, NJ) into sterile, glass, commercial blood collection tubes without anticoagulant. Samples were immediately refrigerated on farm. Serum was subsequently
separated by centrifugation at 3000 rpm for 15 minutes and stored at -20 °C. The length of refrigeration before serum separation ranged from four hours up to three days. This storage and sampling protocol was validated by collecting four tubes of whole blood from 20 periparturient cows and refrigerating for 1, 24, 48 and 72 hours after collection prior to centrifugation. The mean concentration of calcium changed minimally (0.02 mmol/L) after 72 hours of refrigerated storage. All serum was submitted to the Animal Health Laboratory, University of Guelph for analysis of total calcium concentrations (tCa) and was measured using the Cobas Calcium Gen 2 kit (Roche Diagnostics, Indianapolis, IN, USA). The analytical sensitivity of the calcium assay is 0.2mmol/L and the inter-assay control coefficient of variation was 1.49%.

Additional whole blood samples were collected from a subset of 144 cows at 24 and 48 hours after enrollment according to the collection procedure previously listed, and analyzed for total calcium concentration. The sample size was designed to detect a difference of 0.2mmol/L total calcium concentration between groups. These samples represented all the animals that were enrolled from herds 2 and 4 and a convenience sample from herds 1 and 5 when a technician was available to take the samples on weekdays.

Herds were visited weekly on the same day of the week by research technicians. All cows that had been enrolled by farm personnel and were between 3 and 16 DIM had a blood sample collected for blood BHB measurement using the Precision Xtra meter (Abbott Laboratories, Abbott Park, IL). This resulted in all cows being tested twice one week apart. The Precision Xtra meter has been previously validated for use in cattle (Iwersen et al., 2009). Blood was collected from the coccygeal vessels via a 20 gauge x 2.54 cm needle and 3 mL syringe and ketone testing was performed immediately. The blood beta-hydroxybutyrate (BHBA) concentration displayed
was subsequently recorded. At first visit, all cows were assigned a body condition score according to Ferguson et al. (1994).

At the weekly visit, cows between 28-42 DIM were evaluated for vaginal discharge using a Metricheck device (Metricheck, Simcro, New Zealand) validated by McDougall et al. (2007). The vulva of each cow was cleaned and the metricheck inserted and advanced to the cranial vaginal fornix and then slowly retracted caudally. Material that remained on the concave surface of the device was classified according to the scoring system outlined by Sheldon et al. (2006).

Disease, production, reproduction and culling data for each herd were exported from DairyComp305 at the end of the study period. Milk weight, percent fat, percent protein, somatic cell count (SCC) and linear score (LS) were recorded from first, second and third DHI test for each animal enrolled. Projected mature-equivalent 305 day milk yield was collected from third test. Previous lactation mature-equivalent 305 was also obtained for cows that were second parity and greater.

The date of first insemination and date of pregnancy were exported from DairyComp305 at the end of the study. Herds 2 and 6 did not use DairyComp305 on farm to record reproductive events so breeding dates were obtained from farm records. A cut-off date of 7 months (214 days) after the last day of enrollment in the study was used to end the period during which cows could be recorded as pregnant. Dates of removal from the herd and the recorded reason for exit were obtained for each culled animal. All herds were provided with standardized definitions of milk fever, retained placenta, metritis, and displaced abomasum (LeBlanc, 2002). Herds 1, 3, 4, 5 and 7 used DairyComp305 to record disease events and this information was exported. Farm records from herds 2 and 6 were consulted weekly and recorded by researchers.
**Statistical Analysis**

All statistical analysis were performed in SAS (version 9.4, SAS Institute, Cary, North Carolina) considering the cow as the unit of interest.

**Blood Calcium Dynamics Models**

Among the sample of cows with daily blood samples, the outcomes of interest were blood calcium concentration at 24 and 48 hours after enrollment and whether blood calcium concentration was above a defined biologically significant cut point of 2.15mmol/L at 24 and 48 hours. Blood calcium concentrations at 0, 24 and 48 hours were examined for normality. Parity was set as 1, 2 and ≥3. Descriptive statistics were generated using the PROC MEANS and PROC FREQ procedures of SAS. Each variable was examined for association with the selected outcomes using contingency tables and the Chi-square statistic for categorical variables and univariable linear regression for continuous variables using PROC MIXED. Any variable associated with the outcome with $P < 0.2$ was offered to multivariable models. Treatment was retained in all models regardless of $P$ value. Interactions of variables with treatment were tested if biologically plausible and retained if statistically significant ($P < 0.05$). For all models, an autoregressive covariance error structure was utilized.

The effect of treatment on blood calcium concentration over time until 48 hours after enrollment was evaluated using a linear mixed model with cow as a repeated measure (PROC MIXED in SAS) and blood calcium concentrations at 24 and 48 hours after administration were evaluated individually using linear regression models (PROC MIXED in SAS). Parity and blood calcium concentration at the time of enrollment (tCa0) had a Pearson Correlation Coefficient of -0.69 and so only tCa0 was offered to the models. Recognizing that blood calcium concentration is often
not available to producers or practitioners, each model was built a second time with parity as surrogate for tCa0. The effect of treatment on tCa being above the defined cut point of 2.15mmol/L at 24 and 48 hours was evaluated using a logistic regression model (GLIMMIX procedure). This cut point was chosen based on its association with the risk of metritis and neutrophil function (Martinez et al, 2012).

Clinical Disease and Culling Models

The outcomes of interest were the occurrence of retained placenta, displaced abomasum, metritis, hypocalcemia (requiring additional calcium therapy), hyperketonemia at week 1, 2 or both after calving, purulent vaginal discharge at 5 weeks postpartum and removal from the herd within the first 60 days. Producers could give additional Ca if they judged that a cow was entering Stage I hypocalcemia. Metritis was defined as visible systemic illness with fetid vaginal discharge and rectal T > 39.5 °C. Hyperketonemia was defined as BHBA levels ≥ 1.2mmol/L (Raboisson et al., 2014). Cows with a purulent vaginal discharge score ≥2 were considered positive for PVD during analysis. Descriptive statistics were generated using PROC FREQ and PROC MEANS in SAS. Each variable was examined for association with the outcomes and any variable \( P < 0.2 \) was offered to multivariable models. For each model, variables were removed manually by backward stepwise elimination in order of highest \( P \)-value until only variables associated with the outcome remained \( (P < 0.05) \). With each variable removal, evidence of confounding was determined by a change in coefficient for treatment of >20%. Variables meeting these criteria were left in the model regardless of statistical significance.

The effect of Theracalcium® on clinical disease and culling was examined using multivariable logistic regression models (PROC GLIMMIX) with farm offered as a fixed effect. Treatment
was retained in all models regardless of $P$ value. Interactions were tested with treatment and retained if biologically plausible and significant.

**Milk Production and Reproductive Success Models**

The outcomes of interest for milk production were daily milk yield at first, second and third DHI test, daily milk yield over the three first tests in the lactation, and projected mature-equivalent 305 day milk yield at third test. All variables with a univariable association with the outcome ($P \leq 0.2$) in a univariable mixed regression model were offered to the multivariable model and built using the backward elimination process described previously. Daily milk yield at each test and projected mature-equivalent 305 day milk yield at third test were modeled using linear regression. Production over the 3 tests was modeled using linear regression with cow as a repeated measure (PROC MIXED). Treatment was retained in all models regardless of $P$ value and interactions with treatment were retained if biologically plausible and significant.

The probability of pregnancy at first insemination was modeled using a multivariable logistic regression model with farm as a fixed effect (GLIMMIX procedure). Backward elimination was used until only significant variables or their interactions remained ($P < 0.05$). A Cox proportional hazard model (PHREG procedure) considering the time to pregnancy with herd as a fixed effect was used to evaluate the impact of treatment on the interval from calving to subsequent pregnancy. Cows were censored at time of pregnancy, date of culling or reaching the data collection end date. A maximum observation time of 214 days was utilised.

Clinical disease, culling, milk production and reproduction outcomes were examined for all cows enrolled in the study ($n = 984$) and were modeled again for the 657 cows with pre-enrollment
blood samples offering tCa0 as a covariate. The latter models are described if tCa0 was significant in the final model.

All models were initially built testing placebo controls in separate models from the negative controls used for the first component of the study, prior to receiving the placebo. However, no differences were found in any of the models, therefore, placebo controls were combined with negative controls to make one control group for comparison.

RESULTS

Blood Calcium Study

Daily blood samples at 0, 24 and 48 hours were collected from 144 cows from four herds. Fifteen animals that received additional calcium therapy by the farm personnel for exhibiting clinical signs of hypocalcemia were excluded from analysis for a total of 129 animals used in all blood calcium concentration models (Table 2.1). Descriptive statistics for the 129 animals enrolled in the subsample are in Table 2.2. There was no difference in parity (2.20 ± 1.28 vs. 2.10 ± 1.18) or serum total calcium concentration at enrollment (2.07 ±0.28 vs. 2.01 ± 0.24 mmol/L) between treatment and control groups (P > 0.3). However, more cows in the treatment group than the control group had serum Ca ≥ 2.15 mmol/L at enrollment (43% vs. 26%, P < 0.05). At 22 ± 5.9 hours after enrollment, the mean calcium concentration was 2% higher than baseline (initial calcium concentration) for treated cows and 4% lower than baseline for control cows. At 48 hours, both groups were 4% above baseline concentrations. The proportion of cows above a biological threshold at each time point is outlined in Table 2.2. Cumulatively up to 48 hours, 72% of cows tested in this study were below 2.15 mmol/L at one or more of three tests.

Effect of Treatment on Blood Calcium Concentration over Time
The repeated measures model of the effect of treatment on blood calcium concentration until 48 hours after enrollment included treatment ($P < 0.0001$), time ($P < 0.0001$), blood calcium concentration at time 0 ($P < 0.0001$) and a treatment by time interaction ($P = 0.001$). As a result of the treatment by time interaction, total calcium concentration was examined at each time point separately.

Effect of Treatment on Blood Calcium Concentration after 24 Hours

In 96 cows, the 24h blood sample was obtained immediately prior to the second treatment. However, in the remaining 33 cows, the 24h blood sample was collected after the second treatment. Therefore, two separate models were built. For cows that had received one injection, variables included in the model for total blood calcium at 24 hours (tCa24) were treatment, blood calcium concentration at time 0 (tCa0) and a treatment by tCa0 interaction (Table 2.3). Least squares means between groups differed by 0.13 mmol/L. Treatment significantly increased tCa24 (LSM was $2.03 \pm 0.03$ in the treatment group and $1.90 \pm 0.03$ mmol/L in the control group; $P = 0.01$). The interaction of pre-treatment tCa with treatment is illustrated in Figure 2.1. Among Theracalcium® treated cows; there was a greater rise in tCa for cows with lower tCa0 compared to cows with a higher tCa0. In a similar model using parity as a covariate instead of tCa0; least squares means tCa24 was 0.20 mmol/L higher in the treatment group ($2.04 \pm 0.03$ vs. $1.84 \pm 0.03$ mmol/L; $P < 0.001$).

For cows that had received both treatment injections before the 24 hour blood sample (n=33), variables in the model for tCa24 were treatment and tCa0 ($P = 0.005$). Least squares means were $2.17 \pm 0.04$ mmol/L in the treated group and $2.07 \pm 0.05$ mmol/L in the control group ($P = 0.18$). When parity replaced tCa0 as a covariate, the model was similar and tCa tended to be higher in
the treatment group \( (P = 0.07) \) accounting for parity \( (P=0.02) \); LSM were 2.19 ± 0.05 mmol/L and 2.05 ± 0.06 mmol/L, respectively.

**Effect of Treatment on Blood Calcium Concentration after 48 Hours**

After 48 hours there was no effect of treatment on tCa \( (P = 0.46) \) after accounting for tCa0 \( (P < 0.0001) \) or parity \( (P <0.01) \) and farm as a fixed effect \( (P = 0.07) \). Least squares means were 2.12 ± 0.02 mmol/L and 2.09 ± 0.03 mmol/L for the treated and control groups, respectively.

**Effect of Treatment on the Proportion of Cows with Blood Calcium above a Biological Threshold**

Among the cows that had received one injection of treatment at 24 hours \( (n=96) \), there was no significant difference between treatment groups in the proportion of cows \( (36\%) \) with a blood calcium concentration greater than the cut point \( (P = 0.73) \), after accounting for tCa0 \( (P < 0.001) \). Similarly, at 48 hours \( (n=129) \) there was no difference between treatment groups in the proportion of cows above the cut point \( (P = 0.81) \) after accounting for tCa0 \( (P <0.01) \).

**Full Study**

A total of 994 cows were enrolled in the study, however 10 cows did not receive the treatment according to the protocol and were therefore excluded from analysis. The distribution of study animals by parity and treatment is presented in Table 2.5. Thirty-seven animals had been removed from the herd and were unavailable for ketone sampling in week one \( (3 \text{ to } 9 \text{ DIM}) \) and an additional 23 animals were not available at week two \( (10 \text{ to } 16 \text{ DIM}) \). Postpartum at 28 to 42 DIM, 868 animals were available for Metricheck examination with losses due to death \( (n=25) \), culling \( (n=46) \) and sale for dairy \( (n=45) \).
Of the 984 cows enrolled, blood samples were collected from 659 animals prior to treatment. Two blood samples with values close to zero were excluded as laboratory error. The distribution of total blood calcium by parity is displayed in Figure 2.2. The numbers enrolled in each of the treatment and control groups are displayed in Table 2.5.

**Effect of Treatment on Clinical Disease**

There were no differences in the lactational incidence risk between groups for any of the clinical diseases recorded (Table 2.6). The recorded lactational incidence risk (LIR) of retained placenta (RP) in the study population was 6.6%. Treatment had no effect on the incidence of RP ($P = 0.92$) after accounting for farm ($P < 0.0001$), body condition score ($P = 0.06$) and twins ($P < 0.01$). The recorded LIR of metritis in the study was 3.4%. Treatment had no effect on the incidence of metritis ($P = 0.13$) after accounting for parity ($P < 0.01$) and calving difficulty score ($P < 0.01$). There were only 13 displaced abomasum (DA) cases during the study and therefore not enough to model the effect of treatment on DA.

**Effect of Treatment on Additional Calcium Therapy**

There were 65 animals (6.6%) that displayed clinical signs of hypocalcemia after enrollment and required treatment with additional calcium therapy. Therapies were producer or veterinarian directed and included one or more of intravenous, subcutaneous or oral calcium. Of the animals treated, 29 cows (45%) were treated with subcutaneous or oral calcium only and 36 cows (55%) were treated with intravenous calcium with or without subcutaneous or oral calcium products. Variables retained in the model for effect of treatment on hypocalcemia included parity and twins. Cows treated with Theracalcium® were significantly less likely to require additional
calcium therapy than control cows (5.0% vs. 8.4%; $P=0.02$). Third and greater parity cows and cows that had twins were significantly more likely to require additional therapy (Table 2.7).

**Effect of Treatment on Hyperketonemia**

The prevalence of ketosis at each of weeks one and two was 14% with a cumulative incidence of 22% during the first two weeks of lactation. Variables retained in the week 1 postpartum ketosis model included farm ($P<0.0001$), season ($P<0.0001$) and parity ($P<0.0001$). At week 2, farm ($P<0.0001$), parity ($P=0.03$) and ketosis at week 1 ($P<0.0001$) were retained. Treatment had no effect on the prevalence of subclinical ketosis in either week ($P>0.37$), or cumulatively ($P=0.50$) over the two weeks (modeled with identical variables as week 1).

**Effect of Treatment on Purulent Vaginal Discharge**

The mean ± SD at which cows enrolled were examined for purulent vaginal discharge was 33 days (±3.4). The prevalence of purulent vaginal discharge (PVD) was 13%. Treatment had no effect on the prevalence of PVD ($P=0.21$) after accounting for twins ($P<0.01$), parity ($P=0.05$) and having one of RP, DA or metritis ($P<0.0001$).

**Effect of Treatment on Culling**

Within the first 60 days of lactation, 7.8% of animals were culled (n=76). There was no effect of treatment on the odds of being culled in the first 60 days (OR=0.95, CI 0.58 to 1.55) after accounting for body condition score ($P<0.0001$), parity ($P<0.01$) and displaced abomasum ($P<0.01$). Among the 657 animals with a blood sample collected at enrollment, the model included tCa₀ ($P<0.01$) and body condition score ($P<0.0001$) but there was no effect of treatment ($P=0.6$).
Effect of Treatment on Milk Production in Early Lactation

Covariates retained in each of the models for daily milk yield at the first three DHI test days included farm as a fixed effect ($P < 0.0001$), parity ($P < 0.0001$), fat percentage ($P < 0.002$), protein percentage ($P < 0.04$) and linear somatic cell count score ($P < 0.01$). In all three test day models, cows that were in third or greater parity, had a lower percentage fat or protein, or a lower somatic cell count score had greater milk yield. At first test, season ($P = 0.02$), days in milk at test ($P < 0.0001$) and having a transition disease ($P < 0.0001$) were also retained in the model such that cows that calved in the summer, had lower DIM at test-day or had a transition disease made less milk. At second test, cows that calved in the summer produced less milk ($P = 0.0002$) while cows that had ketosis in week 1 or 2 tended to produce more milk ($P = 0.07$). At third test, DIM was retained ($P < 0.0001$) such that as DIM increased, milk production decreased. Treatment had no effect on milk production in any of the three test day models ($P > 0.13$).

For the subset of cows in second or greater lactation (n=576), previous mature equivalent 305-day milk production (me305) was offered to each test day model as a covariate. The variable was significant in all three models ($P < 0.0001$) in addition to the variables listed above. The effect of treatment on milk production was unchanged ($P > 0.45$).

The repeated measures model for milk production in early lactation (all three DHI tests) included farm as a fixed effect ($P < 0.0001$), parity ($P < 0.0001$), percent fat ($P < 0.0001$), percent protein ($P < 0.0001$), linear somatic cell score ($P < 0.0001$), DIM at test day ($P < 0.0001$) and the quadratic term for DIM ($P < 0.0001$). Treatment had no effect on milk production ($P = 0.18$). In a model for animals in second or greater parity, previous me305 was included ($P < 0.0001$) but there was no interaction with treatment or an effect of treatment.
At the third test day, variables in the projected milk yield 305 model included farm as a fixed effect \((P <0.0001)\), parity, \((P <0.0001)\), season \((P = 0.0004)\), percent protein \((P <0.0001)\), percent fat \((P = 0.0008)\), and subclinical ketosis \((P = 0.06)\). There were no interactions with treatment and no effect of treatment \((P = 0.22)\) on projected 305 day milk yield.

**Effect of Treatment on Reproductive Success**

Among the 784 animals that were eligible to conceive after parturition, the probability of pregnancy to first AI was 47%. Treatment had no effect on pregnancy at first insemination \((P = 0.46)\) after accounting for RP \((P = 0.03)\) and body condition score at enrollment \((P = 0.03)\).

The model of the effect of treatment on time to pregnancy model retained the variables farm (as a fixed effect; \(P <0.0001)\), parity \((P <0.001)\), season \((P = 0.03)\) and RP \((P = 0.04)\). Treatment had no effect on time to pregnancy \((P = 0.59)\). The survival curves for time to pregnancy are displayed in Figure 2.3.

**DISCUSSION**

The purpose of this study was to evaluate the effect of Theracalcium® on blood calcium concentrations, clinical disease, culling, milk production in early lactation, and on the probability of pregnancy. This is the first large-scale study to examine the administration of Theracalcium® prophylactically after calving. The population of cows used for the study were purposively chosen from farms willing to participate and comply with the protocol. These were well managed farms with good animal husbandry, attention to diets and animal health. The blood calcium results demonstrate these farms did have hypocalcemia present despite high-quality management.
Using the traditional cut point of 2.0mmol/L, the prevalence of subclinical hypocalcemia approximately 1 to 8 hours after calving was 38% in the animals enrolled with a blood sample at calving. Among third or greater parity cows only, the prevalence of hypocalcemia using this cut point was 57%. Although not a random sample of the population, these findings are similar to those reported in Reinhardt et al. (2011) for second and greater lactation cows. Reinhardt et al. (2011) however reported a prevalence of 25% subclinical hypocalcemia in first lactation animals while we found only 1% of animals with tCa < 2.0mmol/L. It should be noted that Reinhardt et al. (2011) took samples over the first 48 hours while the present study samples were collected at time of enrollment after calving which does not include the typical nadir of blood calcium between 12 and 24 hours after calving (Goff, 2008).

A large majority of the time-of-enrollment samples came from one larger herd so it would be inappropriate to extrapolate these results to the Ontario dairy population. However there are no studies that report the prevalence of subclinical hypocalcemia immediately after calving in Canadian dairy cattle. A previous study collected blood samples once weekly between 1 and 7 days after calving from 24 dairy farms in Ontario and reported that 5.8% of animals were below the same cut point of 2.0mmol/L during this time (Seifi et al, 2011). A window of 1 to 7 DIM may not capture cows that had hypocalcemia initially which resolved within the first 2 to 3 DIM as was evident for many cows in the current study. Martinez et al. (2012) found that cows with blood calcium less than 8.59 mg/dL or 2.15mmol/L at least once during the first 3 DIM had impaired neutrophil function and increased incidence of metritis. Using this cut point, 72% of 129 cows tested repeatedly after calving in our study were below 2.15 mmol/L at one or more of three tests in the 48 hours and therefore at greater risk.
With this subcutaneous prophylactic calcium protocol, blood calcium levels were temporarily increased at 24 hours after treatment. Accounting for pre-treatment blood Ca and its interaction with treatment, on average, one of the two doses of treatment increased blood Ca by 0.13 ± 0.04 mmol/L. Among Theracalcium® treated cows; there was a greater rise in tCa for cows with lower tCa0 compared to cows with a higher tCa0. Biologically, these cows are in greater need of calcium to re-establish calcium homeostasis compared to cows with higher blood calcium at the time of calving. Based on the model (Table 2.3), treatment did not increase tCa24 among cows with a tCa0 ≥ 2.4 mmol/L but there were very few cows in the study with levels this high (n=26, 2.6%).

The goal of prophylactic administration of Ca is to mitigate the calcium nadir that occurs between 12 and 24 hours after calving (Goff, 2008) when calcium demand has spiked and homeostasis strategies are not yet fully functional. Cows may begin to show signs of Stage I milk fever in the range of 1.4-1.9 mmol/L (Oetzel, 2011) but the exact tCa at which a cow exhibits clinical signs is not consistent. According to our results (Table 2.3), cows in this range would experience a 0.18 to 0.33mmol/L rise in tCa in response to subcutaneous Theracalcium® treatment, which may be enough to mitigate early milk fever signs in some cows. This may be reflected in the lower proportion of animals in the treated group that were treated for hypocalcemia.

Throughout the study, producers were able to give additional calcium therapy to enrolled cows only if these cows showed signs of hypocalcemia such as trembling, weakness, or recumbence. It is presumed that the 29 cows treated with subcutaneous or oral calcium only were showing Stage I hypocalcemia signs whereas the 36 cows that received intravenous calcium alone or with subcutaneous or oral calcium may represent cows showing signs of either Stage I or Stage II
hypocalcemia. Producers were blinded to the treatment given to prevent bias for this outcome in particular. Of the 65 animals that received additional Ca therapies for milk fever, 60% were in the control group. The finding that cows treated with Theracalcium® were significantly less likely to require additional calcium therapy shows that the blinded producers observed fewer symptoms of milk fever among these cows.

The increased risk for disease, poorer immune function and production losses associated with lower tCa in the days after calving suggests that mitigating hypocalcemia would be advantageous. Using the cut point of 2.15 mmol/L from Martinez et al. (2012), treated cows were not more likely to be above this cut point at 24 or 48 hours after enrollment. A 0.13mmol/L average rise in tCa from 0 to 24h was insufficient for most cows to reach 2.15mmol/L based on tCa0. Theracalcium® mitigated decline as on average tCa24 were 2% above baseline compared to 4% below in control cows but this did not raise blood calcium to a threshold that is predicted to have effects on immune function and risk of disease based on other reported studies (Chapinal et al., 2011; Martinez et al., 2012).

In accordance with this prediction, there was no effect of treatment on any of the disease, milk production or reproduction outcomes. There are no other subcutaneous calcium supplementation controlled trials with which to compare these results, however Melendez et al. (2002) and Oetzel and Miller (2012) found no effects of two different oral calcium supplements on blood calcium concentration, early lactation health or milk yield in cows supplemented with anionic salts. Oetzel and Miller (2012) reported very low levels of milk fever (0.6%) and hypocalcemia (14%, ionized calcium ≤ 1.0 mmol/L between 8 and 35 h after calving) in the two herds used so there may have been less opportunity to mitigate hypocalcemia. The present study differs from those above as none of the herds fed anionic salts prepartum and the incidence of milk fever (6.6%)
and hypocalcemia (43%, using a similar cut point of total calcium < 2.0 mmol/L between 0 and 8 hours among cows of parity ≥ 2), were considerably higher; however there was still no effect on any of these outcomes.

A tCa of ≤2.2 mmol/L in the first week after calving was associated with 2.7 greater odds of DA in a large sample of herds across Canada and the United States (Chapinal et al., 2011). Prophylactic calcium treatment might therefore be hypothesized to reduce the risk of DA but only Oetzel et al. (1995) found reduced incidence of DA among cows of all parities treated with 3 to 4 doses of CaCl₂ gel (54 g elemental calcium/dose) at 12 hour increments beginning 12 hours before predicted calving (n = 204). Other controlled studies using prophylactic calcium found no effect of treatment on the incidence of DA (Hernandez et al., 1999; Melendez et al., 2002; Oetzel and Miller, 2012). The current study had a very low incidence of DA (1.3%) which was verified at weekly visits by study personnel but did not provide an adequate sample size to examine risk despite enrolling almost 1000 cows.

Martinez et al. (2012) found that cows with tCa < 2.15 mmol/L between 1 and 3 DIM were at increased risk of metritis compared to cows that maintained higher tCa. Therefore calcium therapies that maintain normocalcemia might have an effect on metritis. The incidence of metritis in this study was 3.4% which is low compared to other reported studies (Chapinal et al., 2011; Kelton et al., 1998) and may indicate there was under-reporting despite giving producers a standardized disease definition (systemic illness including fever >39.5°C with fetid discharge) and verifying against treatment records. Cows in the study herds were examined thoroughly and rectal temperature taken only if daily observation indicated illness. This approach may have diagnosed fewer cows than a systematic daily fresh cow exam protocol would find. However with only seven herds, it is possible this is normal for these well managed herds. Prophylactic
calcium treatment with Theracalcium® had no effect on the incidence of metritis. No other controlled calcium prophylaxis studies have reported an effect on metritis including a study that enrolled a group of cows with RP and therefore had greater risk of metritis (Hernandez et al., 1999).

Early lactation is a time of higher risk for cows to leave the herd. Data from 4134 dairy cows in Canada and the United States established a threshold of calcium ≤2.2 mmol/L in the first week after calving to predict culling risk with these cows having 1.5 greater odds of removal from the herd compared to cows above the threshold (Roberts et al., 2012). Similar to Oetzel and Miller (2012), prophylactic calcium treatment with Theracalcium® had no effect on culling although tCa0 was an important predictor of culling risk. The lack of treatment effect on culling is not a surprising result as there was an absence of effect on other transition disease outcomes which likely more directly influence culling risk in early lactation.

Chapinal et al. (2012) found that cows with calcium ≤2.1 mmol/L in the first week after calving had lower average milk production at the first DHIA test with an estimated reduction of 2.6 kg/day. No treatment effects on milk production were identified in the current study. Likewise, Melendez et al (2002) did not find any improvement in milk yield for cows treated with oral CaCl\textsubscript{2} gel fed anionic salts before calving. In contrast, Oetzel and Miller (2012) found that cows with a higher previous lactation mature equivalent milk production (greater than 105% of herd rank) supplemented with oral Ca boluses produced 2.9 kg more milk at first test, but no treatment effects in other subpopulations or on other early lactation outcomes. It is not known if any effect would be realized using Theracalcium® in a population with a lower prevalence of hypocalcemia similar to the herds in Oetzel and Milller (2012) that fed an acidified diet. There might have been
a production effect of treatment if more cows were closer to the thresholds observed by Martinez et al., 2012 and Chapinal et al., 2011.

Minimizing postpartum reproductive disease and promptly returning to cyclicity is essential to ensuring subsequent pregnancy. Caixeta et al. (2015) reported cows that remained normocalcemic in the first three days after calving had higher levels of progesterone in the first 55 days after calving and resumed ovarian activity earlier than hypocalcemic cows. Additionally, in a large North American study (Chapinal et al., 2012), cows with low serum Ca in the week before or after calving had reduced odds of pregnancy at first service. Although milk fever has been associated with PVD (Whiteford and Sheldon, 2005), in the present study, treatment did not affect the prevalence of PVD between 4 and 6 weeks after calving which might be expected as tCa0 was not predictive of PVD in this study. There are no other calcium prophylaxis studies that have examined this outcome. Similarly, there was no effect of treatment on pregnancy at first service or time to pregnancy. Other calcium supplementation studies that have looked at reproductive outcomes have found no effect of calcium treatment. Hernandez et al. (1999) found no effect of CaCl2 gel treatment on conception at first service in a small population of cows (n=60) where one third of cows enrolled had an RP, but study power limited their ability to detect a difference. The large (n = 927) oral calcium bolus supplementation study (Oetzel and Miller, 2012) that looked at time to pregnancy produced analogous results to our findings.

It is unclear whether a larger dose of Theracalcium® than was used in the study protocol would lead to different results. There is no literature available exploring alternative dosing such as increasing the initial dose immediately after calving or repeated administration at different times than used here. The duration of action of most prophylactic products and the blood calcium nadir that occurs between 12 and 24 hours in parturient dairy cows commonly necessitates a second
dose of calcium prophylaxis in this time period (Oetzel, 2013). Among Theracalcium® treated 
cows there was increased tCa 24 hours after initial treatment even in cows that had only received 
120mL of product up to that point, suggesting that the effect is long lasting. The magnitude and 
timing of peak tCa in the preceding 24 hours is unknown but understanding the pharmacokinetics 
of the product is necessary in order to explore alternative dosing strategies. Martinez et al (2012) 
reported that cows with tCa less than 2.15mmol/L at any point in the first 72 hours after calving 
were at risk of compromised immune function and greater risk of metritis, so alternative dosing 
that keeps cows closer to this cut point may have greater effects on early lactation health and 
production. A comparison between the dose and timing used here and larger and/or repeated 
doses with more intensive blood sampling in the first 24 hours should be explored.

CONCLUSIONS

Results of this study demonstrate that prophylactic use of two doses of subcutaneous calcium 
was effective in modestly increasing serum calcium concentrations in the first day after calving 
and reduced the proportion of cows that received supplemental calcium because they exhibited 
clinical signs of hypocalcemia. Under the treatment regime studied, there was no effect of 
supplemental calcium on the risk of disease or culling, milk production or reproductive 
performance.
REFERENCES


Table 2.1: Distribution of Holstein cows enrolled in a randomized controlled trial of an injectable calcium supplement administered within 8 h after calving. Blood samples for measurement of serum total calcium were collected from a subset of animals.

<table>
<thead>
<tr>
<th>Farm</th>
<th>Number of Animals Enrolled in Study</th>
<th>Number of Animals with Blood Sample at Enrollment</th>
<th>Number of Animals with Blood Samples at 24 and 48h after Enrollment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>73</td>
<td>19</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>74</td>
<td>70</td>
<td>65</td>
</tr>
<tr>
<td>3</td>
<td>136</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>34</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>45</td>
<td>31</td>
<td>24</td>
</tr>
<tr>
<td>6</td>
<td>81</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>551</td>
<td>507</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>994</td>
<td>661</td>
<td>129</td>
</tr>
</tbody>
</table>
Table 2.2: Descriptive statistics for subset of 129 animals from 4 herds enrolled in a randomized controlled trial of an injectable calcium supplement administered within 8 h after calving. Blood samples for measurement of serum total calcium were collected at 0, 24 and 48 h relative to enrollment.

<table>
<thead>
<tr>
<th>Variable</th>
<th>N</th>
<th>Mean</th>
<th>Median</th>
<th>Standard Deviation</th>
<th>Min</th>
<th>Max</th>
<th>% Ca ≥ 2.15 mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parity</td>
<td>129</td>
<td>2.15</td>
<td>2.0</td>
<td>1.23</td>
<td>1</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Total calcium at enrollment (mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parity 1</td>
<td>49</td>
<td>2.25</td>
<td>2.24</td>
<td>0.11</td>
<td>2.01</td>
<td>2.53</td>
<td>83.7</td>
</tr>
<tr>
<td>Parity 2</td>
<td>38</td>
<td>2.05</td>
<td>2.09</td>
<td>0.17</td>
<td>1.68</td>
<td>2.31</td>
<td>42.1</td>
</tr>
<tr>
<td>Parity 3+</td>
<td>42</td>
<td>1.80</td>
<td>1.79</td>
<td>0.25</td>
<td>1.31</td>
<td>2.24</td>
<td>11.9</td>
</tr>
<tr>
<td>Total calcium at 24 h after enrollment (mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parity 1</td>
<td>49</td>
<td>2.16</td>
<td>2.15</td>
<td>0.12</td>
<td>1.97</td>
<td>2.41</td>
<td>53.1</td>
</tr>
<tr>
<td>Parity 2</td>
<td>38</td>
<td>2.00</td>
<td>2.04</td>
<td>0.24</td>
<td>1.48</td>
<td>2.46</td>
<td>29.0</td>
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<tr>
<td>Parity 3+</td>
<td>42</td>
<td>1.86</td>
<td>1.88</td>
<td>0.33</td>
<td>1.23</td>
<td>2.56</td>
<td>23.8</td>
</tr>
<tr>
<td>Total calcium at 48 h after enrollment (mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parity 1</td>
<td>49</td>
<td>2.16</td>
<td>2.19</td>
<td>0.15</td>
<td>1.70</td>
<td>2.47</td>
<td>57.1</td>
</tr>
<tr>
<td>Parity 2</td>
<td>37</td>
<td>2.11</td>
<td>2.10</td>
<td>0.15</td>
<td>1.63</td>
<td>2.37</td>
<td>31.6</td>
</tr>
<tr>
<td>Parity 3+</td>
<td>41</td>
<td>2.03</td>
<td>2.08</td>
<td>0.24</td>
<td>1.48</td>
<td>2.43</td>
<td>40.5</td>
</tr>
</tbody>
</table>
**Table 2.3:** Final model for blood calcium concentration 24h after enrollment for 96 cows from 4 herds having received one injection of treatment at time 0.

<table>
<thead>
<tr>
<th>Variable</th>
<th>β</th>
<th>SE</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>0.15</td>
<td>0.24</td>
<td>0.52</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Theracalcium</td>
<td>0.79</td>
<td>0.31</td>
<td>0.01</td>
</tr>
<tr>
<td>Control</td>
<td>referent</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Total Calcium at Time 0 (tCa0)</td>
<td>0.88</td>
<td>0.12</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>tCa0*Theracalcium</td>
<td>-0.32</td>
<td>0.15</td>
<td>0.03</td>
</tr>
<tr>
<td>tCa0*Control</td>
<td>0</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>
Table 2.4: Final model for blood calcium concentration 24h after enrollment for 96 cows from 4 herds having received one injection of treatment at time 0 considering parity as a covariate instead of pre-treatment serum calcium concentration

<table>
<thead>
<tr>
<th>Variable</th>
<th>$\beta$</th>
<th>SE</th>
<th>$P$-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>1.63</td>
<td>0.06</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Theracalcium</td>
<td>0.30</td>
<td>0.08</td>
<td>0.0002</td>
</tr>
<tr>
<td>Control</td>
<td>referent</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Parity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.49</td>
<td>0.08</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2</td>
<td>0.12</td>
<td>0.09</td>
<td>0.16</td>
</tr>
<tr>
<td>3+</td>
<td>referent</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Theracalcium*Parity1</td>
<td>-0.29</td>
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<td>0.06</td>
</tr>
<tr>
<td>Theracalcium*Parity2</td>
<td>-0.02</td>
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<td>0.84</td>
</tr>
<tr>
<td>Theracalcium*Parity3</td>
<td>--</td>
<td>--</td>
<td>--</td>
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<tr>
<td>Control*Parity1</td>
<td>--</td>
<td>--</td>
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<td>--</td>
</tr>
<tr>
<td>Control*Parity3</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
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Table 2.5: Distribution of Holstein cow enrollment by treatment and control groups in a randomized controlled trial of an injectable calcium supplement administered within 8 h after calving.

<table>
<thead>
<tr>
<th>Parity Group</th>
<th>n</th>
<th>Treatment</th>
<th>Placebo Control</th>
<th>Negative Control</th>
<th>Total Control Cows</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>345</td>
<td>180</td>
<td>135</td>
<td>30</td>
<td>165</td>
</tr>
<tr>
<td>2</td>
<td>231</td>
<td>130</td>
<td>87</td>
<td>14</td>
<td>101</td>
</tr>
<tr>
<td>3</td>
<td>408</td>
<td>208</td>
<td>169</td>
<td>31</td>
<td>200</td>
</tr>
<tr>
<td>All Cows</td>
<td>984</td>
<td>518</td>
<td>391</td>
<td>75</td>
<td>466</td>
</tr>
</tbody>
</table>
**Table 2.6:** Description of covariates and disease outcomes in 984 Holstein cows randomly assigned to receive two injections of Theracalcium (total of 240mL SC within approximately 24 h after calving) or placebo.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Treatment n=518</th>
<th>Control n=466</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of Cows</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PARITY</td>
<td></td>
<td></td>
<td>0.43</td>
</tr>
<tr>
<td>1</td>
<td>52</td>
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<td></td>
</tr>
<tr>
<td>2</td>
<td>56</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>3+</td>
<td>51</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>Twins</td>
<td>5</td>
<td>5</td>
<td>0.94</td>
</tr>
<tr>
<td>Dystocia(^1)</td>
<td>17</td>
<td>16</td>
<td>0.71</td>
</tr>
<tr>
<td>RP</td>
<td>7</td>
<td>6</td>
<td>0.84</td>
</tr>
<tr>
<td>Metritis</td>
<td>4</td>
<td>3</td>
<td>0.20</td>
</tr>
<tr>
<td>BCS at enrollment</td>
<td></td>
<td></td>
<td>0.11</td>
</tr>
<tr>
<td>Thin (&lt;2.75)</td>
<td>21</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Moderate (3-3.5)</td>
<td>73</td>
<td>79.0</td>
<td></td>
</tr>
<tr>
<td>Fat (&gt;3.75)</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Received Additional Calcium</td>
<td>5</td>
<td>8</td>
<td>0.03</td>
</tr>
<tr>
<td>Ketosis (BHBA ≥1.2 mmol/L) at Week 1 (3-9DIM)</td>
<td>15</td>
<td>13</td>
<td>0.18</td>
</tr>
<tr>
<td>Ketosis (BHBA ≥1.2 mmol/L) at Week 2 (10-16 DIM)</td>
<td>16</td>
<td>13</td>
<td>0.12</td>
</tr>
<tr>
<td>Ketosis – 3-16 DIM</td>
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<td>20</td>
<td>0.35</td>
</tr>
<tr>
<td>Displaced Abomasum</td>
<td>2</td>
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</tr>
<tr>
<td>Culling ≤60 DIM</td>
<td>8</td>
<td>8</td>
<td>0.81</td>
</tr>
<tr>
<td>Purulent Vaginal Discharge</td>
<td>12</td>
<td>14</td>
<td>0.28</td>
</tr>
</tbody>
</table>

\(^1\) Dystocia defined as a calving difficulty score greater than 1; 1=Unassisted 2= Easy pull (one person, <15 min) 3=Hard pull (>15 min, use of calving jack or multiple people) 4=Caesarian section
Table 2.7: Final model for clinical hypocalcemia requiring additional calcium treatment in 984 Holsteins from 7 herds.

<table>
<thead>
<tr>
<th>Variable</th>
<th>β</th>
<th>SE</th>
<th>P-Value</th>
<th>OR</th>
<th>95% CI</th>
</tr>
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<tr>
<td>Intercept</td>
<td>-0.03</td>
<td>0.40</td>
<td>0.95</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Treatment</td>
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<td></td>
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<tr>
<td>Theracalcium</td>
<td>-0.60</td>
<td>0.28</td>
<td>0.03</td>
<td>0.55</td>
<td>0.32 to 0.95</td>
</tr>
<tr>
<td>Control</td>
<td>referent</td>
<td></td>
<td></td>
<td></td>
<td>--</td>
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<tr>
<td>Parity</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>-3.96</td>
<td>1.01</td>
<td>0.0001</td>
<td>0.02</td>
<td>0.003 to 0.14</td>
</tr>
<tr>
<td>2</td>
<td>-1.54</td>
<td>0.40</td>
<td>0.0001</td>
<td>0.21</td>
<td>0.10 to 0.46</td>
</tr>
<tr>
<td>3+</td>
<td>referent</td>
<td></td>
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<tr>
<td>Twins</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>0</td>
<td>-1.70</td>
<td>0.40</td>
<td>&lt;0.0001</td>
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<td>0.08 to 0.40</td>
</tr>
<tr>
<td>1</td>
<td>referent</td>
<td></td>
<td></td>
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<td>--</td>
</tr>
</tbody>
</table>
**Figure 2.1**: The interaction of injectable calcium supplementation with pre-treatment blood calcium on blood total calcium 24 hours after initial treatment in 96 Holstein cows from 4 herds that had received half (120mL) of the total treatment at time of blood sampling. The LSM for tCa at 24h after initial treatment was 0.13 mmol/L higher in the treatment group.
Figure 2.2: Distribution of serum total calcium concentration by parity at the time of enrollment (approximately 1 to 8 hours after calving) for 657 Holstein cows in a randomized controlled trial of an injectable calcium supplement.
**Figure 2.3:** Time to pregnancy measured with Kaplan-Meier regression in 984 Holstein cows randomly assigned to receive two injections of Theracalcium (total of 240 mL SC within approximately 24h after calving) or placebo. Time to pregnancy was not different ($P = 0.26$) between treatment groups; the median (95% CI) was 108 (96 to 121) days in the Theracalcium group and 96 (89 to 113) days in the control group.
CHAPTER 3

THE EFFECT OF CALCIUM SUPPLEMENTATION ON NEUTROPHIL FUNCTION IN EARLY LACTATION

ABSTRACT

Low total blood calcium concentration after calving has been demonstrated as a risk factor for reduced neutrophil function. The objective of this study was to evaluate if administration of an injectable calcium supplement product at time of calving increased neutrophil oxidative burst or phagocytosis capacity. Cows (n=27) from 4 farms were blocked by parity and randomly assigned to receive either calcium gluconate (35% w/v) in combination with calcium glucoheptonate (10% w/v; Theracalcium, Vétoquinol Canada Inc., Lavaltrie, Quebec) or a placebo (medication vehicle solution with no calcium) within 12 hours after calving and again 24 hours later. Each dose was 120mL injected subcutaneously over two sites. Total serum calcium concentration (tCa), neutrophil oxidative burst and neutrophil phagocytosis capacity were measured from coccygeal blood samples before (time 0) and 72 hours after first treatment. There was no difference in lactation number, total calcium concentration, oxidative burst or phagocytosis at time of enrollment between treatment groups ($P > 0.2$). Of the 27 animals enrolled, 23 were first parity heifers and 6 were multiparous cows. There was no effect of treatment on oxidative burst ($P = 0.64$) or phagocytosis ($P = 0.19$). This preliminary study does not support an effect of supplemental calcium, as given to low parity parturient cows to alter oxidative burst or phagocytosis capacity of neutrophil function.
INTRODUCTION

From the day of calving through the 72 hour period that follows, most dairy cows have reduced circulating blood calcium (Ca) concentrations. This transitory reduction is a result of a sudden substantial demand for calcium from the plasma pool at the onset of colostrum and milk production. Initiation of homeostatic mechanisms to restore circulating blood calcium to normal levels takes several days with the nadir of blood calcium concentrations occurring 12 to 24 hours following parturition (Goff, 2008).

Many essential physiological functions are dependent upon calcium. The most commonly observed functional deficit during hypocalcemia is compromised neurotransmission and muscle contraction. Insufficient calcium leads to impaired acetylcholine release at neuromuscular junctions and the transmission of nerve impulses to muscle fibers is compromised (Oetzel, 2011). This transmission failure results in the flaccid paralysis that is characteristic of severe hypocalcemia.

Ionized calcium (iCa) is also important for cellular messaging in cell metabolism and proliferation through changes in cytosolic iCa concentrations (Saris and Carafoli, 2005). Intracellular calcium signaling is a key element in immune cell activation by way of an influx of calcium from the extracellular space when antigen receptors are triggered (Vig and Kinet, 2009). In vitro, low extracellular Ca\(^{2+}\) was associated with decreased phagocytosis (Ducusin et al., 2003). Kimura et al. (2006) demonstrated that mononuclear cells of periparturient cows have lower intracellular calcium stores resulting in blunted calcium release in response to immune cell activation signals. Cows with clinical hypocalcemia had further reduced intracellular calcium stores and a poorer response compared to normocalcemic cows. Reduced calcium release in
response to an immune cell activation signal likely contributes to periparturient immune suppression (Kimura et al., 2006) but the reduced immune function that is experienced by nearly all transition dairy cows with varying severity (Kehrli et al., 1989) is multifactorial and not well understood (Overton and Waldron, 2004).

Neutrophils are typically the first immune cells to respond to infection and are the primary innate immune cell associated with clearing postpartum uterine bacterial contamination in dairy cows (Hussain, 1989; Gilbert et al., 2007). Similar to other immune cells, an increase in intracellular calcium concentration is an early event in neutrophil activation (Burgos et al., 2011). Martinez et al. (2012) found neutrophil number, oxidative burst and phagocytosis capacity to be reduced among cows with a blood calcium concentration <2.15 mmol/L between 1 and 3 DIM. These cows were also at greater risk of developing metritis postpartum. The link between reduced neutrophil function and hypocalcemia is further supported by the finding that non-parturient cows induced to develop subclinical hypocalcemia had reduced percentage of neutrophils performing phagocytosis and a reduction in oxidative burst activity of neutrophils (Martinez et al., 2014).

Diminished neutrophil function has been associated with metabolic and reproductive disease in the transition period. A decrease in circulating neutrophil oxidative burst activity postpartum has been found in cows that develop metritis (Hammon et al., 2006) and endometritis (Mateus et al., 2002; Hammon et al., 2006). Additionally, neutrophil function has been linked with lower dry matter intake and elevation in plasma non-esterified fatty acids (NEFA) prior to parturition (Hammon et al., 2006). In vitro exposure of neutrophils to elevated levels of NEFA and beta-hydroxy butyrate (BHBA) consistent with subclinical ketosis in dairy cows resulted in reduced oxidative burst function (Scalia et al., 2006; Hoeben et al., 1997). Supporting neutrophil function...
to reduce metabolic and reproductive disease among transition dairy cows is a desirable objective however there are currently no clear methods to achieve this goal.

Prophylactic calcium supplementation is commonly administered to high risk parturient dairy cows to reduce the incidence of clinical hypocalcemia (Degaris and Lean, 2007). Subcutaneous prophylaxis in the form of 500mL of calcium borogluconate (23%) raises blood calcium concentrations for about 6 hours after calving (Goff, 1999). Oral calcium products which are typically composed of calcium chloride in either drench, paste or bolus form have been shown to raise blood calcium levels for about 12 hours after administration (Goff and Horst, 2003; Sampson et al., 2009; Blanc et al., 2014). There are no published studies on the effect of calcium supplementation on immune function parameters.

The objective of this study was to evaluate if administration of an injectable calcium supplement product at time of calving increased neutrophil oxidative burst or phagocytosis capacity.

**MATERIALS AND METHODS**

**Study Population**

A randomized controlled trial was conducted using parturient cows from four commercial dairy farms in Ontario in June 2014. Herds were purposively selected based on proximity to the University of Guelph, willingness to comply with the calcium supplementation protocols and herd size such that there would be cows available to enroll that had calved within the 12 hours prior to the technician’s visit. Producers agreed to refrain from using other forms of prophylactic calcium supplementation in enrolled cows and consented to a study protocol that had been reviewed and approved by the University of Guelph Animal Care Committee. All herds fed a total mixed ration (TMR) and prepartum cows did not receive an anionic dietary supplement.
Study Design and Data Collection

Cows including first parity animals that had calved in the previous 12 hours were enrolled on the first day of the week for three consecutive weeks. Any cows that showed signs of milk fever, injury related to calving or had already received a calcium supplementation product were excluded from enrollment.

Cows were randomly assigned to receive the calcium supplementation product or a placebo. Technicians administered and recorded experimental treatments according to randomized assignment sheets and were blinded to the treatment given. Cow ID, parity, calving ease and time of calving were collected from farm personnel.

Prior to receiving treatment or placebo, whole blood samples were collected for calcium and neutrophil analysis. Whole blood was collected from the coccygeal vein and or artery using a 20 gauge, 1-inch hypodermic needle into sterile, glass, vacuum blood collection tubes without anticoagulant (BD Vacutainer Precision Glide, Becton, Dickinson and Co., Franklin Lakes, NJ). An additional two vacuum tubes were collected with the anticoagulant acid citrate dextrose (ACD; Vacutainer, Becton Dickinson). Blood with preservative was inverted gently 10 times to ensure mixing. All samples were immediately placed on ice for transport.

Cows in the treated group received calcium gluconate (35% w/v) in combination with calcium glucoheptonate (10% w/v) for a total of 9.46 g of calcium (Theracalcium, Vétoquinol Canada Inc., Lavaltrie, Quebec) given in two doses, within 12 hours after calving at enrollment and again 24 hours later. Each dose was 120mL injected over two sites (60mL per site) subcutaneously. Cows in the control group received a similar volume of placebo (medication vehicle solution with no active ingredient) at time of enrollment and 24 hours later.
At three days in milk (DIM), the blood sampling procedure was repeated. Within three hours of collection, blood in tubes without preservative was separated by centrifugation at 1500 g for 15 minutes for serum harvest. All serum was stored at -20 °C and analyzed together. Neutrophil isolation was executed on whole blood with the anticoagulant ACD within 3 hours of collection.

**Calcium Analysis**

All serum was submitted to the Animal Health Laboratory, University of Guelph for determination of total calcium concentration measured using the Cobas Calcium Gen 2 kit (Roche Diagnostics, Indianapolis, IN, USA). The analytical sensitivity of the calcium assay was 0.2mmol/L and the inter-assay control coefficient of variation was 1.49%.

**Neutrophil Isolation**

Eight mL of whole blood with the anticoagulant ACD was diluted with 20 mL of 1× concentrated phosphate buffered saline (PBS) at room temperature, overlaid on 8 mL of Ficoll-Paque PLUS (General Electric Healthcare Bio-Sciences AB, Uppsala, Sweden) at room temperature, and centrifuged at 700 G force for 30 minutes. The plasma and buffy coat were gently removed by pipette and erythrocytes lysed with 6 volumes of sterile cold water using gentle inversion. Three volumes of 3× concentrated PBS were added to the tubes to re-establish osmolarity and centrifuged at 4 °C at 500 G force for 10 min. Supernatant removal was completed by pipette and samples were washed and centrifuged again if visible hemoglobin remained in the cell pellet. Once free of hemoglobin, cell pellets were resuspended in 500uL of 1x PBS and a hemocytometer chamber and trypan blue exclusion was used to assess cell concentration. The pelleted neutrophils were diluted to a concentration of $1 \times 10^6$/mL using 1×
concentrated PBS with 10% filtered fetal bovine serum (FBS, Invitrogen, Burlington, ON, Canada).

**Oxidative Burst Assay**

In a flow cytometry tube (BD Biosciences, Bedford, MA, USA), 2 μM of 2′, 7′-dihydro-dichlorofluorescein-diacetate (H₂DCFDA, Molecular Probes, Eugene, OR, USA) was added to 200 μL of the reconstituted neutrophils. The tubes were then incubated in the dark for 15 min at 37°C under gentle agitation. Subsequently 200 μL of 1× concentrated PBS with 10% FBS was added to control samples and 200 μL of phorbol myristate acetate (PMA, Sigma, St. Louis, MO, USA) diluted in PBS/FBS for a total of 25 ng/mL of PMA was added to stimulate oxidative burst in non-control samples. All tubes were incubated in darkness for a further 15 min at 37°C with gentle agitation and then placed on ice until and protected from light until flow cytometry. The green cellular fluorescence of the H₂DCFDA was measured on a flow cytometer as described below.

**Phagocytosis Assay**

One volume of activated normal cow serum was produced for the full experiment using serum from 20 healthy lactating Holstein cows at the University of Guelph Dairy Research Centre. 100 mg of Zymosan A from *Saccharomyces cerevisiae* (Sigma-Aldrich, St. Louis, MO, USA) was added per 10mL of pooled serum and incubated at 37°C for 60 minutes under gentle rotation followed by centrifugation at 700 G force for 15 minutes. The serum was removed and stored at -80°C.

In a flow cytometry tube, 200 μL of reconstituted neutrophils were incubated with $1 \times 10^6$ fluorescently labeled 1 μm beads (TransFluo-Spheres® Fluorescent Microspheres, Molecular
Probes) and 50 μL of normal cow serum with Zymosan A for 30 min at 37°C in the dark. Each sample had a negative control of neutrophils incubated without fluorescent beads. Following incubation, cells were re-suspended in flow buffer, placed on ice and protected from light until phagocytosis of the fluorescent beads was measured on the flow cytometer.

**Flow Cytometry Analysis**

A flow cytometer (FACScan, Becton Dickinson) with Cell Quest software (Becton Dickinson) was used to measure oxidative burst and phagocytosis. Neutrophil fluorescence was identified on cytograms of forward versus side scatter and a gate was placed around the neutrophil population. A total of 50000 neutrophil events were collected for each assay. H2DCFDA oxidation was measured at 530 nm using FL1 and phagocytosis of fluorescent beads was measured at 645 nm using FL3 on a log scale. Samples were analyzed using FlowJo software (Tree Star, Ashland, OR, USA). For each observation, the shift in the percentage of cells that underwent oxidative burst or phagocytosis was evaluated relative to the negative control. A gate was placed around ≥ 97% of samples without PMA for the oxidative burst analysis and samples without fluorescent beads for the phagocytosis analysis (negative controls). The difference between the negative control and the positive observation for the percentage of cells outside the negative control gate was used to express success or oxidative burst of phagocytosis.

**Statistical Analysis**

All statistical analyses were performed in SAS (version 9.4, SAS Institute, Cary, North Carolina). Descriptive statistics were generated using the PROC MEANS and PROC FREQ procedures of SAS. The outcomes of interest were the difference between the samples and negative control for the mean percent shift in cells that successfully performed oxidative burst
and phagocytosis at 3 DIM. The oxidative burst variable required a natural logarithmic transformation to normalize the data with an adjustment factor of 0.5.

For the neutrophil function outcomes, a mixed model (MIXED procedure in SAS) was used with an autoregressive covariance structure. Univariable analyses were conducted using linear regression for each neutrophil function outcome using a conservative significance level ($P \leq 0.2$). Collinearity between variables was assessed using Pearson correlation coefficients.

Farm, parity and total calcium concentration at the time of enrollment were offered as covariates to each model. Parity was offered as two levels, primiparous or multiparous. Predictor variables that met the significance criteria in the univariable evaluation were placed in a multivariable mixed model for backward stepwise analysis. Variables remained in the final model if the $P$-value was $\leq 0.1$. Due to the variation in the assay, each cow’s oxidative burst or phagocytosis value at 0 DIM was controlled for. Interactions were tested with treatment and retained if biologically plausible and significant ($P \leq 0.05$).

**RESULTS**

A total of 29 animals were enrolled in the experiment. Two animals were unavailable for follow-up samples at three days in milk due to death (n=1) or culling (n=1). There was no difference in lactation number, total calcium concentration, oxidative burst or phagocytosis at time of enrollment between treatment groups ($P > 0.2$) (Table 3.1). Of the 29 animals enrolled, 23 were first parity, 5 were second parity and 1 was third parity. Enrollment occurred on four farms with 11, 1, 3 and 14 cows from farm 1, 2, 3 and 4, respectively. The time between calving and first treatment as well as the interval between injection of the two doses of calcium did not differ between treatment groups ($P = 0.65$).
**Total Calcium Concentrations**

The prevalence of subclinical hypocalcemia at enrollment was 38% using a cut point of 2.15 mmol/L (n=11). A cut point of 2.0mmol/L characterized 24% of cow cows as subclinically hypocalcemic (n=7). After 72 hours there was no difference in mean calcium concentration between groups ($P = 0.62$). The distribution of total calcium concentrations at 3 DIM as a function of calcium concentrations at time of enrollment is displayed in Figure 3.1.

**Effect of Treatment on Oxidative Burst**

Total calcium concentration at enrollment ($P = 0.19$) was offered as a predictor variable to the neutrophil oxidative burst linear regression model and oxidative burst at time of enrollment on day 0 was forced into the model. The final model for the natural log transformation of neutrophil oxidative burst capacity is reported in Table 3.2. There was no effect of treatment on oxidative burst ($P = 0.64$). The association of treatment with oxidative burst is displayed in Figure 3.2 and the change in oxidative burst capacity between day 0 and day 3 for each cow is in Figure 3.3.

**Effect of Treatment on Phagocytosis**

There were no predictor variables (farm, parity or total calcium concentration at enrollment) that screened less than 0.2 in univariable analysis to offer to the neutrophil phagocytosis linear regression model. Phagocytosis at time of enrollment on day 0 was included as a covariate. The final model for neutrophil phagocytosis capacity is reported in Table 3.2. There was no effect of treatment on phagocytosis ($P = 0.19$). The association of treatment with phagocytosis capacity is displayed in Figure 3.4 and the change in phagocytosis between day 0 and 3 postpartum for each cow is in Figure 3.5.
DISCUSSION

It was hypothesized that cows treated with supplemental calcium would have an improvement in neutrophil function that would be manifest as a mitigation of the expected reduction in neutrophil function. The data suggest that supplementation of parturient cows with subcutaneous calcium as given here does not improve neutrophil function. There was no effect of treatment on oxidative burst capacity or phagocytosis of the neutrophils.

The degree of hypocalcemia among the study sample was moderate with 11 animals (39%) with tCa <2.15mmol/L and 7 animals (25%) <2.0mmol/L immediately before treatment, within 12 hours after calving. This is not unexpected considering first lactation animals made up the majority of the sample. Although first lactation animals are less likely to suffer from hypocalcemia and clinical milk fever is rare, they are not exempt from the condition (Goff, 2008). The prevalence in the current study is similar to the sample of parturient cows tested by Reinhardt et al. (2011) in which 25% of first parity cows had tCa <2.0mmol/L at 0 to 48 hours postpartum.

Martinez et al. (2012) found that animals with a total blood calcium < 2.15mmol/L were at risk for poorer neutrophil oxidative burst and phagocytosis capacity. The present study design and power were insufficient to detect or confirm associations between total calcium concentration and immune function or total calcium and transition cow diseases. The goal of this pilot study was to explore whether calcium supplementation after calving could shift neutrophil function.

Circulating neutrophil numbers typically increase on the day of calving with a subsequent reduction in the postpartum period and recovery to pre-calving levels by four weeks postpartum (Cai et al., 1994; Kim et al., 2005). The postpartum reduction is due to the large exodus of
neutrophils to the uterus and mammary gland (Guidry et al., 1976). Despite circulating numbers of neutrophils remaining high until calving, typically neutrophil oxidative burst function is already compromised in the prepartum period and begins declining from 2 weeks before calving, reaching a nadir approximately one week after calving (Kehrli et al., 1989; Detilleux et al., 1995). Conversely, phagocytosis ability has been described to not decline until after calving (Kehrli et al., 1989, Kim et al., 2005), however Sander et al. (2011) found no change in phagocytosis ability through the peripartum period, suggesting it may be possible for capacity to be maintained throughout the transition period. The degree of suppressed immune function varies between cows with high producing cows (Kehrli et al., 1989, Goff and Horst, 1997), older cows (Gilbert et al., 1993) and cows with or that go on to develop uterine disease (Cai et al., 1994; Kim et al., 2005; Hammon et al., 2006) being more greatly affected.

In the present study, overall oxidative burst capacity did not differ between the two time points measured (13.2 ± 17.4 vs. 13.8 ± 13.2% of neutrophils). However, it varied widely among individual cows as seen in Figure 3.4. This is consistent with expectations that some cows will be affected more severely than others (Kehrli et al., 1989; Detilleux et al., 1995). The mean percentage of neutrophils with oxidative burst was low on the day of calving (13.2 ± 17.4) however more than half the cows had lower oxidative burst at 3 DIM than calving, consistent with the nadir of function after calving. Overall phagocytosis capacity increased between the two time points measured (27.0 ± 7.5 vs. 32.6 ± 14.4) but two-thirds of the cows had declining phagocytosis capacity between the two time points. This is consistent with expected results that calving would be associated with increased phagocytosis which declines around 1 week postpartum (Kehrli et al., 1989; Kim et al., 2005; Sander et al., 2011).
Neutrophil lifespan is short-lived with cells lasting no more than a few days before being replaced (Tizard, 2013; Kolaczkowska and Kubes, 2013). After production and differentiation in the bone marrow, it is unclear whether calcium concentrations can be altered in circulating neutrophils. When neutrophils were treated with the anticoagulant ethylene diamine tetraacetic acid (EDTA), an extracellular calcium ion chelator, neutrophil phagocytosis capacity was severely reduced (Ducusin et al., 2001). Previous work has suggested that cows with reduced calcium concentrations in blood are unable to replenish intracellular calcium and therefore have less calcium in the endoplasmic reticulum available to affect cytosolic concentrations, thereby compromising neutrophil activation and generation of reactive oxygen species (Ducusin et al., 2003; Martinez et al., 2012). These studies lead to the hypothesis that raising the total blood calcium concentration would alter this pathway, but this was not seen in the current study. A greater understanding of the mechanism by which neutrophils take up calcium in bone marrow or in circulation is needed to understand the potential to effect improvements in intracellular calcium and in neutrophil function. It is possible the supplementation product did not raise total blood calcium enough to effect change in intracellular calcium concentrations or alternatively that plasma calcium concentration does not affect function of circulating neutrophils. It would be beneficial to measure the intracellular calcium concentration of neutrophils from cows supplemented versus those that were not to establish if higher intracellular calcium was achieved.

A flux of intracellular calcium from the extracellular space to intracellular space is part of neutrophil activation; however calcium-independent signaling pathways by means of protein kinases are also present (Sayeed, 2000). Cytosolic calcium is required to initiate phagocytosis (Sayeed, 2000) as well as the fusion of secondary granules with the phagosomal membrane (Jaconi et al., 1990). Furthermore, cytosolic calcium is required in the production of reactive
oxygen species through activation of NADPH oxidation (Brechard and Tschirhart, 2008). However, as neutrophil activation is not solely calcium dependent, a calcium supplement intervention might not be sufficient to affect neutrophil function when compromised peripartum immunity is multifactorial. Furthermore, if neutrophil oxidative burst function begins declining 2 to 3 weeks before calving, an intervention on the day of calving may be too late to effect change in neutrophil function. As neutrophil lifespan is short and all neutrophils circulating during this time are of lower oxidative burst capacity, increasing blood calcium concentration for 6 to 12 hours through supplementation, even if this increases neutrophil intracellular calcium, might not be enough to effect meaningful improvement in immune defence and the overall health of the cow.

It is not clear if different results would be achieved using a population of cows at greater risk of hypocalcemia such as cows of greater parity. One major limitation of the current study is the lack of multiparous cows in third or greater lactation. The study sample used was a function of cows that had calved on the days selected for enrollment. Several older cows on these farms had already received supplemental calcium which presents a systematic bias in the sample of cows available to be enrolled. As risk of hypocalcemia increases with age, an improvement in immune defence from calcium supplementation might be evident in older cows compared to the current sample. Additionally, a larger sample that includes a greater number of cows at risk of reduced peripartum immunity, such as higher producing cows and those that develop postpartum uterine disease may also yield different results. Finally, interventions that produce a more sustained and earlier increase in blood calcium such as feeding a prepartum diet with anionic salts to achieve a negative dietary cation-anion difference would be worthwhile to investigate. The effect of a more intense calcium supplementation protocol, using a higher dose or repeated administration on
neutrophil function is similarly unknown and would be valuable to explore. Measuring the intracellular calcium concentration of neutrophils from a sample of treated versus untreated cows at higher risk of hypocalcemia and postpartum disease to better understand the ability to increase neutrophil intracellular calcium concentrations would be a practical next step.

CONCLUSIONS

This preliminary study does not support an effect of supplemental calcium, as given to low parity parturient cows to alter oxidative burst or phagocytosis capacity of neutrophil function.
REFERENCES


**Table 3.1:** Total serum calcium concentrations, oxidative burst capacity and phagocytosis capacity on day 0 and day 3 relative to calving measured in 27 dairy cows that received calcium supplementation or a placebo within 12 hours of calving and repeated 24 hours later.

<table>
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<tr>
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<th>Maximum</th>
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</tr>
<tr>
<td>Day 0 (mmol/L)</td>
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<td>2.19</td>
<td>1.48</td>
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<tr>
<td>Day 3 (mmol/L)</td>
<td>2.22</td>
<td>0.23</td>
<td>2.29</td>
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<tr>
<td><strong>Oxidative Burst</strong></td>
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<td>Day 0 (% cells activated)</td>
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<td>17.44</td>
<td>6.5</td>
<td>0</td>
<td>68.3</td>
</tr>
<tr>
<td>Day 3 (% cells activated)</td>
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<td>13.19</td>
<td>7.5</td>
<td>0</td>
<td>42.0</td>
</tr>
<tr>
<td><strong>Phagocytosis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0 (% cells activated)</td>
<td>27.04</td>
<td>7.52</td>
<td>26.5</td>
<td>15.5</td>
<td>49.4</td>
</tr>
<tr>
<td>Day 3 (% cells activated)</td>
<td>32.64</td>
<td>14.38</td>
<td>29.9</td>
<td>0</td>
<td>66.0</td>
</tr>
</tbody>
</table>

Oxidative burst was measured as the percentage of cells activated by phorbol myristate acetate, as evaluated using flow cytometry.

Phagocytosis was measured as the percentage of neutrophils that phagocytosed ≥ 1 fluorescent bead, as evaluated using flow cytometry.
Table 3.2: Final models for the natural logarithm transformation of neutrophil oxidative burst function and phagocytosis measured in 27 dairy cows that received calcium supplementation or a placebo within 12 hours of calving and repeated 24 hours later.

<table>
<thead>
<tr>
<th>Variable</th>
<th>β</th>
<th>SE</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oxidative Burst (ln-transform)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>1.98</td>
<td>0.34</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subcutaneous calcium</td>
<td>-0.20</td>
<td>0.41</td>
<td>0.64</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Oxidative Burst before treatment</td>
<td>0.03</td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Phagocytosis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>13.19</td>
<td>11.00</td>
<td>0.24</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subcutaneous calcium</td>
<td>7.38</td>
<td>5.53</td>
<td>0.19</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Phagocytosis before treatment</td>
<td>0.55</td>
<td>0.37</td>
<td>0.16</td>
</tr>
</tbody>
</table>
Figure 3.1: Total calcium concentrations at 3 DIM as a function of total calcium concentrations before treatment from 27 dairy cows that received calcium supplementation or a placebo within 12 hours of calving and repeated 24 hours later.
Figure 3.2: In vitro mean oxidative burst (% of neutrophils) ± SE in neutrophils of cows treated with subcutaneous calcium or placebo.
**Figure 3.3**: Relative change in neutrophil oxidative burst capacity (%) between 0 and 3 days postpartum for 27 cows treated with subcutaneous calcium (■) or placebo (□).
Figure 3.4: In vitro mean phagocytosis capacity (% of neutrophils) ± SE in neutrophils of cows treated with subcutaneous calcium or placebo.
**Figure 3.5:** Relative change in neutrophil phagocytosis capacity (%) between days 0 and 3 postpartum for 27 cows treated with subcutaneous calcium (■) or placebo (□).
Chapter 4

THE EFFECT OF PREPARTUM FEEDING AND LYING SPACE ON METABOLIC HEALTH AND IMMUNE FUNCTION

ABSTRACT

The determinants of metabolic and reproductive health disorders in the peripartum period and the degree to which feeding and lying space and management can influence health are only partially understood. The objective of this randomized controlled study was to determine if providing non-competitive feeding and lying access in the close-up dry period improves health and immune function. Forty-eight Holstein cows of all parities were randomly assigned to a treatment group of 6 to 10 cows in 1 pen with either 80% cows to stalls and 90 cm of feeding space/cow (understocked) or 120% stocking density and 45 cm of feeding space/cow (overstocked) for 3 weeks before expected calving. Pen size and bunk space were adjusted to maintain space per cow as animals were removed for calving. All cows wore electronic data loggers to monitor daily standing and lying time for the duration of their time in the pen. To assess competitive behavior, 3 days of video recordings representing d 7 to 9 after group formation were reviewed and each competitive displacement at the feedbunk was recorded. A competition index (C_Ind) was calculated for each cow by dividing the number of times the cow displaced another at the feedbunk by the total number of displacements in which the cow was involved. Weekly blood samples measured non-esterified fatty acids (NEFA), beta-hydroxybutyrate (BHB), calcium, glucose, albumin, aspartate aminotransferase (AST), bilirubin, haptoglobin, insulin and insulin like growth factor-1 from 3 weeks before to 5 weeks after calving. Measures of innate immune function (neutrophil phagocytosis and oxidative burst) were
assessed at -2, -1, 1, 2, 3 and 5 weeks relative to calving. Purulent vaginal discharge (PVD) and endometritis were assessed at weeks +3 and +5. Cows in the understocked group spent significantly more time per day lying ($P = 0.02$); the back transformed least squares means (LSM) and 95% CI were 14.8 hours (13.9 to 15.6) vs. 12.8 hours (12.0 to 13.7). Understocked cows were also involved in fewer displacements at the feedbunk (LSM ± SE 21 ± 6 vs. 41 ± 6; $P = 0.02$). Controlling for parity, there was no difference between treatments in BHBA, NEFA, glucose, AST, bilirubin or haptoglobin. Overall there was no treatment effect on phagocytosis throughout the study period but cows with a higher C_Ind in the understocked treatment group had higher oxidative burst function ($P < 0.01$). Cows in the understocked treatment had higher mean albumin and calcium over the study ($P < 0.05$) and tended to have lower liver triacylglycerol content at week 3 ($P = 0.06$). At 5 weeks postpartum, 7% of cows had PVD and 33% of cows were diagnosed with endometritis based on $> 5\%$ neutrophils. There was no effect of treatment on endometritis. Despite greater displacements at the feedbunk and lower lying time, the expected harmful effects of crowding and competition were mostly not seen. While this does not refute the importance of access to feeding and lying space, these results indicate that metabolic and reproductive health is more complex than can be explained solely by exposure to what are understood to be best practices.

**INTRODUCTION**

The peripartum period, a well-recognized time of challenge to the dairy cow, is characterized by a period of negative energy balance (NEB), diminished dry matter intake, insulin resistance, lipolysis, and weight loss of varying severity among cows (Goff and Horst, 1997, De Koster and Opsomer, 2013). Furthermore, diminished immune function typifies the peripartum period, a time when the majority of cows have bacterial contamination of the uterus that must be promptly
eliminated (Sheldon et al., 2002). The innate immune system, specifically neutrophils as the first responders, is the primary means of cellular defense against bacterial colonization in the uterus. Impairment of neutrophil function has been documented beginning two weeks before calving, reaching a nadir one week postpartum with slow recovery in the early weeks of lactation (Kehrli et al., 1989). Mitigating this immune suppression would be auspicious as it contributes to common production-limiting diseases important to animal welfare and profitability.

Differences in innate immune function in the weeks ahead of calving have been recognized in several reproductive diseases. Kimura et al. (2002) identified changes in neutrophil function and cytokine levels two weeks before calving in cows that developed retained placenta postpartum. Reduced migration and killing activity through neutrophil phagocytosis and oxidative burst function has been associated with metritis and endometritis (Hammon et al, 2006). Endometritis is associated with impaired innate immune function (Sheldon et al., 2009) and measurable changes in phagocytosis and pro-inflammatory mediators prepartum (Kim et al. 2005). These changes are present weeks before disease becomes manifest, coincident with the onset of insulin resistance and lipolysis, at least in cows at higher risk of disease.

A postpartum inflammatory response associated with parturition is common in dairy cows, even those without signs of systemic disease. Deregulation of this normal response is accompanied by excessive proinflammatory cytokine release which has potential effects on liver function, nutrient partitioning, dry matter intake, and reproductive activity (Bertoni et al., 2008). The relationships between fat and energy metabolism, inflammation and immune function are beginning to be described. The immense metabolic challenge that dairy cattle experience in the periparturient period can disrupt the balance between resolution of the normal inflammatory response and uterine disease (Sordillo et al., 2009). Wathes et al. (2009) demonstrated that cows
in severe negative energy balance (NEB) had delayed endometrium repair and ongoing uterine inflammation two weeks after calving compared to cows with mild NEB. Cows in greater negative energy balance as measured by diminished DMI, higher NEFA and higher beta-hydroxybutyrate (BHB), and in particular those that go on to have metritis or endometritis have more pronounced impairment of at least some immune functions (Hammon et al., 2006; Galvão et al., 2010). High NEFA concentrations are a risk factor for ketosis and fatty liver and may also have direct effects on neutrophil function as demonstrated in vitro by Scalia et al. (2006). Graugnard et al. (2012) found phagocytosis capacity of neutrophils was lower prepartum in cows that consumed more dietary energy than required for maintenance and fetal growth during the close up dry period compared to cows fed a controlled energy diet, although by one week post-calving both groups had similar phagocytic function. Despite the emerging understanding of these relationships, among cows with similar nutrition and management it is unclear what determines the incidence of metabolic and reproductive disease.

Animal management, particularly the social environment of cows in the prepartum period may be important to better understanding and preventing disease in dairy cattle. An environment that limits DMI and lying time may have enduring effects on transition cow health. Proudfoot et al. (2009) demonstrated that with increased competition in transition pens, displacements at the feedbunk increased, cows ate less in the week before calving and spent more time standing in the week after calving. Prepartum pens often do not have a stable group structure and additions may be weekly or more frequently. After regrouping, lactating dairy cows moved to a new pen had reduced resting time and feed intake and more displacements from the feed bunk (von Keyserlingk et al., 2008). Cook and Nordlund (2004) have suggested that weekly additions to close-up pens result in similar disruption of social interactions among prepartum cows. The
relationships among cow grouping, group size, bunk space and competition for feed have been reviewed with the conclusion that bunk space not be limited, in order to avoid dry matter intake reductions (Grant and Albright, 2001). Huzzey et al. (2007) found cows with severe metritis ate 2 to 6 kg DM less than healthy cows in the 2 to 3 weeks prior to clinical signs of disease. Lower feed intake prepartum is associated with increased NEFA and hepatic lipid accumulation (Grummer, 1993). Increased liver triacylglycerol content in the first two weeks after calving is associated with impaired neutrophil function (Zerbe et al., 2000), emphasizing again the relationship between the metabolic and immunological state. Whether impairment of immune function or reproductive disease risk can be influenced through management has not been well tested despite the recognized associations between a competitive environment and dry matter intake.

The objective of this study was to test whether providing non-competitive access to feeding and lying modifies metabolic health and immune function. A secondary objective was to determine if differences in metabolic health and immune function could be explained using a measure of social status in the group. The hypothesis was that providing greater feeding and lying space would reduce social stress and thereby improve measures of adaption to negative energy balance and of innate immune function.

**MATERIALS AND METHODS**

*Animals, Housing and Management*

A total of twenty-one nulliparous and 27 parous (total n = 48, parity 2.0 ± 1.2) Holstein dairy cows housed in prepartum groups in a free-stall facility at the University of Guelph Dairy Research Centre (Guelph, ON, Canada) were enrolled in this study. Cows were managed
according to the guidelines set by the Canadian Council on Animal Care (2009) and subject to an Animal Utilization Protocol approved by the University of Guelph Animal Care Committee.

Cows were enrolled in the study 21 to 28 days prior to expected calving date and monitored until 5 weeks after calving from October 2012 to October 2013. Weeks were corrected to actual calving date. Groups consisted of 6 to 10 cows depending on the availability of calvings at the farm. Prior to group formation and after calving, cows were housed individually in tie stalls. The pre-calving diet was a controlled energy TMR diet (Net Energy Lactation= 1.37 MCal/kg DM) delivered once daily at 0600. After calving, all cows were fed a TMR ration three times a day (0900, 1300, and 1500 h) and were milked twice daily (0530 and 1600 h). The diets fed were identical between treatment groups pre- and postpartum (Table 4.1 and 4.2). A sample size of 20 cows per group was planned based on a difference of 15 units (SD = 16) between treatments for the percentage of cells activated by phorbol myristate acetate in an oxidative burst assay or percent of neutrophils that phagocytosed ≥ 1 fluorescent bead, both evaluated by flow cytometry.

**Treatments**

Each group was randomly assigned to be “understocked” or “overstocked”. Understocked cows were housed at 80% stocking density (cows to stalls) and 90 cm of bunk space provided for each cow in the group. Overstocked cows were housed at 120% stocking density and 45 cm of bunk space per cow. The facilities in the barn and the numbers of cows available at one time only allowed for sequential (rather than concurrent) treatment groups. The time and parity composition of each experimental group is outlined in Table 4.3. Groups of 6 to 10 cows and heifers with expected calving dates with a range of 8 days were formed and moved together to the study pen 21 to 28 days before expected calving. No animals were added to groups once formed. Cows were moved from the pen to an individual maternity box stall one to two days
before expected calving and were monitored until 5 weeks after calving in tie-stalls. The minimum number of cows in a treatment at the end of a study group was 2; at which time both remaining animals were moved to maternity pens. The same prepartum pen was used for all consecutive groups and was adjusted to the group assignment. A diagram of the prepartum pen is included in Figure 4.1. As animals were removed from the pen at calving, the stall number and bunk space were adjusted to maintain these standards. The pen used was a gated off portion of a two-row free-stall pen (stalls measuring 132 x 188 cm) with alleys of rubberized concrete (239 x 343 cm). The surface of each stall comprised a rubber crumb-filled mattress (Pasture Mat®, Promat, Ltd., Seaforth, ON) which were covered daily with clean chopped straw. The feeding space was a feed rail and gates were used to change the space allotted depending on treatment and cow number.

Clinical Disease Recording and Definitions

Retained placenta was defined as the presence of the placenta at 24 hours after calving and was diagnosed by research barn staff. Metritis was diagnosed based on clinical signs by the herd veterinarian and diagnosed when body temperature was ≥ 39.5 with fetid vaginal discharge. Cows were observed daily but not systematically examined for body temperature. Displaced abomasum was diagnosed and corrected by the herd veterinarian.

Rumination Time

While housed in the prepartum experimental pen, all cows were fitted with automated rumination monitoring devices (HR-Tags, SCR Engineers LTD., Netanya, IL, USA) which have been previously validated by Schirmann et al. (2009). Collars that hold the tags were applied to cows the day they entered the treatment pen and were used to measure rumination time during the
duration of time in the pen. The tags continuously record the time spent ruminating in 2 h intervals using rumination and regurgitation sounds detected by a small microphone in the tag. Tags were placed on the upper left side of the cow’s neck according to manufacturer directions. Raw rumination data were amassed by an automatic reader mounted above the water trough, recorded each time the cow drank and saved with the associated software. Outputs provided the rumination time per two hour interval which was summed into a daily value.

*Lying Behavior*

Daily lying behaviour was collected using a 3-axis accelerometer (HOBO Pendant G Logger, Hoskin Scientific LTD, British Columbia, Canada). The accelerometers were secured on the right hind leg of the cow on the day of entry to the pen to assess lying behaviour of the cow at 1 min intervals as validated by Ledgerwood et al., 2010. The device was removed when the cow was moved to the calving pen and data were downloaded using Hoboware® Lite (Onset Computer Corporation, Bourne, MA, USA) and compiled using SAS 9.4 (UBC AWP, 2013).

*Social Behavior*

Competitive behaviour at the feedbunk was monitored by digital video surveillance (Panasonic camera WV-CP504 and lens WV-LZA61/2S, Panasonic Canada Inc., Mississauga, Ontario, Canada) connected to a recording system (GeoVision, UVS 1240E2, GeoVision Inc, USAVisionSys, California, USA). Two cameras were mounted 3 m above the feed rail so that the entire length of the feed bunk was visible. Cows in the pen were marked with unique alphanumeric symbols using cow paint or hair dye if the cow was predominantly black haired. Cow paint was reapplied twice weekly. Competitive behaviour was assessed over three consecutive days (d 7 to 9 after group formation) for cows that were feeding. To be considered
feeding, the cow’s neck collar had to be visible beyond the top rail on the feed alley side of the pen. A displacement was recorded when a cow’s head (actor) made contact with another cow’s head that was feeding (reactor) and as a result of the interaction, the reactor completely withdrew her head from the feedbunk. Each cow were assigned a competition index score (C_Ind) to describe the proportion of successful displacements relative to overall interactions as previously described by Galindo and Broom (2000). The competition index was calculated as follows:

\[ C_{\text{Ind}} = \frac{\# \text{ of times cow is the actor}}{\# \text{ of times cow is the actor} + \# \text{ of times cow is the reactor}} \]

Cows were categorized as low ranking (index < 0.4), middle ranking (index 0.4 to 0.6) or high ranking (index > 0.6). Video was reviewed by two individuals and inter-observer reliability assessed using the raw score for total interactions \((r^2=0.90)\).

**Blood Metabolites**

On a weekly basis, with the first sample taken immediately prior to cows entering the treatment pen on the day of group assembly, whole blood was collected from the coccygeal vein and or artery using a 20 gauge, 1-inch hypodermic needle into sterile, glass, vacuum blood collection tubes without anticoagulant (BD Vacutainer Precision Glide, Becton, Dickinson and Co., Franklin Lakes, NJ). In tandem, two 8.5 mL vacuum tubes of blood were collected with the anticoagulant acid citrate dextrose (ACD; Vacutainer, Becton Dickinson) for neutrophil isolation at weeks -2, -1, 1, 2, 3, and 5. Blood with preservative was inverted gently 10 times to ensure mixing. All samples were placed on ice for transport and within three hours of sampling, serum was harvested from whole blood without anticoagulant by centrifugation at 1500 g for 15 minutes. All serum was separated into four aliquots and stored at -20°C.
Serum from each week of enrollment as well as the day of calving was submitted to the Animal Health Laboratory, University of Guelph for measurement of NEFA, BHBA, total calcium, glucose, albumin, aspartate amino-transferase (AST), total bilirubin and haptoglobin concentrations. Analysis was performed using an auto-chemistry analyzer (Cobas 6000 c 501, Roche Diagnostics, Indianapolis, IN, USA). Glucose concentrations were measured using the Roche GLUC3 kit (Roche Diagnostics). The analytical sensitivity of the glucose assay is 0.1 mmol/L, and the inter-assay control coefficient of variation was 1.13%. The NEFA and BHBA concentrations were determined using Randox NEFA and Randox BHBA kits (Randox Laboratories Canada Ltd., Mississauga, ON, Canada). The analytical sensitivity of both assays is 0.1 mmol/L and the inter-assay control coefficients of variation were 4.3% and 2.5% for NEFA and BHBA, respectively. Total calcium concentration was measured using the Cobas Calcium Gen 2 kit (Roche Diagnostics, Indianapolis, IN, USA). The analytical sensitivity of the calcium assay is 0.2 mmol/L and the inter-assay control coefficient of variation was 2.2%. Total bilirubin and AST concentrations were measured using Cobas BILTS and Cobas ASTL kits (Roche Diagnostics, Indianapolis, IN, USA). The analytical sensitivity of the total bilirubin assay is 1.7 µmol/L and the inter-assay control coefficient of variation was 3.2%. The analytical sensitivity of the AST assay is 5 U/L and the inter-assay control coefficient of variation was 1.9%. Albumin concentrations were measured using Cobas ALB2 kit (Roche Diagnostics, Indianapolis, IN, USA) with an analytical sensitivity of 2 g/L and the inter-assay control coefficient of variation was 1.9%. Hemoglobin binding capacity was used to measure haptoglobin concentrations. A methemoglobin reagent was made on-site according to the method described by Makimura and Suzuki (1982) and Skinner et al. (1991). The analytical sensitivity of the haptoglobin assay is 0.03 g/L and the inter-assay control coefficient of variation was 5.5%.
The quantitative determination of insulin in bovine serum was performed in our lab using a bovine Insulin ELISA (Mercodia AB, Uppsala, Sweden). The detection limit of the assay is 0.025µg/L and the intra-assay and inter-assay coefficients of variation were 7.4% and 11.9%, respectively. The quantitative determination of IGF-1 was performed using a human IGF-1 immunoassay (Quantikine ELISA, RnDSystems, Minneapolis, MN, USA). The detection limit of the assay is 0.026µg/L and the intra-assay and inter-assay coefficients of variation were 7.1% and 8.4%, respectively. The insulin and IGF-1 ELISA microplates were read with a spectrophotometer (EON; Biotek, Winooski, VT, USA).

**Glucose Tolerance Test**

A simplified glucose tolerance test (GTT) was performed at 1 week prior to expected calving date using the procedure described by Matteo et al. (2009). An intravenous bolus of 0.25g/kg BW dextrose (Dextrose 50%, Vétquinol Canada Inc., Lavaltrie, QC) was given via jugular venipuncture over 2 minutes. Blood samples were collected immediately before the dextrose infusion from the jugular vein (0 min) and at 10 and 80 minutes after infusion from the coccygeal vein or artery for blood glucose and insulin concentrations. These tubes were immediately placed on ice. The ratio of blood glucose concentrations at 80 min to 0 min was calculated and examined at two cut points, 1.05 and 1.2 to assess insulin resistance.

**Neutrophil Function Assays**

Neutrophil isolation, oxidative burst function and phagocytosis assays, and flow cytometry analysis were performed as described in Chapter 3.
Uterine Health Parameters

At weeks 3 and 5 after parturition, all cows enrolled were examined for vaginal discharge using a disposable vaginal speculum (JorVet Jorgensen Labs, Loveland, CO, USA). Discharge observed was scored according to the scoring system outlined in Sheldon et al. (2006). Separate cervical and uterine cytology samples were collected immediately after visual inspection to assess cervicitis and endometritis. Using a cytobrush (VWR CanLab, Mississauga, ON, Canada) and modified AI rod, two samples were obtained, the first from directly beyond the most caudal ring of the cervix and the second from the uterine body. Using the procedure described in Dubuc et al. (2010), cytology slides were generated, stained and examined for the number of neutrophils per 100 nucleated cells (neutrophil to epithelial cell ratio). The cytobrush end was snipped off and placed in TRIzol (Life Technologies, Carlsbad, California) and frozen at -80°C for gene expression analysis.

Body Condition Score and Milk Yield

All cows were scored for body condition on the day of group assembly at 3 weeks before calving and at the last sample collection at 5 weeks after calving using the scoring system described by Ferguson et al. (1994).

Milk yield was recorded using meters in the milking parlor daily throughout the 5 weeks postpartum.

Liver Biopsies

At week 1 and 3 after calving, a liver biopsy was performed while cows were restrained in a standing chute. Briefly, the skin over the last three ribs on the right side at the level of the greater
trochanter was shaved and disinfected using a three step scrub with chlorhexidine gluconate (Germi-stat 4%, Germiphene Corporation, Brantford, Ontario, Canada), 70% isopropyl alcohol (Commercial Alcohols, Brampton, Ontario, Canada) and chlorhexidine gluconate solution 0.5% in 70% isopropyl alcohol (Baxedin Pre-Op, Omega Laboratories Limited, Montréal, Quebec, Canada). The skin and body wall were anesthetized with 10mL of 2% lidocaine (Lidocaine Neat, Wyeth Animal Health, Guelph, Ontario, Canada). A stab incision was made through the skin in the area of the right 11th intercostal space where it crosses the line from midhumerus to tuber coxae and at the level of the greater trochanter. A 30cm by 8mm diameter trocar biopsy tool was passed through the incision, inserted into the liver and approximately 2g of liver tissue was collected. The skin incision was closed with 35mm skin staples (3M Precise PGX Disposable Skin Stapler, London, Ontario, Canada). Tissue was distributed to two vials, one of which contained the reagent TRIzol. All samples were immediately frozen in liquid nitrogen and stored at -80°C.

**Tissue Triacylglycerol Concentration**

Liver biopsies were analyzed for triacylglycerol concentration at the Mammalian NutriPhysioGenomics laboratory, Department of Animal Sciences, University of Illinois. A total of 50 mg of tissue was first homogenized in 1.5 mL of PBS/10 mM EDTA using a hand-held homogenizer (Tissue-Tearor, Biospec Products). Subsequently, 200 µL of GPBS-EDTA along with 3 mL of isopropanol-hexane-water (80:20:2 vol/vol) were added to each sample and the mixture incubated covered with aluminum foil for 30 min at room temperature. One mL of hexane-diethyl ether (1:1) was then added to each sample followed by vortexing and incubation for 10 min at room temperature (protected from light). One mL of water was added to each sample to separate the lipid phase and the mixture was vortexed. Samples were incubated and
covered with aluminum foil for ~20 min at room temperature. The organic phase was then aspirated and placed into glass vials, prior to evaporation under a stream of N gas. An 8-point TAG standard was prepared with Infinity TG reagent (cat#10010509, Cayman Chemicals). Each sample was mixed with 540 µL of Infinity TG reagent prior to vortexing. A total of 160 µL of this sample mixture was pipetted into a flat-bottom 96-well plastic microplate. The plate was incubated for 15 min at 37°C prior to determining absorbance at 540 nm using a microplate reader. Concentration of TAG was calculated from the standard curve.

*Liver and Uterine Gene Expression*

Transcriptome profiling of genes from liver biopsies and cytobrush ends preserved in TRIzol was performed at Mammalian NutriPhysioGenomics laboratory, Department of Animal Sciences, University of Illinois according to procedures previously reported in Osorio et al., 2014. A list of all genes analyzed is reported in Table 4.7.

*Statistical Analysis*

All statistical analyses were performed in SAS (version 9.4, SAS Institute, Cary, North Carolina) considering the cow as the experimental unit with experimental block offered as a random effect. The outcomes of interest included the effect of treatment group on the behaviour measures daily lying time, daily rumination, and displacements at the feedbunk. A logit transformation was used to normalize lying time. Daily lying and rumination time were adjusted to actual day of calving. Day -1 and -2 were excluded from lying time analysis as cows were moved to the maternity pen at this time. Rumination data were also censored at day -2 as collars were no longer read automatically once cows left the group pen. Data before day -24 was excluded due to too few observations. Daily measurements of each were modeled using linear mixed models (PROC
MIXED) with the repeated measures statement. Parity, set as 1, 2 and ≥ 3, body condition at enrollment, set as ≤ 3.5 and ≥ 3.75, and a lameness event recorded in the cow’s health record during the far off dry period before enrollment were offered as covariates to all models. Interactions between treatment and each covariate were tested and retained where significant.

The effect of treatment group on the metabolic parameters (measured weekly) BHB, NEFA, glucose, insulin, IGF-1, calcium, AST, total bilirubin, albumin, and haptoglobin were modeled using linear mixed models (PROC MIXED) with the repeated measures statement. The effect of treatment group on the difference between samples and their negative controls in the mean percent shift in cells that successfully performed oxidative burst or phagocytosis was also modeled using linear mixed models (PROC MIXED) with the repeated measures statement. BHBA, NEFA, haptoglobin, liver triacylglycerol, oxidative burst and phagocytosis were normalized using a log transformation. Parity and body condition at enrollment were offered as covariates to all models. Competition index was offered as a covariate to all metabolic and immune parameter models. Correlation between covariates was examined using Pearson and Spearman correlation coefficients. Where significant as a covariate, the model including competition index is reported. All data with a log transformation were back transformed for ease of interpretation.

Descriptive statistics were generated using PROC FREQ and PROC MEANS in SAS. Each variable was examined for association with the outcomes and any variable associated with the outcome at $P < 0.2$ was offered to multivariable models. Variables were removed manually by backward stepwise elimination in order of highest $P$-value until only variables associated with the outcome remained ($P < 0.1$). With each variable removal, evidence of confounding was determined by a change in coefficient for treatment of > 20%. Interactions between treatment...
group and each covariate were tested and retained where significant. For all models, the
covariance error structure yielding the lowest Akaike’s Information Criterion value was utilized,
choosing from autoregressive, heterogeneous or toeplitz.

The probability of endometritis and cervicitis between treatment groups was evaluated by
Fisher’s exact test. The probability of being insulin resistant at two different cut points for the
ratio of blood glucose concentrations at 80 min to 0 min following a glucose tolerance test was
also evaluated by Fisher’s exact test.

Area under the curve (AUC) was calculated for insulin using insulin measured from each serum
sample taken during the glucose tolerance test. Total area using the trapezoidal rule was
calculated (Cardoso et al., 2013), log transformed to improve normality and modeled using a
linear mixed model (PROC MIXED).

Daily milk yield was collapsed into weekly means and modeled using a linear mixed model
(PROC MIXED) with the repeated measures statement.

Gene expression results were normalized using the geometric mean of 3 internal control genes
and the real-time quantitative PCR data were log₂ transformed to improve normality. Each gene
was assayed in triplicate and a mean of the three log₂ transformed values was used for statistical
analysis. Data were analyzed using PROC MIXED repeated measures. The statistical models
included time (week 1 and 3), treatment (understocked and overstocked) and the time x treatment
interaction as fixed effects. All data were back transformed for ease of interpretation.
RESULTS

A total of 48 cows were enrolled in the study. Parity, clinical disease and cytological results from the study population are reported in Table 4.4. There were too few cases of clinical disease to produce a meaningful analysis. Correlation was tested between covariates to be offered to the models (parity, body condition and competition index score) but in no case was the correlation coefficient above 0.5.

Effect of Treatment on Behaviour Measures

Over the three weeks in the prepartum pen, lying time averaged $13.6 \pm 2.7 \text{ h/d}$ among all cows. The final mixed linear regression model for the logit transformation of total daily lying time during the three weeks before calving is summarized in Table 4.5. Cows in the understocked group spent significantly more time lying per day ($P = 0.02$) accounting for parity ($P < 0.001$), days from calving ($P < 0.001$), cows with a lameness diagnosis ($P = 0.10$) and a treatment by lameness interaction ($P = 0.04$). The least squares means for the proportion of the day spent lying were 61.6% (95% CI 57.9% to 65.2%) for understocked and 53.5% (95% CI 50.2% to 56.9%) for overstocked cows which equates to 14.8 hours (95% CI 13.9 to 15.6) and 12.8 hours (95% CI 12.0 to 13.7), respectively. First parity animals spent less time lying than third parity, and second parity tended to spend less time than third parity cows. Cows with a lameness diagnosis spent more time lying (1.2 h) if they were in the understocked group. Predicted time lying per day during the treatment period among cows without a lameness diagnosis is displayed in Figure 4.2. The mean variation in daily lying time was $79 \pm 48 \text{ min}$ for understocked cows and $98 \pm 38 \text{ min}$ for overstocked cows and did not differ between treatments ($P = 0.14$).
Over three days of continuous sampling, the mean number of total interactions (actor + reactor interactions) at the feedbunk were 82 (± 41) and 44 (± 23) for overstocked and understocked cows respectively. In a linear mixed model, understocked cows participated in significantly fewer total interactions ($P = 0.04$) accounting for parity. Second lactation cows participated in significantly more total interactions than third parity cows at the feedbunk. Total actor and total reactor interactions were modeled independently offering treatment only. For every displacement, there is 1 actor and 1 reactor, therefore treatment only models were identical for total actor and total reactor interactions. Cows in the overstocked group had a significantly higher frequency of displacements from the feedbunk ($P = 0.02$), and were involved in nearly twice the number of displacements (41 ± 6 versus 21 ± 6). Parity confounded this relationship such that total actor interactions over three days did not differ between groups ($P = 0.28$) accounting for parity ($P = 0.06$) and the least squares means were 39 ± 6 for overstocked and 29 ± 7 for understocked. Second parity cows tended to participate in more actor interactions. For the total number of reactor interactions, upon removal of the parity variable there was a change of > 20% in the coefficient of treatment, verifying confounding was present. Over three days of continuous observation, total reactor interactions did not differ significantly ($P = 0.21$) accounting for parity ($P = 0.44$). The least squares means were 38 ± 6 for the overstocked group and 25 ± 7 for the understocked group. On days 7 to 9, there was no difference in the mean time to visit the feedbunk after fresh feed delivery ($P = 0.78$) between groups or the proportion of cows that did not arrive within 10 minutes after fresh feed delivery ($P = 0.84$).

Daily rumination was recorded during the three weeks in the prepartum pen and averaged 7.7 ± 1.9 hours for all cows in the trial. Rumination detectors were not functional for the first pen of cows, therefore rumination was recorded from 16 understocked cows and 26 overstocked cows.
There was no difference in daily time spent ruminating between treatments ($P = 0.18$) accounting for days from calving ($P = 0.01$). Daily ruminating time least squares means accounting for days from calving were $7.2 \pm 0.3$ h and $7.8 \pm 0.2$ h for understocked and overstocked cows, respectively.

*Effect of Treatment on Metabolic Health*

There was no difference between treatment groups for BHBA over time ($P = 0.32$) accounting for parity, sampling week and experimental block as a random effect. Back transformed least squares means for BHBA were $717.8$ umol/L (95% CI 574.6 to 896.6) and $633.6$ umol/L (95% CI 633.5 to 778.9), for understocked and overstocked cows, respectively. Least squares means for each treatment group by week are displayed in Appendix 4.1. Similarly, NEFA did not differ between treatment groups ($P = 0.61$) accounting for parity, sampling week and experimental block as a random effect. Back transformed least squares means for NEFA were $0.29$ mmol/L (95% CI 0.21 to 0.41) and $0.32$ mmol/L (95% CI 0.24 to 0.45) and are displayed in Appendix 4.2.

Glucose did not differ between treatment groups ($P = 0.69$) accounting for sampling week, parity and experimental block as a random effect; least squares means were $3.14 \pm 0.1$ mmol/L and $3.24 \pm 0.1$ mmol/L for understocked and overstocked cows, respectively. Competition index of the cow was significant when offered as a covariate ($P = 0.03$) with no change to other variables in the model. Glucose was higher among first parity animals compared to third and greater parity cows and in the postpartum weeks with the highest levels being recorded on the day of calving. Glucose tended to be lower overall among animals with a low competition index (< 0.4) relative to a high competition index (> 0.6). Least squares means for each treatment group by week are
Stocking treatment had no effect on serum insulin concentration \((P = 0.59)\) after accounting for sampling week, parity and experimental block as a random effect. Competition index was significant when offered as a covariate \((P = 0.002)\) with no change to the other variables in the model. Insulin declined over the prepartum period and stayed low throughout the postpartum period. First parity cows had higher insulin concentrations and low competition index cows had lower insulin concentrations over time. Back transformed least squares means were 0.24 \(\mu\)g/L (95% CI 0.17 to 0.34) and 0.27 \(\mu\)g/L (95% CI 0.19 to 0.38) for understocked and overstocked respectively. Least squares means for each treatment group by week are displayed in Appendix 4.5 and for each competition index are displayed in Appendix 4.6. Insulin like growth factor-1 did not differ significantly between treatments \((P = 0.72)\) accounting for sampling week, parity and experimental block. Competition index level of the cow was significant when offered as a covariate \((P < 0.0001)\) with no change to the rest of the model. Back transformed least squares means were 0.57 \(\mu\)g/L (95% CI 0.46, 0.70) and 0.55 \(\mu\)g/L (95% CI 0.45, 0.67), for understocked and overstocked, respectively. First parity cows had higher IGF-1 concentrations and low competition index cows had lower IGF-1 concentrations over time Least squares means for each treatment group by week are displayed in Appendix 4.7 and for each competition index level in Appendix 4.8.

The acute phase proteins haptoglobin and albumin were assessed. There was no significant difference in haptoglobin concentrations between groups \((P = 0.84)\) accounting for sampling week and the random effect of experimental block. Least squares means were 0.09 g/L (95% CI 0.05 to 0.15) for understocked and 0.09 g/L (95% CI 0.06 to 0.16) for overstocked cows
Serum albumin concentrations tended to be higher in the understocked cows throughout the study period ($P = 0.06$) accounting for sampling week, parity, body condition score at enrollment, a treatment by week interaction ($P = 0.06$) and the random effect of experimental block. There was no effect of treatment when albumin was modeled at each sampling week ($P > 0.10$) accounting for parity, body condition score and the random effect of experimental block. Least squares means for each treatment group by week are displayed in Appendix 4.10.

Serum total calcium differed significantly between the two treatment groups ($P = 0.03$) accounting for sampling week, parity and experimental block as a random effect. Third and greater parity cows had significantly lower calcium and calcium was significantly lower at week 0 and 1. There was no treatment by week interaction. Least squares means for understocked and overstocked cows were $2.37 \pm 0.02$ mmol/L and $2.29 \pm 0.02$ mmol/L and are displayed in Appendix 4.11. As bound calcium is mostly bound to albumin, albumin concentration was offered as a covariate; serum total calcium did not differ between treatment groups ($P = 0.40$) accounting for week, parity and albumin concentration. Least squares means were $2.34 \pm 0.03$ mmol/L and $2.31 \pm 0.02$ mmol/L for understocked and overstocked, respectively.

Liver health was assessed using serum AST and total bilirubin concentration. AST did not differ between treatment groups ($P = 0.57$) accounting for sampling week, body condition score at enrollment and experimental block as a random effect. Competition index level of the cow was significant when offered as a covariate ($P = 0.03$) with no change to the other effects in the model other than increasing the significance of body condition score ($P = 0.01$). Least squares means for understocked and overstocked cows were $64.4 \pm 0.5.3$ U/L and $69.9 \pm 5.3$ U/L (Appendix 4.12). Similarly, there was no difference between treatments for total bilirubin ($P =$
accounting for sampling week, parity and experimental block. Least squares means for understocked and overstocked cows were 1.94 ± 0.67 umol/L and 2.29 ± 0.65 umol/L (Appendix 4.13).

**Effect of Treatment on Liver Triacylglycerol**

Liver biopsies were successfully collected from 31 cows at week 1 (days 2 to 7 postpartum) and 30 cows from week 3 (days 15 to 21 postpartum). There was an insufficient amount of liver for analysis for one biopsy from each week generating a total of 30 and 29 samples analyzed from week 1 and 3, respectively. At week 1 the mean ± SD for TAG content was 2.89 ± 2.96% in the understocked treatment and 3.79 ± 4.02% in the overstocked treatment. There were 17% of understocked cows and 28% of overstocked cows that had TAG greater than 5% indicating at least moderate fatty liver ($P = 0.67$; Bobe et al., 2004). There was no significant difference in the log transform of triacylglycerol (TAG) content between treatment groups ($P = 0.70$) with experimental block as a random effect. At week 3 the mean ± SD for TAG content was 2.85 ± 3.27% in the understocked and 5.43 ± 5.63% in the overstocked treatment. In week 3 14% of understocked cows and 43% of overstocked cows had TAG greater than 5% ($P = 0.21$). Understocked cows tended to have lower log transform of triacylglycerol content ($P = 0.09$) with pen as a random effect. There was no difference between groups in the change in log liver triacylglycerol content between samples one and three ($P = 0.15$) with pen as a random effect.

**Effect of Treatment on Indicators of Insulin Resistance**

The ratio of blood glucose concentrations at 80 min to 0 min from the modified glucose tolerance tests was examined at the cut points 1.05 and 1.2 and cows above the cut point were defined as insulin resistant. There was no difference between understocked and overstocked cows for the
proportion of cows above the cut point 1.05 (67% vs. 69%; \( P = 0.99 \)) or 1.2 (44% vs. 36%; \( P = 0.75 \)). Area under the curve was calculated for the insulin measurements from the 0, 10 and 80 minute blood samples. There was no significant difference for the area under the insulin curve between treatment groups \( (P = 0.17) \) accounting for parity \( (P = 0.06) \).

Effect of Treatment on Immune Function

Log transformed oxidative burst function did not differ between treatment groups \( (P = 0.69) \) accounting for sampling week and experimental block as a random effect. When competition index was offered to the model, there was a significant competition index level by treatment interaction \( (P = 0.01) \) such that oxidative burst function was greater among overstocked cows with a moderate or high competition index compared to low competition understocked cows or overstocked cows (Table 4.6). Predicted values for each treatment and competition index level are displayed in Figure 4.3. Oxidative burst declined over the prepartum period reaching its nadir in the first week after calving. Least squares means were 23.7% (95% CI: 7.07 to 79.1) and 18.3% (95% CI: 5.5 to 60.9) for understocked and overstocked, respectively (Appendix 4.14), and the means by competition index are displayed in Appendix 4.15. Log transformed phagocytosis also did not differ between treatment groups \( (P = 0.87) \) accounting for sampling week, parity, a treatment by week interaction \( (P = 0.003) \) and pen as a random effect (Appendix 4.16). There was no effect of treatment when the log transformation of phagocytosis was modeled at each sampling week \( (P > 0.17) \) accounting for parity and the random effect of pen.

Effect of Treatment on Uterine Health Parameters

At five weeks postpartum, 46 cows were available for vaginoscopy and 7% of these cows \( (n=3) \) were diagnosed with purulent vaginal discharge. Concurrently, cervical cytology was performed
on 44 cows of which 14% (n=6) were classified as having cervicitis, and endometrial cytology performed on 43 cows, of which 33% (n=14) were classified as having endometritis. All cows that had metritis after calving (n=5) were diagnosed with endometritis by cytology at week 5 with the exception of one cow from which a cytology sample was not successfully collected. A difference in the incidence of cervicitis \( (P = 0.68) \) or endometritis \( (P = 0.10) \) at week 5 was not detected.

*Effect of Treatment on Milk Yield*

Daily milk yield was collapsed into weekly averages and compared between groups during the first 5 weeks postpartum while cows were enrolled in the experiment. There was no significant difference between understocked and overstocked cows in daily milk yield \( (P = 0.83) \) accounting for parity and week. Over the first five weeks, the least squares means estimates for each group were 35.6 ± 1.3 and 35.2 ± 1.1 litres/day for understocked and overstocked cows, respectively.

*Effect of Treatment on Uterine and Liver Gene Expression*

A list of all genes tested is included in Table 4.7. There were no treatment, time (week) or interaction (treatment by week) effects \( (P > 0.05) \) for genes associated with ketogenesis, cyclooxygenase (inflammation) or metabolic transcription factors (Table 4.8). XBP1 transcript abundance, an indicator of endoplasmic reticulum stress was affected by treatment with greater expression in understocked cows \( (P = 0.049) \). FGF21 transcript abundance, a hepatokine triggered by stress, had greater expression in understocked cows \( (P = 0.01) \). The transcript abundance of two gluconeogenesis genes, PCK1 and FBP1 had significant treatment by time interactions \( (P < 0.05) \) such that expression increased among understocked cows and decreased among overstocked cows from week 1 to week 3. DGAT1, a gene related to lipoprotein and
TAG metabolism, also had a significant treatment by time interaction ($P < 0.05$) such that expression increased in overstocked cows and decreased in understocked cows between weeks 1 and 3.

Expression of several genes decreased over time regardless of treatment including the gluconeogenesis gene PC, the fatty acid oxidation gene ACADVL, the acute phase proteins HP, SAA2, SAA3, the inflammation related gene STAT3, the hepatokine ANGPTL4, the insulin signaling gene INSR, and the lipoprotein and TAG metabolism gene MTTP.

There were no treatment or interaction (treatment by week) effects ($P>0.05$) for any of the uterine genes measured (Table 4.7). IL1β, IL6, IL8 and TLR4 had significant time effects ($P < 0.05$) and back transformed results are reported in Table 4.9.

**DISCUSSION**

The purpose of this study was to investigate if reducing competition for feeding and lying space alters metabolic health and immune function. Housing prepartum cows at 120% cows to stalls with 45 cm bunk space compared to 80% cows to stalls with 90 cm bunk space produced greater competition at the feedbunk and reduced lying time; however there were few changes to metabolic and immune health parameters. Cows exposed to the lower stocking density had modestly improved albumin and calcium concentrations during the peripartum period and tended to have lower liver triacylglycerol content at three weeks postpartum. Within the understocked treatment, cows of higher social rank had higher oxidative burst compared to low social status cows.

The current recommendations for transition cow housing outlined in the Canadian Code of Practice for Dairy Cattle advocate pregnant dry cows be given 76 cm of linear bunk space and no
more than 100% cows to stalls for transition cows (DFC-NFACC, 2009). The stocking densities used in the experiment (80% cows to stalls and 90 cm bunk space/cow vs. 120% cows to stalls and 45 cm bunk space/cow) are encompassed in the wide range of prepartum pen stocking densities seen on commercial dairies (Cook and Nordlund, 2004). The social environment the subjects experienced in the two pens was evidently different and more competitive for the overstocked groups. Cows in the overstocked group spent significantly less time lying per day and video analysis revealed these cows were subject to significantly more displacements at the feedbunk. Not surprisingly, heifers spent less time lying per day than multiparous cows and participated in a similar number of displacements regardless of which group they were in. Heifers did not experience the same advantage in the understocked pen as multiparous cows, perhaps because they are smaller than pen counterparts and more likely to be lower in the dominance hierarchy. However, the correlation coefficient between parity and competition index score was 0.21 indicating the two variables were not highly correlated and do not fully explain the heifer experience.

Daily rumination time did not differ between groups. Schirmann et al. (2012) found no relationship between daily ruminating and daily lying time in 42 Holstein dry cows in group pens at a research dairy. As cows were able to perform this behaviour whether standing or lying, it is not surprising that it did not vary between groups even though overstocked cows spent less time lying. Dry matter intake is recognized as important in determining the extent of negative energy balance in the postpartum period (Drackley, 2005). The current experiment did not measure DMI on an individual or group basis due to the design of the pen and daily summaries of rumination behaviour are considered a poor indicator of DMI (Schirmann et al., 2012). Overstocked cows were subject to increased displacements at the feedbunk; however other feeding behaviours were
not characterized. Given the importance of DMI and its effects on NEB, this information may provide insight on the individual cow experience in the pen.

Daily time budgets have been described for lactating dairy cows (Gomez and Cook, 2010; Grant and Albright, 2001) but less information is available for dry cows. Lactating dairy cows are characterized to spend approximately 3 to 5 hours/day eating, 12 to 14 hours/day lying, 0.5 hours/day drinking and 7 to 10 hours/day ruminating while either standing or lying (Grant and Albright, 2001). Among transition cows, Huzzey et al. (2006) reported prepartum cows stood for 12.3 hours/day in free-stall housing and similar time has been reported for cows housed in tie-stalls (12.4 h/d; Dechamps et al., 1989). In the week before calving, prepartum cows exposed to two different stocking densities stood between 10 and 11.7 h/d (Proudfoot et al., 2009), suggesting lying times for dry cows range from 12.3 to 14 hours per day and are similar to lactating cows. Feeding time is not widely reported for non-lactating cows. Huzzey et al. (2012) reported a mean of 230 min/d spent feeding among 40 late-gestation cows housed in a free-stall barn. Feeding time described for 12 peripartum free-stall housed cows was on average 86.8 ± 2.95 min/d precalving and 61.7 ± 2.95 min/d postpartum (Huzzey et al., 2006). These numbers suggest that prepartum cows likely feed for less time per day than lactating cows. Based on the extent of crowding in the overstocked group, theoretically there would still be enough time in the day for cows to perform all of these behaviours for the desired length. However, dairy cattle have been described to be allelomimetic, preferring to perform the same activity at the same time as herd mates, including resting, eating and drinking (Miller and Wood-Gush, 1991). Feeding behaviour in particular is highly synchronized in housed cattle with fresh feed delivery as the primary factor that stimulates eating (von Keyerlingk and Weary, 2009). Therefore, even though the stocking density in the overcrowded pens theoretically allowed all cows adequate time in the
day to perform desired behaviour, not being able to do so collectively potentially limited the time eating and lying for some animals. Although eating time was not recorded, lying time was reduced in overstocked groups.

Despite the environment being less competitive at the feedbunk and understocked cows having more time to rest, there were few metabolic, immunological or reproductive advantages found in the understocked cows during the transition period. There was no difference between any of the metabolic parameters examined over the periparturient period except calcium and albumin. Cows in the understocked group had higher calcium and albumin over time, however these differences were small and it is unclear whether these are biologically important.

Albumin is a negative acute phase protein which decreases during inflammation. Hepatic synthesis of negative acute phase proteins is reduced and positive acute phase protein synthesis amplified during the acute phase, mediated by IL-6 and TNFα (Fleck, 1989; Bertoni et al., 2008). Burke et al. (2010) and Green et al. (2009) found albumin to be slightly but significantly lower through the transition period in cows that were diagnosed with endometritis at 42 days. A similar magnitude of difference (1 – 1.5 g/L) was seen between understocked and overstocked cows in the current study as was seen between cows that later did or did not have endometritis (Burke et al., 2010). There was no difference between treatment groups in the prevalence of endometritis at week 5 in the present study, however the study was not designed to have the statistical power to detect such a difference. Haptoglobin, the only positive acute phase protein measured did not differ between treatments, so evidence for a difference in systemic inflammation status between groups is limited.
Indicators of inflammation assessed by liver gene expression were not different between groups, showing a transient inflammatory state after calving that lessened over time, consistent with resolving an acute phase response. From week 1 to 3, expression of HP, CP, SAA2 and SAA3 declined and ALB increased as would be expected. A lack of change or difference between groups in other inflammation transcription regulators STAT3 and NFKB1 supports the finding that both groups demonstrated the expected transient adaptive inflammatory response to parturition and lactation.

Albumin is a component of a liver activity index proposed by Bertoni and Trevisi (2013) which considers the pattern of change of some negative acute-phase proteins in the first month of lactation to assess metabolic health and predict lactation and reproductive performance. Lower albumin concentrations may be indicative of impaired liver function or evidence of liver fat infiltration. Although other serum markers of liver health (AST and total bilirubin) and production of the positive acute phase protein haptoglobin were not different between groups, liver triacylglycerol content did tend to be lower among understocked cows when sampled in the third week after calving. According to a review by Bobe et al. (2004), > 5% triacylglycerol content is consistent with moderate fatty liver. At week 3, 14% of understocked cows and 43% of overstocked cows had TAG greater than 5%. Other markers of energy status and adaptation to NEB such as NEFA, BHB, glucose, insulin and IGF-1 were not different between groups so it is unclear why hepatic uptake of lipids tended to be higher among overstocked cows. Body condition score at enrollment was not a significant covariate in the triacylglycerol content models at week 1 or 3. As previously discussed, DMI was not captured in the current study as facility design did not permit individual intakes to be measured in a group fed at a post and rail feedbunk; however such data may have provided some insight into these apparent differences.
Like albumin, calcium exhibited a treatment effect. As bound calcium is mostly bound to albumin, albumin concentration was explored as a confounder for the calcium finding. Accounting for albumin, the treatment effect on calcium disappears, supporting it as a confounder. This suggests that treatment did not have a direct effect on calcium metabolism, but might have on liver metabolism, specifically albumin as previously discussed. Although calcium concentration on the day of calving has been suggested to be indicative of DMI, both treatments were fed the same non-acidified prepartum ration and feedbunk management and frequency of feed push up were identical between groups. Dry matter intake was not measured so it was not possible to probe whether the difference in serum Ca concentration might be attributable to DMI, however a difference in liver metabolism is the more likely explanation.

Liver tissue was collected to profile mRNA expression of key metabolic genes associated with fatty acid oxidation, ketogenesis, lipoprotein and triacylglycerol metabolism, insulin signalling and inflammation. Greater expression of hepatokines (FGF21 and ANGPTL4) in liver tissue is associated with worse NEB (Khan et al., 2014). ANGPTL4 was similar between groups, higher at week 1 indicating NEB and less at week 3 demonstrating some adaptation. Expression of FGF21 was lower at both time points in overstocked cows ($P = 0.01$) suggesting possible differences in this aspect of stress response among overstocked cows. Khan et al. (2014) demonstrated that feeding 140% of calculated net energy of lactation ($NE_L$) requirements during the dry period compared to 100% $NE_L$ is associated with more severe NEB postpartum, higher NEFA concentration and resulted in greater hepatic expression of FGF21. Greater expression of the genes APOB, MTTP and DGAT1 is associated with increased lipoprotein synthesis and reduced accumulation of liver triacylglycerol (Loor, 2010). APOB did not differ between groups or over time whereas MTTP decreased between week 1 and 3 for both treatments. DGAT1 had a
significant treatment by week interaction such that expression was similar between groups at week 1, but decreased at week 3 in understocked cows and increased in overstocked cows, consistent with a more metabolically active liver in the overstocked group. The differences noted are of a similar magnitude to cows fed different prepartum diets with gene expression performed in the same laboratory (Khan et al., 2014).

As previously stated, other serum indicators of energy status did not differ between groups and liver biopsies suggested that fatty liver was more prevalent among overstocked cows which are inconsistent with the data on FGF21 and DGAT1 expression. Expression of XBP1 was greater at week 1 for understocked than overstocked cows. Deficiency of XBP1 and resulting ER stress has been associated with increased sensitivity to inflammation, and XBP1 transcription is downregulated when dietary energy is supplied in excess of requirements (Loor, 2010). This finding contrasts our data on FGF21 expression. Bionaz et al., (2013) hypothesized that PPARA and its targets (ACOX1, CPT1A, HMGCS2, ACADVL and FGF21) are upregulated in association with increased circulating NEFA concentrations to coordinate LCFA oxidation and ketogenesis. There was no significant difference between weeks 1 and 3 among groups for PPARAα or its targets other than FGF21, suggesting LCFA oxidation and ketogenesis did not differ between groups or over time.

Expression of PCK1 and FBP1, two genes that are associated with gluconeogenesis, showed a treatment by time interaction such that they increased for understocked cows and decreased for overstocked cows between weeks 1 and 3. Greater expression of these and other gluconeogenesis genes (PC, PDK4) is associated with increased gluconeogenic capacity of the liver, suggesting understocked cows were greater prepared to generate glucose from non-carbohydrate substrates, however this did not translate to a difference in milk production.
Recommendations in the literature state that overcrowding should be avoided in transition pens, stocking at no more than 1:1 cows to stalls, and pen additions should be limited (Grant and Albright, 2001; Cook and Nordlund, 2004). Nordlund et al. (2006) notes that lactating cattle will generally not fill more than 80% of 24 inch headlocks, therefore 30 inches (76 cm) per cow should be offered and wider gestating cows might benefit from even more. These recommendations although commonly cited, are not well supported with transition cow data (Cook and Nordlund, 2004). There is very little published in the literature examining metabolic health of transition cows associated with housing environment. Two recent studies that examined prepartum housing similarly found little metabolic advantage with housing strategies intended to reduce social stress. Silva et al. (2013a) examined a stable all-in-all-out (AIAO) pen with no further cow additions throughout the close up period versus a traditional prepartum pen stocked at 100% stalls and 91.6% headlocks with weekly additions to re-establish and maintain this stocking density (n = 567) and found treatment did not affect the incidence of retained fetal membranes or metritis, or the concentrations of NEFA or BHB. A second study conducted by the same research group utilized 728 prepartum Jersey cows and examined stocking pens at 80% versus 100% headlock stocking density and 86% versus 109% stalls with twice weekly additions and found that treatment did not affect the incidence of retained fetal membranes, metritis, purulent vaginal discharge, or the concentrations of NEFA or BHB.

The effect of housing treatment on innate immune function parameters was a key objective of the present study. Silva et al. (2013b) evaluated phagocytic and oxidative burst activity of neutrophils among a subgroup of 68 Jersey cows from the larger study of 567 animals housed in either an AIAO or a traditional close up group and found no effect of treatment on the percentage of neutrophils positive for phagocytosis or oxidative burst. Similarly, we found no main effect of
lying and feeding space allowance on the same measures of neutrophil function. When competition index category was offered to the oxidative burst model in the present study, a treatment by C_Ind interaction showed that while oxidative burst function was similar between competition categories in the overstocked groups, cows in the understocked group with a higher competition index score (0.4-0.6 and >0.6), had greater oxidative burst function than low ranking cows. Cows in the understocked group with a low competition score (<0.4) did not differ from the overstocked cows suggesting that the benefit of reduced competition did not extend to them. There are no other studies that have reported immune function relative to group social status.

Social hierarchy is established through physical and non-physical interactions and regrouping forces cows to constantly re-establish this order, intensifying aggressive and submissive behaviours (von Keyerslingk et al., 2008). Assuming that social status of a cow would be unchanged regardless of treatment group, it seems in a stable group with greater lying and feeding space, cows higher in the social hierarchy were able to extract an advantage from lower competition, yet the space allowances provided in this study were not sufficient to compensate for the effect of lower competitive success at the feedbunk.

Competition index was associated with several metabolites including glucose, insulin, IGF-1, and AST concentrations. The effects included a tendency for glucose to be lower among cows with a low competition index compared to a high competition index, whereas insulin and IGF-1 were significantly lower among cows with a low competition index compared to a high competition index. Low index cows also had significantly lower serum glucose and insulin concentrations than cows with a moderate competition index. The differences in insulin and IGF-1 were primarily prepartum while glucose was different prepartum and postpartum. Typically, postpartum homeorhetic changes partition glucose towards the mammary gland for energy and
lactose synthesis (Lucy, 2008). As a result of this partitioning, blood glucose concentrations are low postpartum despite increased gluconeogenesis in the liver in response to increased blood growth hormone (GH) concentrations. Low glucose concentrations are associated with low insulin concentrations and partial insulin resistance (Lucy, 2008). Growth hormone binds to GH receptor in the liver to initiate secretion of IGF-1, which acts on the hypothalamus and pituitary to control GH secretion via negative feedback. In the prepartum period, circulating glucose, insulin and IGF-1 concentrations are associated with energy status. Although elevated levels are not ideal because cows overfed energy in the prepartum close up period have greater concentrations of glucose and insulin (Janovick et al., 2011), low prepartum glucose has been associated with increased risk of disease for clinical mastitis (Schwegler et al., 2013). The risk of metritis decreased as prepartum IGF-1 increased (Giuliodori et al., 2013). Energy balance and plasma concentrations of glucose, insulin, and IGF-1 during the dry period were higher in cows with luteal activity in the first 3 weeks postpartum (early ovulatory cows) compared to anovulatory cows (Castro et al., 2012). Using the same competition index, Huzzey et al. (2012) found late gestation cows in the low success group had a different physiological profile than high success cows, with greater average daily fecal cortisol metabolite concentrations, greater NEFA concentrations and a difference in peak insulin response to a glucose tolerance test. The association between lower status cows and these metabolites in the current study suggests energy metabolism was different for these cows and a larger study might find this translates into a difference in the incidence of reproductive disease.

A common criticism of housing research is the small scale at which it is performed in research facilities (Cook and Nordlund, 2004). The close-up prepartum groups assembled were small (6 to 10 cows) but would be typical of many Canadian dairy herds. With small groups, the number of
additional cows in the pen between an understocked and overstocked group was only 2 to 4. This number is easily consistent with calving pattern variabilities that happen on many small dairies, resulting in fluctuating stocking densities. Many North American dairies are much larger than this however and whether these results would be replicated in a larger pen is unknown. The three larger experiments examining social group stability and feeding space (Silva et al., 2013a, 2013b, 2014) were conducted on a single commercial dairy with pens holding more than 40 cows per replicate but failed to find significant effects on metabolic and innate immune function when examining stocking density and group stability independently. The interaction that we found between competitive success at the feedbunk and space allowance for neutrophil oxidative burst points to nuances and conditioned effects of feed and lying space. Examining metabolic and immune function parameters in relation to social status in a larger group would be worthwhile. While it would be challenging to study, there may be interactions among the effects of space allowances, social group stability and competitive success for resources. Whether space allotment could be increased to some level that all cows regardless of social status experience an improvement in immune function is not known. It is possible that regardless of space allotted, the inherent social hierarchy and its expression by cows would always result in some cows being disadvantaged.

Despite greater competition at the feedbunk and reduced lying time, the expected harmful effects of crowding and competition were mostly not seen. One difference between the overcrowding in this research study and crowding that occurs on commercial dairies is the length of time cows were exposed. Study subjects were in a zero competition environment (tie stalls) for the far off dry period before commencing the study and for the five weeks after calving while they continued to be assessed. Crowding that occurs throughout the far off, close up and fresh period
may have worse effects and elicit metabolic and immune function differences that were not seen in the current experiment or those reported by Silva et al. (2013a, 2013b, 2014).

It has been suggested that the goal of the transition housing environment be to provide for the needs of each cow so that she can behave as a herd animal: eating, resting and socializing with the herd and without fear (Cook and Nordlund, 2004). In this experiment, the degree of competitive pressure induced by the lying and feeding space allowances reduced lying time and changed displacements at the feedbunk but did not appreciably alter metabolic and immune health parameters, although there were conditional effects on neutrophil oxidative burst function.

CONCLUSIONS

A non-competitive housing strategy of 80% cows to stalls and 90 cm of bunk space modestly improved albumin and calcium concentrations during the peripartum period and improved the innate immune parameter oxidative burst function among cows with higher social status compared to low social status cows. The housing strategy failed to improve other markers of metabolic health, however, cows exposed to the non-competitive housing strategy tended to have lower liver triacylglycerol content at three weeks postpartum. The hypothesis that providing greater feeding and lying space would reduce social stress and thereby improve measures of adaption to negative energy balance and of innate immune function was not proven. On average, crowding in a small, stable group through the close-up dry period without crowding or social instability in the postpartum period did not negatively affect the metabolic health and immune function of cows. These results indicate that metabolic health and immune function are more complex than can be explained solely by exposure to what are understood to be best housing practices.
REFERENCES


Table 4.1: Composition of feed for prepartum (d -24 ± 3 to 0) and postpartum (d 1 to 35) diets for 48 Holstein cows randomly assigned to be understocked (80% cows to stalls and 90 cm of feeding space per cow) or overstocked (120% cows to stalls and 45 cm of feeding space per cow) for 21 to 28 days prepartum.

<table>
<thead>
<tr>
<th>Ingredient (%)</th>
<th>Prepartum</th>
<th>Postpartum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Straw</td>
<td>29.2</td>
<td>3.8</td>
</tr>
<tr>
<td>Haylage</td>
<td>4.8</td>
<td>26.9</td>
</tr>
<tr>
<td>Corn silage</td>
<td>40.9</td>
<td>28.7</td>
</tr>
<tr>
<td>High moisture corn</td>
<td>--</td>
<td>24.4</td>
</tr>
<tr>
<td>Concentrate mix</td>
<td>25.1</td>
<td>16.2</td>
</tr>
</tbody>
</table>

Composition of concentrate mix (based on 1000kg mix)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Prepartum</th>
<th>Postpartum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tri Pro Gold&lt;sup&gt;1&lt;/sup&gt;</td>
<td>345.60</td>
<td>248.70</td>
</tr>
<tr>
<td>Wheat Shorts</td>
<td>294.53</td>
<td>--</td>
</tr>
<tr>
<td>Canola Meal</td>
<td>130.1</td>
<td>--</td>
</tr>
<tr>
<td>Soybean Meal</td>
<td>73.8</td>
<td>295.41</td>
</tr>
<tr>
<td>Hi Pro Corn Gluten Meal</td>
<td>--</td>
<td>187.20</td>
</tr>
<tr>
<td>Calcium Carbonate</td>
<td>51.70</td>
<td>42.60</td>
</tr>
<tr>
<td>Monocalcium Phosphate</td>
<td>23.60</td>
<td>41.60</td>
</tr>
<tr>
<td>Sodium Sesquicarbonate</td>
<td>--</td>
<td>29.60</td>
</tr>
<tr>
<td>Fish Meal</td>
<td>--</td>
<td>29.60</td>
</tr>
<tr>
<td>Yeast</td>
<td>16.50</td>
<td>13.30</td>
</tr>
<tr>
<td>Tallow</td>
<td>30.00</td>
<td>24.00</td>
</tr>
<tr>
<td>Molasses</td>
<td>--</td>
<td>24.00</td>
</tr>
<tr>
<td>Fine salt</td>
<td>10.10</td>
<td>26.10</td>
</tr>
<tr>
<td>DCAD +ve Potassium Carbonate</td>
<td>--</td>
<td>15.40</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>9.10</td>
<td>--</td>
</tr>
<tr>
<td>Magnesium Oxide</td>
<td>8.90</td>
<td>9.20</td>
</tr>
<tr>
<td>FFM Org Ruminant Micro&lt;sup&gt;2&lt;/sup&gt;</td>
<td>4.68</td>
<td>6.52</td>
</tr>
<tr>
<td>Monensin</td>
<td>0.44</td>
<td>0.36</td>
</tr>
<tr>
<td>Sel-Plex 2000&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.41</td>
<td>0.17</td>
</tr>
<tr>
<td>Sulphur flour 99.5%</td>
<td>0.41</td>
<td>1.10</td>
</tr>
<tr>
<td>Rovimix Biotin 20 000&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.13</td>
<td>--</td>
</tr>
<tr>
<td>MetaSmart&lt;sup&gt;5&lt;/sup&gt;</td>
<td>--</td>
<td>5.14</td>
</tr>
</tbody>
</table>

<sup>1</sup>Tri Pro Gold- Bypass soybean meal  
<sup>2</sup>FFM Org Ruminant Micro- Trace mineral and vitamin premix containing 5 organic micro minerals (zinc, manganese, copper, cobalt and selenium)  
<sup>3</sup>Sel-plex 2000 – Organic selenium  
<sup>4</sup>Rovamix Biotin 20 000- Water soluble B vitamin (20 000 mcg/kg)  
<sup>5</sup>MetaSmart- Rumen protected methionine
Table 4.2: Nutrient analysis of feed for prepartum (d -24 ± 3 to 0) and postpartum (d 1 to 35) diets for 48 Holstein cows randomly assigned to be understocked (80% cows to stalls and 90 cm of feeding space per cow) or overstocked (120% cows to stalls and 45 cm of feeding space per cow) for 21 to 28 days prepartum.

<table>
<thead>
<tr>
<th>Item, % of DM (unless otherwise noted)</th>
<th>Prepartum</th>
<th>Postpartum</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM%</td>
<td>53.24</td>
<td>48.47</td>
</tr>
<tr>
<td>NE&lt;sub&gt;L&lt;/sub&gt;, Mcal/kg</td>
<td>1.37</td>
<td>1.57</td>
</tr>
<tr>
<td>CP</td>
<td>13.53</td>
<td>16.74</td>
</tr>
<tr>
<td>ADF</td>
<td>28.83</td>
<td>18.15</td>
</tr>
<tr>
<td>NDF</td>
<td>48.02</td>
<td>32.02</td>
</tr>
<tr>
<td>Ash</td>
<td>8.63</td>
<td>8.24</td>
</tr>
<tr>
<td>Calcium</td>
<td>1.03</td>
<td>0.91</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>0.45</td>
<td>0.45</td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.39</td>
<td>0.30</td>
</tr>
<tr>
<td>Potassium</td>
<td>1.08</td>
<td>1.2</td>
</tr>
<tr>
<td>Sulfur</td>
<td>0.19</td>
<td>0.24</td>
</tr>
<tr>
<td>Sodium</td>
<td>0.16</td>
<td>0.36</td>
</tr>
<tr>
<td>Chlorine</td>
<td>0.50</td>
<td>0.54</td>
</tr>
</tbody>
</table>
Table 4.3: Description of sequential treatment groups randomly assigned to be understocked (80% stocking density (cows to stalls) and 90 cm of bunk space per cow) or overstocked (120% stocking density and 45 cm of bunk space per cow) formed 21 to 28 days before expected calving.

<table>
<thead>
<tr>
<th>Experimental Block</th>
<th>Treatment</th>
<th>Number Primiparous</th>
<th>Number Multiparous</th>
<th>Start Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Understocked</td>
<td>1</td>
<td>5</td>
<td>18/10/2012</td>
</tr>
<tr>
<td>2</td>
<td>Overstocked</td>
<td>4</td>
<td>5</td>
<td>27/12/2012</td>
</tr>
<tr>
<td>3</td>
<td>Understocked</td>
<td>2</td>
<td>4</td>
<td>05/03/2013</td>
</tr>
<tr>
<td>4</td>
<td>Overstocked</td>
<td>4</td>
<td>3</td>
<td>01/05/2013</td>
</tr>
<tr>
<td>5</td>
<td>Understocked</td>
<td>5</td>
<td>5</td>
<td>26/06/2013</td>
</tr>
<tr>
<td>6</td>
<td>Overstocked</td>
<td>4</td>
<td>6</td>
<td>31/07/2013</td>
</tr>
</tbody>
</table>
Table 4.4: Summary of observed diseases in 48 Holstein dairy cows randomly assigned to overstocked (120% cows to stalls and 45 cm of feeding space per cow) or understocked (80% cows to stalls and 90 cm of feeding space per cow) housing cohorts for 3 weeks prior to calving and followed until 5 weeks after calving.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total Cases</th>
<th>% of total cows</th>
<th>Number of cases in understocked treatment</th>
<th>Number of cases in overstocked treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retained placenta</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Metritis</td>
<td>5</td>
<td>10</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Left displaced abomasum</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Purulent vaginal discharge at 3 wk after calving</td>
<td>9</td>
<td>20</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Purulent vaginal discharge at 5 wk after calving</td>
<td>3</td>
<td>7</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Endometritis at Week 3</td>
<td>17</td>
<td>39</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Endometritis at Week 5</td>
<td>14</td>
<td>33</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>Cervicitis at Week 3</td>
<td>9</td>
<td>23</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Cervicitis at Week 5</td>
<td>6</td>
<td>14</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

1 Retained placenta was diagnosed by farm staff if placenta still present after 24 hours.

2 Metritis (T ≥ 39.5 and fetid discharge) and left displaced abomasum were diagnosed based on clinical signs by the herd veterinarian.

3 Purulent vaginal discharge at week 3 and 5 was diagnosed by vaginoscopy using a score of 2 or 3 according to Sheldon et al. (2006).

4 Endometritis and cervicitis were diagnosed with cytology at week 3 and 5 postpartum as ≥ 6% neutrophils in cell population.
Table 4.5: Final model for the logit transformation of daily lying time (proportion of the day spent lying) recorded by a 3-axis accelerometer (Hobo datalogger) for day -24 to -3 before calving for cows that were in understocked (80% cows to stalls and 90 cm of bunk space per cow) or overstocked (120% cows to stalls and 45 cm of bunk space per cow) groups for 3 weeks prepartum.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Category</th>
<th>B</th>
<th>P</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td></td>
<td>0.07</td>
<td>0.52</td>
<td>-0.2, 0.34</td>
</tr>
<tr>
<td>Treatment (Trt)</td>
<td>Understocked</td>
<td>0.29</td>
<td>0.02</td>
<td>0.07, 0.50</td>
</tr>
<tr>
<td></td>
<td>Overstocked</td>
<td>Ref</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days from</td>
<td></td>
<td>-0.02</td>
<td>&lt;.0001</td>
<td>-0.03, -0.01</td>
</tr>
<tr>
<td>Calving</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parity</td>
<td>1</td>
<td>-0.35</td>
<td>&lt;0.001</td>
<td>-0.53, -0.17</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>-0.19</td>
<td>0.10</td>
<td>-0.42, 0.04</td>
</tr>
<tr>
<td></td>
<td>3+</td>
<td>Ref</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lameness</td>
<td></td>
<td>-0.07</td>
<td>0.78</td>
<td>-0.57, 0.42</td>
</tr>
<tr>
<td>Lameness*Trt</td>
<td>Understocked</td>
<td>0.66</td>
<td>0.04</td>
<td>0.03, 1.28</td>
</tr>
<tr>
<td></td>
<td>Overstocked</td>
<td>Ref</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4.6: Final model for weekly log transform of oxidative burst function for 48 Holstein dairy cows randomly assigned to overstocked (120% cows to stalls and 45 cm of feeding space per cow) or understocked (80% cows to stalls and 90 cm of feeding space per cow) housing cohorts for 3 weeks prior to calving and followed until 5 weeks after calving.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Categories</th>
<th>B</th>
<th>SE</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td></td>
<td>2.90</td>
<td>0.46</td>
<td>0.003</td>
</tr>
<tr>
<td>Treatment (Trt)</td>
<td>Understocked</td>
<td>0.54</td>
<td>0.60</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>Overstocked</td>
<td>Referent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week</td>
<td>-2</td>
<td>Referent</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-1</td>
<td>-0.34</td>
<td>0.27</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>-0.68</td>
<td>0.26</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.009</td>
<td>0.30</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.16</td>
<td>0.29</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.63</td>
<td>0.30</td>
<td>0.03</td>
</tr>
<tr>
<td>Competition Index (C_Ind)</td>
<td>&lt;0.4</td>
<td>-0.02</td>
<td>0.19</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>0.4-0.6</td>
<td>-0.13</td>
<td>0.32</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>≥0.6</td>
<td>Referent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment*C_Ind Interaction</td>
<td>Understocked*C_Ind &lt;0.4</td>
<td>-0.79</td>
<td>0.28</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>Understocked*C_Ind 0.4-0.6</td>
<td>0.27</td>
<td>0.41</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>Understocked*C_Ind &gt;0.6</td>
<td>0</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Overstocked*C_Ind &lt;0.4</td>
<td>0</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Overstocked*C_Ind 0.4-0.6</td>
<td>0</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Overstocked*C_Ind &gt;0.6</td>
<td>0</td>
<td>--</td>
<td></td>
</tr>
</tbody>
</table>
**Table 4.7:** Abbreviations and descriptions of the genes analyzed for 31 Holstein dairy cows randomly assigned to overstocked (120% cows to stalls and 45 cm of feeding space per cow) or understocked (80% cows to stalls and 90 cm of feeding space per cow) housing cohorts for 3 weeks prior to calving.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Analyzed in liver tissue</strong></td>
<td></td>
</tr>
<tr>
<td>ACADVL</td>
<td>Acyl-coenzyme A dehydrogenase very long chain</td>
</tr>
<tr>
<td>ACOX1</td>
<td>Acyl-CoA oxidase 1, palmitoyl</td>
</tr>
<tr>
<td>AKT1</td>
<td>v-akt murine thymoma viral oncogene homolog</td>
</tr>
<tr>
<td>ALB</td>
<td>Albumin</td>
</tr>
<tr>
<td>ANGPTL4</td>
<td>Angiopoietin-like 4</td>
</tr>
<tr>
<td>APOB</td>
<td>Apolipoprotein B</td>
</tr>
<tr>
<td>CP</td>
<td>Ceruloplasmin</td>
</tr>
<tr>
<td>CPT1A</td>
<td>Carnitine palmitoyltransferase 1A</td>
</tr>
<tr>
<td>DGAT1</td>
<td>Diacylglycerol O-acyltransferase 1</td>
</tr>
<tr>
<td>FBP1</td>
<td>Fructose-1,6-bisphosphatase 1</td>
</tr>
<tr>
<td>FGF21</td>
<td>Fibroblast growth factor 21</td>
</tr>
<tr>
<td>HMGCS2</td>
<td>3-hydroxy-3-methylglutaryl-coenzyme A synthase 1</td>
</tr>
<tr>
<td>HP</td>
<td>Haptoglobin</td>
</tr>
<tr>
<td>INSR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>IRS1</td>
<td>Insulin receptor substrate 1</td>
</tr>
<tr>
<td>MLYCD</td>
<td>Malonyl-CoA decarboxylase</td>
</tr>
<tr>
<td>MTTP</td>
<td>Microsomal Triacylglycerol transfer protein</td>
</tr>
<tr>
<td>NFKB1</td>
<td>Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1</td>
</tr>
<tr>
<td>PC</td>
<td>Pyruvate carboxylase</td>
</tr>
<tr>
<td>PCK1</td>
<td>Phosphoenolpyruvate carboxykinase 1</td>
</tr>
<tr>
<td>PDK4</td>
<td>Pyruvate dehydrogenase kinase, isozyme 4</td>
</tr>
<tr>
<td>PPARA</td>
<td>Peroxisome proliferator activated receptor-α</td>
</tr>
<tr>
<td>PTGS2</td>
<td>Prostaglandin-endoperoxide synthase 2 (COX2)</td>
</tr>
<tr>
<td>RXRA</td>
<td>Retinoid X receptor, α</td>
</tr>
<tr>
<td>SAA2</td>
<td>Serum amyloid A 2</td>
</tr>
<tr>
<td>SAA3</td>
<td>Serum amyloid A 3</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>XBP1</td>
<td>X-box binding protein 1</td>
</tr>
<tr>
<td><strong>Analyzed from uterine body swabs</strong></td>
<td></td>
</tr>
<tr>
<td>IL1β</td>
<td>Interleukin 1 beta</td>
</tr>
<tr>
<td>IL6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>IL8</td>
<td>Interleukin 8</td>
</tr>
<tr>
<td>IL10</td>
<td>Interleukin 10</td>
</tr>
<tr>
<td>MUC1</td>
<td>Mucin 1, cell surface associated</td>
</tr>
<tr>
<td>TLR4</td>
<td>Toll like receptor 4</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td><strong>Housekeeping genes for liver and uterus</strong></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>RPS9</td>
<td>Ribosomal Protein S9</td>
</tr>
<tr>
<td>UXT</td>
<td>Ubiquitously-Expressed, Prefoldin-Like Chaperone</td>
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Table 4.8: Hepatic gene expression (log2- back transformed mean fold change relative to geometric mean RNA abundance of 3 reference housekeeping genes) for 30 Holstein dairy cows randomly assigned to overstocked (120% cows to stalls and 45 cm of feeding space per cow) or understocked (80% cows to stalls and 90 cm of feeding space per cow) housing cohorts for 3 weeks prior to calving.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Understocked</th>
<th>Overstocked</th>
<th>SEM</th>
<th>TRT</th>
<th>TRT*Week</th>
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<tr>
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<td>Wk1</td>
<td>Wk3</td>
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Table 4.9: Uterine gene expression (log2- back transformed mean fold-change relative to geometric mean RNA abundance of 3 reference housekeeping genes) for 30 Holstein dairy cows randomly assigned to overstocked (120% cows to stalls and 45 cm of feeding space per cow) or understocked (80% cows to stalls and 90 cm of feeding space per cow) housing cohorts for 3 weeks prior to calving.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Understocked</th>
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<th>Overstocked</th>
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<td>Wk3</td>
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<td>Week</td>
<td>TRT*Week</td>
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<tr>
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<td>0.03</td>
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<tr>
<td>MUC1</td>
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<tr>
<td>IL10</td>
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<td>0.54</td>
<td>0.49</td>
<td>0.25</td>
<td>0.26</td>
<td></td>
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</tbody>
</table>
Figure 4.1: Diagram of pen layout for groups of 6 to 10 cows that were understocked or overstocked for the close up dry period and sampled weekly. Gates were used to modify the space allotted for feeding and lying depending on stocking density and cow number. Understocked cows had 90 cm of feeding space per cow and lying spaces were stocked at 80% cows to stalls. Overstocked cows had 45 cm of feeding space per cow and lying spaces were stocked at 120% cows to stalls.
Figure 4.2: Predicted lying time in cows with no lameness event that were understocked or overstocked for 3-4 weeks prepartum. Understocked cows had 90 cm of feeding space per cow and lying spaces were stocked at 80% cows to stalls. Overstocked cows had 45 cm of feeding space per cow and lying spaces were stocked at 120% cows to stalls.
Figure 4.3: Predicted oxidative burst function in cows that were understocked or overstocked for the close up dry period. Understocked cows had 90 cm of feeding space per cow and lying spaces were stocked at 80% cows to stalls. Overstocked cows had 45 cm of feeding space per cow and lying spaces were stocked at 120% cows to stalls.

A competition index (CInd) was calculated for each cow by dividing the number of times the cow displaced another at the FB by the total number of displacements the cow was involved in, either as an actor or reactor. Cows were divided into 3 subgroups based on their CInd: high success (HS: CInd ≥0.6), medium success (0.4 ≤ CInd <0.6), and low success (LS: CInd <0.4).
Appendix 4.1: Least squares means (accounting for parity, sampling week and experimental block as a random effect) back transformed (± 95% confidence interval) of BHB concentrations for 48 Holstein dairy cows that were understocked or overstocked for the close up dry period and sampled weekly. Understocked cows had 90 cm of feeding space per cow and lying spaces were stocked at 80% cows to stalls. Overstocked cows had 45 cm of feeding space per cow and lying spaces were stocked at 120% cows to stalls.
Appendix 4.2: Least squares means (accounting for parity, sampling week and experimental block as a random effect) back transformed (± 95% confidence interval) of NEFA concentrations for 48 Holstein dairy cows that were understocked or overstocked for the close up dry period and sampled weekly. Understocked cows had 90 cm of feeding space per cow and lying spaces were stocked at 80% cows to stalls. Overstocked cows had 45 cm of feeding space per cow and lying spaces were stocked at 120% cows to stalls.
**Appendix 4.3:** Least squares means (± SE; accounting for sampling week, parity and experimental block) of glucose concentrations for 48 Holstein dairy cows that were understocked or overstocked for the close up dry period and sampled weekly. Understocked cows had 90 cm of feeding space per cow and lying spaces were stocked at 80% cows to stalls. Overstocked cows had 45 cm of feeding space per cow and lying spaces were stocked at 120% cows to stalls.
Appendix 4.4: Least squares means (±SE; accounting for sampling week, parity, competition index and experimental block) of glucose concentrations for 48 Holstein dairy cows assessed by competition index score. Cows were understocked or overstocked for the close up dry period and sampled weekly. Understocked cows had 90 cm of feeding space per cow and lying spaces were stocked at 80% cows to stalls. Overstocked cows had 45 cm of feeding space per cow and lying spaces were stocked at 120% cows to stalls.

The competition index (CInd) was calculated for each cow by dividing the number of times the cow displaced another at the FB by the total number of displacements the cow was involved in, either as an actor or reactor. Cows were divided into 3 subgroups based on their CInd: high success (HS: CInd ≥ 0.6), medium success (0.4 ≤ CInd < 0.6), and low success (LS: CInd < 0.4).
Appendix 4.5: Least squares means back transformed (± 95% confidence interval; accounting for sampling week, parity and experimental block as a random effect) of insulin concentrations for 48 Holstein dairy cows that were understocked or overstocked for the close up dry period and sampled weekly. Understocked cows had 90 cm of feeding space per cow and lying spaces were stocked at 80% cows to stalls. Overstocked cows had 45 cm of feeding space per cow and lying spaces were stocked at 120% cows to stalls.
Appendix 4.6: Least squares means back transformed (±confidence intervals; accounting for sampling week, parity, competition index and experimental block as a random effect) of insulin concentrations for 48 Holstein dairy cows assessed by competition index score. Cows were understocked or overstocked for the close up dry period and sampled weekly. Understocked cows had 90 cm of feeding space per cow and lying spaces were stocked at 80% cows to stalls. Overstocked cows had 45 cm of feeding space per cow and lying spaces were stocked at 120% cows to stalls.

The competition index (CInd) was calculated for each cow by dividing the number of times the cow displaced another at the FB by the total number of displacements the cow was involved in, either as an actor or reactor. Cows were divided into 3 subgroups based on their CInd: high success (HS: CInd ≥0.6), medium success (0.4 ≤ CInd <0.6), and low success (LS: CInd <0.4).
Appendix 4.7: Least squares means back transformed (± 95% confidence interval; accounting for sampling week, parity and experimental block as a random effect) of IGF-1 concentrations for 48 Holstein dairy cows that were understocked or overstocked for the close up dry period and sampled weekly. Understocked cows had 90 cm of feeding space per cow and lying spaces were stocked at 80% cows to stalls. Overstocked cows had 45 cm of feeding space per cow and lying spaces were stocked at 120% cows to stalls.
Appendix 4.8: Least square means back transformed (±confidence intervals; accounting for sampling week, parity, competition index and experimental block as a random effect) of IGF-1 concentrations for 48 Holstein dairy cows assessed by competition index score. Cows were understocked or overstocked for the close up dry period and sampled weekly. Understocked cows had 90 cm of feeding space per cow and lying spaces were stocked at 80% cows to stalls. Overstocked cows had 45 cm of feeding space per cow and lying spaces were stocked at 120% cows to stalls.

The competition index (CInd) was calculated for each cow by dividing the number of times the cow displaced another at the FB by the total number of displacements the cow was involved in, either as an actor or reactor. Cows were divided into 3 subgroups based on their CInd: high success (HS: CInd ≥0.6), medium success (0.4 ≤ CInd <0.6), and low success (LS: CInd <0.4).
Appendix 4.9: Least squares means back transformed (± 95% confidence interval; accounting for sampling week and experimental block as a random effect) of haptoglobin concentrations for 48 Holstein dairy cows that were understocked or overstocked for the close up dry period and sampled weekly. Understocked cows had 90 cm of feeding space per cow and lying spaces were stocked at 80% cows to stalls. Overstocked cows had 45 cm of feeding space per cow and lying spaces were stocked at 120% cows to stalls.
Appendix 4.10: Least squares means (± SE; accounting for sampling week, parity, body condition score at enrollment, a treatment by week interaction and experimental block as a random effect) of albumin concentrations for 48 Holstein dairy cows that were understocked or overstocked for the close up dry period and sampled weekly. Understocked cows had 90 cm of feeding space per cow and lying spaces were stocked at 80% cows to stalls. Overstocked cows had 45 cm of feeding space per cow and lying spaces were stocked at 120% cows to stalls.
Appendix 4.11: Least squares means (± SE; accounting for sampling week, parity and experimental block as a random effect) of total calcium concentrations for 48 Holstein dairy cows that were understocked or overstocked for the close up dry period and sampled weekly. Understocked cows had 90 cm of feeding space per cow and lying spaces were stocked at 80% cows to stalls. Overstocked cows had 45 cm of feeding space per cow and lying spaces were stocked at 120% cows to stalls.
Appendix 4.12: Least squares means (± SE; accounting for sampling week, body condition score at enrollment and experimental block as a random effect) of AST concentrations for 48 Holstein dairy cows that were understocked or overstocked for the close up dry period and sampled weekly. Understocked cows had 90 cm of feeding space per cow and lying spaces were stocked at 80% cows to stalls. Overstocked cows had 45 cm of feeding space per cow and lying spaces were stocked at 120% cows to stalls.
Appendix 4.13: Least squares means (± SE) accounting for sampling week, parity and experimental block as a random effect) of total bilirubin concentrations for 48 Holstein dairy cows that were understocked or overstocked for the close up dry period and sampled weekly. Understocked cows had 90 cm of feeding space per cow and lying spaces were stocked at 80% cows to stalls. Overstocked cows had 45 cm of feeding space per cow and lying spaces were stocked at 120% cows to stalls.
**Appendix 4.14:** Unadjusted mean (±SE) of oxidative burst activity for 48 Holstein dairy cows that were understocked or overstocked for the close up dry period and sampled weekly. Understocked cows had 90 cm of feeding space per cow and lying spaces were stocked at 80% cows to stalls. Overstocked cows had 45 cm of feeding space per cow and lying spaces were stocked at 120% cows to stalls.
Appendix 4.15: Unadjusted mean (±SE) of oxidative burst activity for 48 Holstein dairy cows assessed by competition index score. Cows were understocked or overstocked for the close up dry period and sampled weekly. Understocked cows had 90 cm of feeding space per cow and lying spaces were stocked at 80% cows to stalls. Overstocked cows had 45 cm of feeding space per cow and lying spaces were stocked at 120% cows to stalls.

A competition index (CInd) was calculated for each cow by dividing the number of times the cow displaced another at the FB by the total number of displacements the cow was involved in, either as an actor or reactor. Cows were divided into 3 subgroups based on their CInd: high success (HS: CInd ≥0.6), medium success (0.4 ≤ CInd <0.6), and low success (LS: CInd <0.4).
Appendix 4.16: Unadjusted mean (±SE) of phagocytosis for 48 Holstein dairy cows that were understocked or overstocked for the close up dry period and sampled weekly. Understocked cows had 90 cm of feeding space per cow and lying spaces were stocked at 80% cows to stalls. Overstocked cows had 45 cm of feeding space per cow and lying spaces were stocked at 120% cows to stalls.
Chapter 5

GENERAL CONCLUSIONS

The periparturient period, during which dairy cattle make the transition from the pregnant, non-lactating state to the non-pregnant, lactating state, is characterized by a disproportionate occurrence of health problems for dairy cows. Most infectious and metabolic disorders occur during this time while cows must make dramatic adaptations to meet the demands of milk production. Minimizing the incidence of peripartum disease is essential, as a successful transition contributes substantially to profitability of the cow through the lactation. The importance of this time has made it a primary focus of research, with many management strategies and interventions investigated to improve success. While not an exhaustive list, strategies include nutrition programs, management of animal grouping, monitoring programs and prophylactic treatment with glucose precursors, calcium sources, and antibiotics.

The overall goal of the research described in this thesis was to contribute to the understanding of the interactions among metabolic and immune system health and management of dairy cows in the peripartum period. Several common management interventions that are recommended by veterinarians and advisors for the benefit of peripartum cows were explored to improve the evidence base for their endorsement.

The first objective was to evaluate the effect of a subcutaneous calcium supplementation product, Theracalcium®, when given prophylactically. The primary outcomes of interest were blood calcium concentrations at 24 and 48 hours after treatment, the incidence of clinical disease and culling, milk production in early lactation and the probability of pregnancy at first insemination. Although widely used in Canada, there is no peer-reviewed literature on the efficacy of this
product in the treatment of clinical or subclinical hypocalcemia. Furthermore, there are no published large scale trials on the effects of any prophylactic subcutaneous calcium products. Recent publications have reported associations between lower blood calcium concentrations around calving and subsequent health and production, including increased risk of metritis (Martinez et al., 2012), increased odds of displaced abomasum (Chapinal et al., 2011), greater odds of culling (Roberts et al., 2012) and reduced milk yield in early lactation (Chapinal et al., 2012). This has prompted many to seek interventions that maintain normocalcemia and mitigate subclinical hypocalcemia rather than just clinical signs of hypocalcemia. As discussed in Chapter 2, there is limited evidence for the ability of any form of calcium supplementation to alter subsequent health and production. In a subsample of cows with blood samples taken at 24 and 48 h after treatment, the supplemental calcium modestly increased serum calcium concentrations in the first day after calving, with greater effect in cows with lower pre-treatment calcium. Our large scale study found the prophylactic use of two doses of subcutaneous calcium reduced the proportion of cows that received supplemental calcium for clinical signs of hypocalcemia but had no effect on the risk of disease or culling, milk production or reproductive performance. This study suggests the prophylactic treatment was effective at raising blood calcium which may be enough to mitigate or prevent visible signs of hypocalcemia in some cows, but on average was insufficient for most cows to reach 2.15 mmol/L which has been associated with effects on immune function and risk of disease in other studies (Chapinal et al., 2011, Martinez et al., 2012).

There are several limitations with this study. Information regarding the blood calcium dynamics of this product is limited and in the current study blood samples were not collected before 24 h. The best dosing regime is unknown and there may be different results if the product were given
differently than used in our current study. Blood calcium concentrations and subsequent disease and production might be altered if an optimal administration regime is identified. Second, the cows used in the trial were from farms purposively chosen to participate based on geographic location and willingness to comply with the study protocol. There may be bias from the high management level and willingness to participate in research such that these farms do not represent the larger population in which the product would be used. Furthermore, one farm contributed 55% of the cows enrolled in the trial. Among the five farms from which pre-treatment blood samples were available (again largely over-representing the same large dairy), there was a considerable number of cows (59%) with total calcium below the proposed target of 2.15 mmol/L which suggests that even in well-managed farms there are significant numbers of cows below the ideal cut point, including some first parity animals. There were no herds included in the study feeding a prepartum diet with anionic salts and it is not known what the effect of the product would be on blood calcium levels in a population with fewer hypocalcemic cows.

The second objective was to evaluate if administration of a calcium supplement at calving increased neutrophil oxidative burst or phagocytosis capacity. Although many recommendations for prophylactic calcium products center on multiparous cows at greater risk for clinical hypocalcemia, there is a growing acceptance that subclinical hypocalcemia and reduced immune function may be experienced by cows of all parities and prevention strategies might be worthwhile in younger cows as well. Hypocalcemia in dairy cows is linked to reduced immunological capacity during the peripartum period. An increase in intracellular calcium concentration is an early event in neutrophil activation (Burgos et al., 2011) and reduced calcium release from the extracellular space likely contributes to periparturient immune suppression.
Cows with blood calcium concentrations below 2.15 mmol/L at any point in the first three days in milk had impaired neutrophil oxidative burst and phagocytosis capacity compared to cows that maintained blood calcium above 2.15 mmol/L (Martinez et al., 2012).

Diminished neutrophil function has been associated with metabolic and reproductive disease in the transition period. There are no published studies on the effect of calcium supplementation on immune function parameters. In our pilot trial administering a subcutaneous calcium supplementation product to 27 cows, there was no improvement in neutrophil function as measured by oxidative burst capacity or phagocytosis of the neutrophils.

One major limitation of the present study is the lack of cows in third or greater lactation. There was a systematic bias in the sample of cows available to be enrolled as older cows on the farms had already received supplemental calcium, leaving only first and second parity cows to enroll. While others have demonstrated that calcium status and neutrophil function are linked, as the risk of hypocalcemia increases with age, an improvement in immune defence from calcium supplementation might be more evident in older cows. A second limitation is that the sample size was insufficient to detect associations between total calcium concentration and immune function (i.e. to replicate the findings of Martinez et al., 2012). It is difficult to understand if a risk factor could be modified without confirming its presence in the population.

Interventions instituted at the time of calving may be thought of as “Band-Aid” tools with limited ability to effect significant change in the metabolic or immune health of cows. With increasing recognition of subclinical disease entities including subclinical hypocalcemia and subclinical ketosis, mitigation of such health disorders likely requires attention in the prepartum period when immune function and dry matter intake (DMI) begin to be affected.
The final objective of this thesis was to test whether reducing social stress by providing non-competitive access to feeding and lying modifies metabolic health and immune function, and if differences in metabolic health and immune function could be explained using a measure of social status in the group. Standards of prepartum housing vary widely among farms and recommendations for peripartum cows made by dairy advisors are largely based on data from lactating cows, ease of management, or inherent beliefs. There are limited data to support the commonly proposed best practices but there is consensus that an environment that limits DMI and lying time may have enduring effects on transition cow health. Conditions that increase stress such as reduced access to feeding and lying are believed to exacerbate negative energy balance and decrease immune function. General instructions “to minimize stressor-induced limitations in DMI” (Drackley et al., 2005) although accurate and well intentioned, are difficult to translate into evidence-based recommendations. Furthermore, the interactions between many stressors or variables in the pen environment limit advisors’ ability to understand what changes may have the greatest impacts or why one strategy is successful on one dairy without being successful on another.

In our own study, crowding in a small, stable group through the close-up dry period without crowding or social instability in the postpartum period did not negatively affect the metabolic health and immune function of cows. We did measure an improvement in the innate immune parameter oxidative burst function among cows with higher social status compared to low social status cows when understocked, suggesting this housing strategy might be more advantageous for some cows. Although we hypothesized that the consequences of stocking density would differ for dominant versus submissive cows, the understocked treatment did not benefit submissive cows as hoped. The minimal differences between the understocked and overstocked
groups do not necessarily suggest that there would be no effects on health in an average commercial dairy stocking cows at these levels. Rather, in an environment where many other factors were well managed including the quality of the prepartum diet, feed availability, social stability, and zero competition before or after the close-up dry period, cows were able to compensate for the increased competition of crowding up to 120%.

**Future Directions**

After decades of hypocalcemia research, milk fever incidence still ranges from 0 to 13.5% in Ontario (McLaren et al., 2006) and in the first week after calving, a third of cows in a large North American study tested below cut-points associated with increased risk of disease and production losses (Chapinal et al., 2011, Chapinal et al., 2012). For these reasons, it is likely that calcium supplementation products will continue to be used by producers despite evidence that blood calcium levels are only temporarily affected by this subcutaneous product and oral products (Goff, 1999, Blanc et al., 2014) and there is limited ability to effect improvements in cow health. The ability of these products to alter immune parameters such as neutrophil function is still not clear. There would be value in measuring the intracellular calcium concentration of neutrophils from a sample of treated versus untreated cows to better understand if the product tested or others are able to make a difference in neutrophil intracellular calcium concentrations. Furthermore, targeting this among cows at greater risk of hypocalcemia (older cows) and cows at greater risk of reduced immune function (e.g. higher producing cows and cows with risk factors for postpartum uterine disease) may yield a better understanding of the mechanisms at play. Interventions with the ability to alter the metabolic state of the cow and produce a more sustained and earlier increase in blood calcium, such as feeding a prepartum diet with anionic salts to achieve a negative dietary cation-anion difference would be worth investigating in relation to
neutrophil function. The current body of calcium supplementation research, including our large scale trial using the product Theracalcium® emphasize that veterinarians and advisors need to avoid thinking of this as a time of calving problem but rather address it preventatively throughout the peripartum period.

Similarly, peripartum housing recommendations will likely not change from targeting no more than 1:1 cows to stalls and 30 inches of bunk space per cow. A constraint of this study for its results to be applied to commercial dairies is the fact that crowding was temporary. Although there may be situations on farm where crowding is brief, many dairies that are overcrowded are likely crowded both pre- and postpartum. Furthermore, although the prepartum group might be socially stable, fresh cow groups often have daily additions as new cows calve, resulting in a fluctuating social structure when cows are most vulnerable. Taking these other factors into account would be necessary in order to choose the target stocking density in the prepartum pen. An interesting next step to better understand the duration of exposure to the stress of crowding would be to repeat the current housing study but with 50% of the groups entering a similarly crowded group fresh pen.

The social hierarchy of dairy cows means there will always be cows at the bottom. Many strategies designed to lower social stress are based on the theory that stressors might be worse for some cows, and lower rank cows would benefit the most from lower competition or stable groups. The finding that improved immune function was achieved by higher rank cows when understocked, but low rank understocked cows did not differ from overstocked cows suggests that low rank cows couldn’t attain the same advantage. Research including our own that energy metabolism is altered among low rank (subordinate) cows indicates that future studies should center on improving the experience for these cows, but exactly how to achieve this is not clear.


