Associations Among Neutrophil Function, Metabolic Indicators, and Reproductive Health in Dairy Cows

by

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This thesis is an investigation of the interactions of insulin resistance (IR), metabolic markers, neutrophil function, and reproductive health in peripartum dairy cows, including the evaluation of a hand-held glucometer for diagnosis of IR. The neutrophil functions of interest were oxidative burst and phagocytosis capacity, and reproductive diseases were endometritis and cervicitis. A total of 81 Holstein cows were enrolled 3 wk prior to expected calving date from November 2010 until October 2011, and were followed until 5 wk postpartum. Known markers of IR, neutrophil function, and disease were monitored through this period. The hand-held glucometer was identified as a useful alternative to laboratory measurements of glucose. Markers of IR influenced phagocytosis capacity and reproductive disease. High haptoglobin concentrations were associated with increased risk of reproductive disease and diminished oxidative burst function. Metabolically related inhibition of neutrophil function may be important in development of reproductive disease.
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CHAPTER ONE

LITERATURE REVIEW

In the peripartum period (PP), typically considered as three weeks before and after calving, dairy cows experience the transition from pregnancy to metabolically demanding production of large quantities of milk. This transition period is challenging because cows simultaneously experience mammary gland development, parturition, rumen adaptation to diet changes, negative energy and protein balance, as well as changes in hormone profiles and adaptations in metabolism of glucose and fatty acids (Goff and Horst, 1997; Overton and Waldron, 2004). Cows in early lactation undergo a period of insulin resistance (IR; Hayirli, 2006), reduced dry matter intake (DMI), negative energy balance (NEB), lipolysis, and weight loss (Goff and Horst, 1997). With these concurrent changes, it is not surprising that during this time dairy cows also experience immune suppression and increased susceptibility to infectious and metabolic diseases.

Reduced immune function is experienced by all cows, to varying degrees, during the PP and generally extends one week before and after parturition (Kehrli et al., 1989; Cai et al., 1994; Detilleux et al., 1994). Peripartum immune function is multifactorial, difficult to evaluate, and not well understood (Overton and Waldron, 2004), however there is evidence to suggest that its decreased function is a natural occurrence (Sordillo and Aitken, 2009; Sordillo et al., 2009). Cows with reduced immunity are more vulnerable to transition diseases, such as mastitis and metritis, which have a substantial economic impact on the dairy industry due to both reduced production and reproductive efficiency (Bonizzi et al., 2003). Compromised function of adaptive or innate immunity contributes to the risk of several transition diseases (Detilleux et al., 1995; Mallard et al., 1998). Due to the transient nature of the PP and the infections commonly
experienced at this time, the prompt response of innate immunity, particularly by neutrophils, is critical in reducing the incidence and severity of retained placenta, metritis, mastitis, and endometritis (Cai et al., 1994).

**Overview of Peripartum Immune Function**

*Adaptive Immunity.* Adaptive immunity is a specific and lasting response to foreign antigens encountered by the cow, and is compromised during the transition period (Vankampen and Mallard, 1997; Mallard et al., 1998). Both B and T cell branches of adaptive immunity are influenced by parturition (Vankampen et al., 1999). B cell populations remain stable prepartum, and decrease briefly after calving (Vankampen and Mallard, 1997). Serum antibody levels remain normal through the transition period, with the exception of shortly prior to parturition, when levels of immunoglobulin G (IgG), the most abundant antibody, drop (Mallard et al., 1983; Detilleux et al., 1995). This decline in circulating IgG occurs concurrently with an increase in lacteal IgG, due to immunoglobulin enrichment of the colostrum (Mallard et al., 1983; Vankampen and Mallard, 1997). Low IgG in the blood results in less adaptive immune protection, potentially increasing susceptibility to disease (Detilleux et al., 1994; 1995).

During pregnancy, cell mediated immunity is down-regulated to prevent the rejection of the growing fetus (Weinberg, 1987). Decreased cell mediated immunity results from the decline in the circulating population of lymphocytes, particularly T cells (Mallard et al, 1998). T cell concentrations are lower in transition cows than in non-pregnant, non-lactating cows (Vankampen and Mallard, 1997). The reduced concentration of circulating T cells during the PP is due to changes in circulating hormones, such as progesterones, estrogens, and corticosteroids, and other factors associated with peripartum stress and lactational demands (Goff and Horst, 1997; Vankampen and Mallard, 1997).
Hypocalcemia is a condition commonly experienced by dairy cows in the PP due to the high calcium demands of lactogenesis (Reinhardt et al., 1988; Goff and Horst, 1997) that impacts immune function. Calcium is important in immune cell activation, acting as a second messenger of signal transduction (Grafton and Thwaite, 2001; Lewis, 2001). Cells that have a greater initial peak, or flux, in calcium concentrations following an activation signal are more responsive and functional (Partiseti et al., 1994; Baus et al., 1996). Kimura et al. (2006) demonstrated that peripheral blood mononuclear cells in periparturient cows have decreased calcium flux in response to activation, in part due to depleted intracellular calcium stores, and that these effects were worse in cows with more severe hypocalcemia. Calcium fluxes are also used in neutrophils for intracellular signaling of activation (Krause et al., 1990). Insufficient calcium is an important mechanism for suppression of lymphocytes, and may also play a role in reduced function of innate immune cells, including neutrophils.

The adaptive immune system is extraordinarily efficient at identifying and eliminating specific pathogens, however there is a considerable delay in action. It can take up to two weeks for a full adaptive immune response to be mounted, including activation of adequate numbers of naïve T and B cells or achievement of protective immunoglobulin titres (Mallard et al., 1983; Mallard et al., 1998). Although adaptive immunity is important in transition diseases such as mastitis (Mallard et al., 1998), due to the relative brevity of the PP, the severity of infections commonly experienced during this time, and the implications transition diseases have on reproduction and production, a timely immune response is critical.

**Innate Immunity.** Innate immunity is the first line of defense against injury or infection. Although the innate immune system is not able to target specific pathogens, it responds quickly to identify and eliminate infected, damaged, or non-self cells (Kehrli et al., 1989; Cai et al.,
1994), a feature that is critical during the transition period of a dairy cow. Innate immunity is comprised of numerous components, including physical barriers, cytokines, and immune cells, which work together to eliminate pathogens (Kehrli et al., 1989; Bonizzi et al., 2003). The expeditious recruitment of functional immune components to the site of infection is critical for bacterial clearance (Galvão et al., 2010). Due to ubiquitous bacterial contamination around calving, the uterus relies initially on the defenses contributed by innate, rather than adaptive immunity (King et al., 2003; Sheldon and Dobson, 2004; Sheldon et al., 2006). All innate immunity components are important and are subjected to varying degrees of impaired function in the PP caused by physical damage and altered regulation related to parturition (Bonizzi et al., 2003). Perhaps the most important constituent, however, are the neutrophils, as they are typically the first immune cells to reach the site of infection, where they kill pathogens via phagocytosis and lysis (Kehrli et al., 1989; Cai et al., 1994; Detilleux et al., Meglia et al., 2005). Neutrophils employ migration directed by chemotaxis, phagocytosis, and lysis using reactive oxygen species (ROS) through the oxidative burst process to eliminate bacterial loads and cellular debris (Klucinski et al., 1988; Cai et al., 1994). Normal functioning of neutrophils is imperative for combating disease during the PP due to the increased exposure to pathogens, particularly in the reproductive tract and mammary glands (Kehrli et al., 1989; Kim et al., 2005; Gilbert et al., 2007).

**Postpartum Uterine Status**

Although the lumen of the uterus is sterile during pregnancy (Földi et al., 2006), at parturition bacteria from the environment or vagina can enter the uterus through the open cervix (Elliott et al., 1968; Földi et al., 2006; Griffin et al., 1974; Noakes et al., 1991). Post partum bacterial contamination of the uterus is routine, with over 90% of cows affected (Elliott et al.,
1968; Sheldon et al., 2002; Sheldon and Dobson, 2004). Despite the high proportion of postpartum cows experiencing this bacterial contamination, not all develop clinical uterine disease. The reported prevalence of puerperal metritis ranges from less than 10% (Leblanc et al., 2002) to 37% (Bartlett et al., 1986; Markusfeld, 1987; Peeler et al., 1994), and that of endometritis 30 days after parturition from 12% (Dubuc et al., 2010a) to 53% (Gilbert et al., 2005). Cervicitis has recently been recognized as a condition discreet from, but related to endometritis, with 42% of animals reported to experience cervicitis (Deguillaume et al., 2012). The variation in the prevalence of these diseases is likely due in part to variability in disease definition and diagnostic techniques (Sheldon et al., 2006). The mechanisms by which some cows develop uterine disease, while others remain healthy when bacterial contamination of the uterus postpartum is ubiquitous are not entirely understood, but are likely multifactorial.

Difference in expression of genes for cytokines associated with inflammatory response has been found in cows that have endometritis (Herath et al., 2009; Sheldon et al., 2009). Inflammation is a process critical in defense against pathogens as it is involved in the activation of both the innate and adaptive immune system, however the inability to adequately regulate the different factors of inflammation may predispose these animals to disease. Excessive production of pro-inflammatory mediators is likely a key factor in cows that develop endometritis (Sheldon et al., 2009). In the week after calving, cows have increased gene expression of pro-inflammatory mediators such as interleukins (IL-1α, IL-1β, and IL-6; Herath et al., 2009). Cows that develop endometritis have higher ratios of mRNA for IL-1α or IL-1β to the anti-inflammatory cytokine IL-10 one week postpartum (Herath et al., 2009). A recent study elucidated that monocytes in cows with metritis had increased un-stimulated gene expression of TNFα, IL-1β, and IL-6, and decreased gene expression of TNFα after bacterial stimulation
(Galvão et al., 2012). This diminished expression of pro-inflammatory cytokines by monocytes likely contributes to reduced chemotaxis and activation of neutrophils, possibly predisposing cows to disease. More research is required to investigate the links between pro-inflammatory status and uterine disease, particularly the involvement of neutrophils.

Neutrophils are the primary innate immune cell associated with clearing bacterial contamination from the uterus (Hussain, 1989; Gilbert et al., 2007). Cows that have a greater influx of neutrophils to the uterus on the day of calving have lower rates of positive bacterial culture, as compared to cows with less migration of neutrophils to the endometrium (Gilbert et al., 2007). High producing dairy cows (Kehrli et al., 1989; Goff and Horst, 1997; Mallard et al., 1998) and those that develop retained placenta (Kimura et al., 2002) or uterine disease (Cai et al., 1994; Kim et al., 2005; Hammon et al., 2006; Galvão et al., 2010) experience compromised neutrophil function during the PP. Reduced neutrophil function is associated with endocrine and metabolic changes around calving (Meglia et al., 2005), which will be discussed in detail below.

**Peripartum Metabolic Changes**

During the PP, cows go through a time of NEB where the energy demands of maintenance, growth, and lactation exceed energy consumed, typically from just after calving until approximately 6 weeks postpartum (Butler et al., 1981; Doepel et al., 2002), although animals may go into NEB prior to parturition (Grummer et al., 2004). The NEB during the PP can be partially attributed to the decrease in DMI, which results in a substantial decrease in circulating glucose concentrations, contributing to increased body fat mobilization in the form of non-esterified fatty acids (NEFA), and the accumulation of byproducts of incomplete NEFA oxidation, i.e. ketones such as β-hydroxybutyric acid (BHBA; Vazquez-Añon et al., 1994).
Cows that experience metabolic disturbances or health problems in early lactation may produce less milk than healthy cows, have reduced reproductive performance, or be more likely to be culled, resulting in economic losses for dairy farmers (Drackley, 1999). This pattern is particularly true of puerperal metritis (Overton and Fetrow, 2008; Wittrock et al., 2010). Endometritis results in substantial reduction of reproductive performance (Kim and Kang, 2003; Dubuc et al., 2011). Cows that develop uterine disease after parturition on average experience more profound NEB that precedes disease than those that remain healthy, as measured by decreased DMI and increased NEFA and BHBA (Hammon et al., 2006; Galvão et al., 2010).

**Insulin Resistance**

Insulin resistance (IR) is a state in which a physiological level of insulin produces a less than normal biological response (Kahn et al., 1978). Insulin resistance can result from decreased production or secretion of insulin in response to glucose, a diminished ability of normally insulin-sensitive tissues to respond to insulin, or both (Kahn et al., 1978; Sano et al., 1991). Inflammation can exacerbate IR, as many mediators of inflammation block intracellular signaling of insulin receptor substrate proteins (Hotamisligil, 2006), a process made worse by lipolysis. A number of physiological circumstances can result in IR, including gestation, lactation, and malnutrition (Hayirli, 2006), which can be experienced in the PP with malnutrition occurring in the form of NEB.

Insulin is a hormone that is involved in glucose homeostasis. The primary role of insulin is to increase glucose absorption of cells by up-regulating, enhancing, and permitting the function of insulin-dependent glucose transporters (Brockman and Laarveld, 1986). These insulin-dependent glucose transporters are located in insulin-sensitive tissues, such as muscles and adipose, which have a lowered responsiveness to insulin in late gestation and early lactation.
(Kronfeld, 1982; Sano et al., 1991). Energy source requirements of insulin-sensitive tissues are more flexible and can shift from glucose to other fuels, including metabolites like NEFA and BHBA (Heitmann et al., 1987). The energy source used in these tissues is dependent on the relationship between circulating glucose and insulin concentrations.

The metabolic transition of the PP results in alterations in circulating concentrations of glucose and glucose homeostasis-related hormones as energy demands change. Circulating glucose concentrations drop from approximately 3.25 mmol/L in the dry period to 2.60 mmol/L 4 days after calving, and despite a steady increase, levels do not recover to precalving levels until after 40 days postpartum (Bossaert et al., 2008). This decrease in glucose concentrations is in part due to inadequate DMI, but a more important contributor is increased clearance of glucose from the blood due to nutrient partitioning that directs glucose towards insulin-insensitive tissues, primarily the mammary gland (Bell and Bauman, 1997). Insulin, and its close relative insulin-like growth factor I (IGF-I), concentrations also drop around calving (Doepel et al. 2002; Holtenius et al., 2003), with IGF-1 still not recovered to prepartum levels 4 weeks after calving (Dopel et al., 2002) and insulin restored 10 to 15 weeks into lactation (Holtenius and Holtenius, 2007). Nadir levels of both insulin and glucose are reached between 8 and 12 days postpartum (Bossaert et al., 2008). Whereas low glucose levels result from partitioning to the mammary gland, low insulin concentrations appear to be due to reduced secretion (Bossaert et al., 2008). Low peripheral concentrations of insulin induce a glucose sparing status, enhancing glucose availability for the mammary gland (van Knegsel et al., 2007; Bossaert et al., 2008). Low insulin concentration contributes to lipolysis (Spicer and Echternkamp, 1995) with excessive lipid mobilization exacerbating IR (Kerestes et al., 2009). Low peripheral insulin and glucose
concentrations create an IR environment, which is characterized in part by elevated NEFA and BHBA.

Insulin resistance is commonly observed beginning in the final weeks of gestation and continuing into early lactation (Baird, 1981; Hayirli, 2006). The duration of IR in early lactation has not been well studied, but considering that NEB can extend as far as 6 weeks postpartum (Butler et al., 1981; Doepel et al., 2002, Grummer et al., 2004), it is probable that IR can be experienced for the same approximate length of time. During late gestation, glucose utilization by peripheral tissues is diminished as numbers of glucose transporters in insulin-sensitive tissues, such as the liver and muscles, are reduced (Thorens, 1996). This suppression of glucose use by insulin-sensitive tissues allows transfer of glucose from dam to fetus, which is an insulin-independent process, and the glucose demands of the fetus increase in late gestation (Prior and Christenson, 1978; Faulkner and Pollock, 1990). Although fetal glucose requirements are an important cause of prepartum IR, more sustained and deeper IR occurs postpartum due to a greater discrepancy in energy demands and availability.

Homeorhesis in early lactation results in a coordination of the metabolism to support the physiological shift in the cow (Bauman and Currie, 1980). The glucose demands of the mammary gland are insulin-independent (Laarveld et al., 1981; Reynolds et al., 2003) and high relative to other species (Rose et al., 1997). By four days postpartum, the glucose demand of the mammary gland triples (Bell, 1995), with upwards of 80% of glucose cleared from circulation by the mammary gland (Rose et al., 1997). Due to the energy demands of lactogenesis, circulating glucose levels are low because of nutrient partitioning, resulting in a drop in insulin concentrations and reduced insulin sensitivity of peripheral tissues (Bell and Bauman, 1997). Under homeostatic conditions, glucose and insulin regulate adipose tissue metabolism of lipids,
however during early lactation, lipolysis in the adipose tissue is unaffected by glucose (Metz and van den Bergh, 1977) or insulin (Knopp et al., 1973). It is speculated that this mechanism of peripheral tissue IR involves alterations of key enzymes and adipocyte receptors involved in homeostatic signaling, such as the number of insulin receptors (Bauman and Currie, 1980).

Increased circulating concentration of growth hormone is in part responsible for the resistance of peripheral tissues to insulin (Bell and Bauman, 1997). After calving, cows also enter a state of NEB in which they are unable to consume enough feed to support the demands of lactation and maintenance (Bauman and Currie, 1980; Bauman, 2000). The metabolic profile during early lactation is characterized by low serum concentrations of insulin, plasma glucose, and high concentrations of glucagon, NEFA, and BHBA (Herbein et al., 1985; Vazquez-Añon et al., 1994). This postpartum metabolic phenomenon, in combination with the decreased insulin sensitivity during late pregnancy, results in exacerbated IR for the cow.

Peripartum IR and NEB are so closely related that it is difficult to completely disentangle the concepts. High circulating levels of NEFA due to mobilization of fat after parturition are a hallmark of NEB, with peak levels being reached between days 3 and 12 postpartum (Bossaert et al., 2008). Pires et al. (2007) demonstrated that experimental hyperlipidemia, achieved through intravenous infusion of tallow triacylglycerol, results in increased IR, likely due to persistently high NEFA. Subsequently, it was hypothesized that elevated NEFA during the PP may be a key factor in triggering IR (Pires et al., 2007). While sustained high levels of NEFA may result in prolonging IR during the PP, it is clear that IR begins in late gestation. Therefore, it is unlikely that NEFA is the sole trigger of IR, but rather that NEFA are a consequence and promoter of sustained IR coupled with NEB experienced by dairy cows postpartum. Although the exact relationship of high NEFA and IR in the PP is not clear, it is known that cows with greater
circulating NEFA are at increased risk of retained placenta, metritis (Kaneen et al., 1997; Ospina et al., 2010; Chapinal et al., 2011), and subclinical endometritis (Galvão et al., 2010). The circulating hormones and metabolites that reflect IR and NEB, in combination with hormone levels associated with the PP, are also inhibitory to normal neutrophil function.

_Evaluating Insulin Resistance_. There are a number of different methods by which to measure IR. The traditional way to measure IR is through a glucose tolerance test (GTT). This procedure consists of a bolus infusion of a 50% glucose solution intravenously after fasting, and blood samples taken at regular intervals to monitor the change in circulating concentrations of glucose and insulin (Lozner et al., 1941). Insulin resistance is then evaluated based on both the ability to clear glucose from circulation and the amount of insulin secreted (peak concentration or area under the curve) in response to high levels of glucose (Holtenius et al., 2003). Other parameters for glucose tolerance evaluation using the GTT include insulin and glucose basal and peak concentrations, plasma disappearance rate, area under the curve, and ratio of plasma glucose to insulin (Opsomer et al., 1999; Hayirli et al., 2001; Holtenius et al., 2003). The inability to recover glucose concentrations to a normal level within a given period of time, or an insufficient release of insulin, both reflect IR. While this procedure provides detailed insight into how an individual responds to a glucose challenge, it is a labour intensive process and is not suitable for large scale research studies or clinical use. Therefore an alternative way to measure IR, and an ability to do so cow-side, would be a beneficial research tool. Simplified and modified versions of the traditional GTT have been used in dairy cows to produce data complementary to the traditional GTT (Staufenbiel et al., 1992). Matteo et al. (2009) recognized that dairy cows that could be defined as not IR using the traditional GTT method had recovered to normal circulating glucose concentrations by 80 minutes after infusion, and they therefore have
proposed using the ratio of blood glucose at 80 minutes after infusion to that before as an indicator of IR, suggesting a cut point of 1.05. While it does appear that insulin responsive animals do recover to normal glucose concentrations by 80 minutes after glucose infusion, it remains unclear whether 1.05 is a meaningful ratio in terms of predicting disease.

Another alternate method to the GTT for measuring responsiveness to insulin is the Revised Quantitative Insulin Sensitivity Check Index (RQUICKI). In humans this method utilizes plasma concentrations of glucose, insulin, and NEFA to achieve linear correlations of 0.86 with euglycemic hyperinsulinemic clamp, a more intensive measure of IR (Rabasa-Lhoret et al., 2003). The use of RQUICKI in dairy cattle is novel, with one study reporting an agreement between RQUICKI and body condition score (BCS), a more traditional form of IR assessment (Holtenius and Holtenius, 2007). The authors of this study, however, reported only a significant negative linear relationship between BCS and RQUICKI, but did not provide a correlation value. A second study found no relationship between RQUICKI, calculated at each time point from 18 days before until 70 days after calving, and GTT, performed between 18 and 22 days before, and on days 7 and 60-70 after calving (Kerestes et al., 2009). The same study reported that while RQUICKI reflected some indicators of IR, it was also sensitive to different diseases and physiological states that are characterized by severe inflammation and should be implemented with caution (Kerestes et al., 2009). Further investigation is required to determine when and whether this tool can be used successfully in dairy cattle research.

**Peripartum Neutrophil Function**

High producing dairy cows (Kehrli et al., 1989; Gilbert et al, 1993; Goff and Horst, 1997; Mallard et al., 1998) and especially those that develop uterine disease (Cai et al., 1994; Kim et
al., 2005; Hammon et al., 2006) experience a reduction in neutrophil function around calving, including diminished migration, phagocytosis, and oxidative burst capacity.

Circulating counts of neutrophils increase at calving, subsequently dropping off 1 week postpartum, and then return to normal over the following 3 weeks (Cai et al., 1994; Hussain and Daniel, 1992; Mateus et al., 2002; Kim et al., 2005). Circulating numbers of neutrophils, as well as their chemotactic proficiency are reduced during the week of calving as compared with 4 weeks before or after parturition (Nagahata et al., 1988). At calving, there is a brief increase in circulating neutrophils as their ability to migrate is inhibited by the rise in cortisol (Preisler et al., 2000). Phagocytic ability of neutrophils has also been observed to increase around calving, and decreases shortly after (Nagahata et al., 1988; Kehrli et al., 1989; Kim et al., 2005). Postpartum, the count drops back down as neutrophils undergo migration towards the mammary gland and the uterus (Guidry et al., 1976). Cows with endometritis had a greater percentage of neutrophils present in the lumen of the uterus at the time of diagnosis than at spontaneous recovery, and the number of neutrophils present was positively correlated with the level of bacterial contamination of the uterus (Mateus et al., 2002). The influx of the neutrophils to the uterus occurred simultaneously with a decrease in circulating neutrophils (Hussain and Daniel, 1992; Mateus et al., 2002), suggesting successful migration to the point of infection. In dairy cows, neutrophils isolated from the uterus have reduced chemotactic, phagocytic, and killing ability as compared to neutrophils in peripheral blood (Subandrio et al., 2000). Although migratory function may be diminished in the PP, neutrophils are still able to reach the uterus when bacterial contamination is at its highest, suggesting that uterine disease is more likely a result of compromised phagocytosis and oxidative burst functions, particularly at the site of infection, than impaired migration.
**Neutrophils and Peripartum Endocrine Changes.** Neutrophil competence is influenced by changes in progesterone, estrogen, and cortisol during the PP. Progesterone is the dominant hormone during pregnancy, and generally is thought to reduce immune function, due to its role in supporting and preventing the rejection of the fetus (Weinberg, 1987; Bonizzi et al., 2003). These immunosuppressive actions are mostly indirect through the down regulation of T cell function, including impairment of proliferation and secretion of cytokines, and modulating the inflammatory response, to prevent fetal rejection (Weinberg, 1987). Specifically, exposure of T cells to concentrations of progesterone similar to those found in a term human placenta reduced the production and effects of IL-1, a mediator of T cell proliferation in response to antigens (Stites et al., 1983). Exposure to physiological levels of progesterone resulted in impaired neutrophil oxidative burst capacity (Roth et al., 1983; Chaveiro and Moreira da Silva, 2010) but not random migration (Roth et al., 1983). Phagocytic ability of neutrophils has been reported to decrease in ovarectomized mares treated with progesterone (Watson et al., 1987), although the mechanism was unclear. This effect on oxidative burst and phagocytosis has negative repercussions for neutrophil capability to respond to infection prior to parturition. Despite the evidence implicating progesterone in the impairment of functions important for normal neutrophil bactericidal activity, other factors likely also contribute. Progesterone concentrations fall immediately prior to calving, and remain very low for several weeks after calving (Detilleux et al., 1995; Goff and Horst, 1997; Vankampen and Mallard, 1997); consequently other factors must be contributing to altered transition period immune competence.

Estrogen has been linked with the functionality of the immune system. Circulating concentrations of estradiol increase substantially towards the end of gestation, peaking just prior to parturition, thereafter dropping off quickly (Radcliff et al., 2003). Estrogen, in the past, was
considered to enhance immunity, especially with regards to uterine infection (Blalock, 1994). Recent research, however, has found that cows that develop uterine disease, such as metritis, had higher circulating levels of estradiol on the day of calving than those that remain healthy (Galvão et al., 2010), suggesting a link between high estradiol concentration and diminished immune function. Physiological levels of estradiol-17β have been implicated in the reduction of chemotaxis of human neutrophils through a receptor-dependent mechanism (Ito et al., 1995). Estradiol-17β also decreases oxidative burst activity in bovine neutrophils, although this result has only been demonstrated at supra-physiologic concentrations (Chaveiro and Moreira da Silva, 2010). At higher concentrations, such as those associated with very late pregnancy, estradiol also alters opsonization and presentation of antigens (Wyle and Kent, 1977), which could negatively impact neutrophil phagocytosis. Both estrogen receptor protein and mRNA have been found in neutrophils, suggesting that estradiol-17β may exert an immunomodulating influence after binding to its receptor (Lamote et al., 2006). More research is required to elucidate the mechanisms by which estradiol impacts immune function in vivo during the PP. Circulating levels of estradiol at calving appear to be an important factor in the development of uterine disease through decreased neutrophil chemotaxis, identification of pathogens, and possibly oxidative burst capacity.

Cortisol is a powerful immunosuppressant as well as a metabolic modulator. Levels of cortisol increase at parturition and remain high for 1 day before decreasing (Goff and Horst, 1997). Cortisol impacts immune function by suppressing antibody and cytokine production (Khansari et al., 1990) of the inflammatory response, important components of innate immunity (Goff and Horst, 1997; Mallard et al., 1998). In addition to interfering with inflammation, cortisol also directly depresses neutrophil function. Exposure of neutrophils to high circulating
levels of cortisol, as observed at parturition, contributes to decreased migration and phagocytic ability (Wyle and Kent, 1977; Nagahata et al., 1988). Cows that developed metritis had greater concentrations of cortisol and reduced neutrophil glycogen stores at calving than cows without metritis (Galvão et al., 2010). The greater concentrations of cortisol in primiparous cows could contribute to the higher incidence of puerperal metritis experienced by these animals (Goshen and Shpiegel, 2006). Neutrophil expression of L-selectin, a surface adhesion molecule involved in leukocyte movement from blood vessels into tissues, is decreased by dexamethasone, a potent synthetic glucocorticoid, which negatively impacts migration (Priesler, 2000). Cortisol influences neutrophil function through suppression of inflammation, regulation of glucose, and reduction of migration capabilities.

**Neutrophils and Peripartum Metabolic Changes.** The shifts in energy metabolism related to late gestation and early lactation contribute to diminished immune function during the PP. Through a process called homeorhesis, normal homeostatic mechanisms of dairy cows are temporarily superseded to allow the partitioning of nutrients to support milk production (Bauman, 2000). The increased energy requirements of the fetus and growing mammary gland in late gestation, while not considered trivial, are not large enough to compromise energy balance (Grummer, 2008). However, the 30-35% reduction in DMI commonly observed prior to calving (Hayirli et al., 2002), in combination with the energy requirements of prepartum mammary and fetal growth, may deplete prepartum energy. The energetic demands of late gestation and early lactogenesis result in IR (Holtenius et al., 2003; Hayirli, 2006) and NEB (Detilleux et al., 1995; Meglia et al., 2005). Insulin resistance and NEB impair peripheral blood neutrophil function (Hoeben et al., 1997; Zerbe et al., 2000). Multiparous cows that develop uterine disease had
greater drops in DMI, increased NEFA and BHBA, and decreased blood neutrophil killing ability (Hammon et al., 2006) and phagocytosis (Kim et al., 2005).

Neutrophil function is negatively impacted by IR, in part through hypoinsulinemia and hypoglycemia. Insulin, in combination with IGF-I, has enhancing effects on neutrophil function. Bovine leukocytes possess receptors for both insulin and IGF-I (Nielsen et al., 2003). Exposure to insulin and IGF-I prior to activation appears to enhance neutrophil competency (Nielsen et al., 2003). Balteskard et al. (1998) demonstrated enhanced neutrophil phagocytosis and oxidative burst in piglets primed with IGF-I. These results are consistent with observations made in bovine neutrophils (Nielsen et al., 2003). In humans, neutrophils that were primed with insulin had improved oxidative burst functions (Spagnoli et al., 1995). More research is required to clearly elucidate the influence of insulin, IGF-I, and IR on bovine neutrophils. It would be expected, however, that during the PP, bovine neutrophils do not have the benefit of exposure to insulin and IGF-I due to low peripheral concentrations, contributing to suboptimal function. In addition to low insulin and IGF-I levels during peripartum IR, there are reduced circulating concentrations of glucose.

Peripheral concentrations of glucose are low during the PP as glucose is partitioned primarily for lactogenesis (Holtenius et al., 2003; Hayirli, 2006), which contributes to compromised neutrophil function. Hypoglycemia precedes depletion of neutrophil glycogen stores, which are severely depleted in all cows during the early PP (Galvão et al., 2010). Neutrophils rely primarily on glucose uptake or glycolysis for chemotaxis, however glycogen stores are necessary for phagocytosis and oxidative burst, even in the presence of exogenous glucose (Kuehl and Eagan, 1980; Borregaard and Herlin, 1982; Weisdorf et al., 1982). The low glucose levels experienced in the PP (Vazquez-Añon et al., 1994) could directly compromise
chemotactic ability, and prematurely deplete glycogen stores, thereby impairing phagocytosis and oxidative burst functions, resulting in elevated susceptibility to disease. Galvão et al. (2010) found that multiparous cows with uterine disease had reduced circulating glucose concentrations and experienced a greater depletion of glycogen concentrations in their circulating neutrophils than those that remained healthy from calving to 42 days postpartum.

Circulating concentrations of glucagon, another hormone involved in the regulation of glucose homeostasis, increase when peripheral levels of glucose are low (Deitch and Bridges, 1987). Glucagon is responsible for increasing circulating glucose through the breakdown of glycogen and can negatively impact neutrophil chemotaxis and microbial killing activity (Deitch and Bridges, 1987), although the mechanism is unclear. In the first 3 weeks postpartum, cows have decreased circulating concentrations of glucose as well as depleted neutrophil glycogen, which contribute to the reduction in immune function at that time.

Acute hyperglycemia is observed in the hours around calving. This transient exposure to high glucose concentrations impairs neutrophil function and increases the risk of infection (Blondet and Beilman, 2007), even though glucose is an important energy source for these cells (Weisdorf et al., 1982; Ohtsuka et al., 2006). Acute hyperglycemia can inhibit both production of reactive oxygen species and phagocytosis (Neilson and Hindson, 1989; Weekers et al., 2003). Type 2 diabetes in humans is a metabolic syndrome similar to the IR experienced by PP cows, except that type 2 diabetes patients have chronic hyperglycemia. It has been found in these human patients, that glucose interferes with the proteins responsible for opsonization, a process critical to the success of phagocytosis (Davidson et al., 1984). Although most of the IR phase in PP dairy cows is associated with postpartum hypoglycemia, the acute hyperglycemia experience near calving could contribute to the diminished phagocytic ability of neutrophils postpartum.
Impaired peripartum DMI contributes to increased NEFA and BHBA generation, which in turn compromises neutrophil function. Dry matter intake decreases around parturition contributing to NEB (Grummer et al., 2004) and associations have been found between low DMI prepartum and the development of metritis postpartum (Huzzey et al., 2007). Low DMI has been implicated in the reduction of neutrophil killing ability, not only after parturition but up to 3 weeks prior, in cows that developed metritis and endometritis (Hammon et al., 2006). Decreased DMI has also been associated with compromised neutrophil chemotaxis (Cai et al., 1994). Low DMI incurs these deleterious effects on neutrophils through lipolysis to meet energy demands, resulting in increased NEFA and BHBA levels, which interfere with normal neutrophil activity (Rukkwamsuk et al., 1999).

Elevated NEFA in circulation during the PP in response to NEB can lead to the impairment of neutrophils. When concentrations of NEFA are high, estrogen enhances deposition of triglycerides in the liver (Goff and Horst, 1997). This accumulation of triglycerides results in liver damage, compromising glucose production and exacerbating IR (Goff and Horst, 1997; Fenwick et al., 2008), and has been associated with decreased phagocytosis and oxidative burst function of neutrophils in circulation (Zerbe et al., 2000). These alterations in neutrophil function may be due, in part, to the increased concentrations of circulating NEFA. Oxidative burst activity has been negatively associated with high concentrations of NEFA in vivo, although the correlation was only moderate, with an R-value of 0.44 (Hammon et al., 2006). An in vivo study investigating the direct effects of varying concentrations of NEFA on neutrophil oxidative burst and phagocytosis function reported no effect on phagocytosis and a decrease in oxidative burst function at concentrations up to 0.5 mM (Scalia et al., 2006). Neutrophil oxidative burst function is less sensitive to increasing concentrations of NEFA than peripheral blood.
mononuclear cells, but may be negatively impacted by the high levels associated with the PP (Ster et al., 2012). Unactivated neutrophils are able to metabolize NEFA through oxidization or incorporate and store NEFA as triacylglycerol (Burns et al., 1976; Phillips et al., 1986). These attributes may make neutrophils more resilient to high levels of NEFA. Further research is required to determine how elevated NEFA, in combination with other markers of NEB, influence neutrophil function in vivo.

Increased levels of BHBA in response to peripartum NEB (Rukkwamsuk et al., 1999; Goff and Horst, 1997) predisposed cows to severe mastitis (Kremer et al., 1993) and endometritis (Dubuc et al., 2010a), and were associated with impaired neutrophil function (Klucinski et al., 1988; Suriyasathaporn et al., 2000). Diminished neutrophil chemotaxis (Suriyasathaporn et al., 1999), phagocytosis (Klucinski et al., 1988) and oxidative burst function (Hammon et al., 2006) were associated with exposure to elevated BHBA (Hoeben et al., 1997). An in vitro study demonstrated that concentrations of BHBA similar to those experienced by cows with subclinical ketosis had a small inhibitory effect on neutrophil oxidative burst (Hoeben et al., 1997). Elevated BHBA also appears to induce leucopenia, or reduced circulating concentrations of leukocytes, including neutrophils (Kremer et al., 1993). Additionally, exposure of neutrophils to concentrations of BHBA associated with subclinical and clinical ketosis interferes with the extracellular killing ability of neutrophils extracellular traps (Grinberg et al., 2008). The combination of decreased functionality and neutrophil population results in compromised efficacy.

Peripartum IR and NEB, along with associated concentrations of hormones and metabolites, result in severely compromised phagocytosis and oxidative burst capacity in neutrophils, which appears to be the primary cause of diminished immune function in dairy cows.
during transition. Although research has been completed to investigate how neutrophils are influenced by different hormones and metabolites associated with IR and NEB, many of these studies have been completed \textit{in vitro}, and therefore do not reflect the status of the peripartum cow as a system. Further research is required to determine how the metabolic status and neutrophil function interact in the peripartum dairy cow, in relation to development of uterine disease.

\textbf{Risk Factors}

Reduced immune function, especially that of neutrophils, is an important problem in the dairy industry as affected animals are more susceptible to disease. The transition diseases that result from poor immune function, such as metritis and mastitis, have economic implications as both production and reproduction efficiency can be severely reduced (Bonizzi et al., 2003). Identification of risk factors for diminished immune function is important in minimizing the impacts on health. Risk factors are primarily related to susceptibility of animals to IR and NEB in the early PP. Two important risk factors associated with compromised immune function are age and prepartum BCS.

Parity is an important factor in determining a cow’s ability to clear bacterial infections. Primiparous animals have had, theoretically, less exposure to fewer pathogens than multiparous cows, and therefore will have more naïve adaptive immunity, resulting in a slower response (Mallard et al., 1983). As previously discussed, however, the role of adaptive immunity during the PP is considerably less important than innate immunity. Neutrophils from primiparous cows have greater microbial killing capacity than those from multiparous cows (Mehrzad et al., 2002). Neutrophils from primiparous cows may have better function due to less severe NEB, as lactational demands (Kehrli et al., 2989; Llamas Moya et al., 2008) and differences in DMI.
between healthy and sick animals (Wittrock et al., 2011) are lower in heifers. Multiparous cows experience greater NEB, and therefore have greater concentrations of potentially toxic energy metabolites (i.e. NEFA and BHBA; Rukkwamsuk et al., 1999), resulting in more severe suppression of neutrophil function as compared with heifers.

Prepartum BCS contributes to immune competence during the PP. Overweight cows (BCS > 4) are at greater risk of immune dysfunction due to increased fat mobilization (Reid et al., 1986; Hoeben et al., 1993; Goff and Horst, 1997) and are more likely to experience IR (Holtenius et al., 2003). Greater lipolysis in obese cows results in increased circulating concentrations of NEFA and BHBA (Klucinski et al., 1988), which contribute both directly and indirectly to neutrophil dysfunction, as discussed previously, increasing their susceptibility to infectious disease.

Age and amount of BCS lost after calving are two risk factors that predispose dairy cows to compromised immune function in the PP. Risk factors associated with incidence of metritis include high haptoglobin (Huzzey et al., 2007), metabolic status, dystocia, retained placenta (Dubuc et al., 2010a), and parity (Bruun et al., 2002). Endometritis risk factors include metabolic disorders, retained placenta, parity (Kim and Kang, 2003), hyperketonemia and low BCS at calving (Dubuc et al., 2010a). Awareness of these, and other determinants that predispose cows to reduced immune function and subsequent disease, is important in guiding future research and development of management strategies.

**Management Implications**

Both endocrine and metabolic environments during the PP modulate the functionality of the immune system. Increased IR and NEB, along with the resulting increases in NEFA and BHBA, contribute to impaired neutrophil function. Neutrophils are vital in the prevention of
infectious transition diseases, and therefore management practices should be developed to promote normal neutrophil function. Neutrophil function is closely tied to IR, DMI and NEB, and therefore managing for energy balance will support immunity. Management for NEB, such as feeding a more palatable peripartum diet that is higher in energy to minimize fat mobilization (Grummer, 1993), may help to minimize IR and circulating levels of NEFA and BHBA, thereby minimizing the negative consequences these metabolic factors have on neutrophil function.

There will always be a degree of compromised immune function during the transition period due to necessary gestational down regulation of immunity (Weinberg, 1987) and altered immune response due to genetic selection of high producing cows (Mallard et al., 1998). The depth of NEB and immune suppression, however, can be mitigated by ensuring that cows are not overconditioned at dry off and minimizing fat mobilization after calving (Reid et al., 1986; Grummer et al., 2004; Grummer, 2008).

**Research Objectives**

The primary goal of this research project was to investigate how insulin resistance influences neutrophil function, and the impacts of those interactions on uterine disease in dairy cows during the peripartum period.

The first objective of the study was to validate a method for practical assessment of insulin resistance in peripartum dairy cows. This assessment included validation of a cow-side glucose meter.

The second objective of this study was to characterize the associations of insulin resistance and markers of adaptation to negative energy balance and lipolysis with innate immune function (phagocytosis and oxidation by neutrophils) in peripartum dairy cows. Uterine
disease was evaluated in relation to the associations of neutrophil function and markers of insulin resistance and negative energy balance.
CHAPTER TWO

EVALUATION OF AN ELECTRONIC COW-SIDE GLUCOSE METER FOR DIAGNOSING INSULIN RESISTANCE IN PERIPARTUM DAIRY COWS

INTRODUCTION

The period around calving is a time of metabolic stress as dairy cows transition into the energetically demanding process of lactation (Goff and Horst, 1997; Overton and Waldron, 2004). During early lactation, dry matter intake increases, but the energy demands of milk production are greater than the energy consumed, causing a state of negative energy balance (NEB; Butler et al., 1981; Goff and Horst, 1997). Beginning in late gestation and continuing into early lactation, cows are insulin resistant (IR) as glucose produced is partitioned primarily to the mammary gland (Hayirli, 2006). In response to IR and NEB, fat stores are mobilized to provide alternate energy sources, resulting in increased circulating concentrations of the metabolites of this process: non-esterified fatty acids and β-hydroxybutyric acid (BHBA; Herbein et al., 1985; Vazquez-Añon et al., 1994).

Mobilization of fat also results in the release of many pro-inflammatory mediators, such as tumor necrosis factor-α and interleukin (IL)-6 (Tilg and Moschen, 2005), which block, among other effects, intracellular signaling of insulin receptor substrate proteins (Hotamisligi, 2006), thus contributing to IR. Although inflammation contributes to the perpetuation of IR, NEB related to lactation demands is likely a much greater contributor to IR. After calving a cow experiences a cycle of IR and inflammation, with IR initiated and sustained due to NEB, resulting in fat mobilization and release of pro-inflammatory mediators, further exacerbating IR. Many of the inflammatory mediators associated with breakdown of adipose tissue have been implicated in uterine diseases. Excessive expression of genes for pro-inflammatory mediators
was a key factor in cows that develop endometritis (Sheldon et al., 2009). Cows with endometritis had increased mRNA expression of proinflammatory cytokines IL-1 and IL-6 (Herath et al., 2009). Although the cause of the inflammatory state of cows with endometritis has not been elucidated, IR and the subsequent mobilization of fat may be complicit in the development of diseases postpartum. Evaluating IR in the peripartum cow may provide an opportunity to identify animals at risk for developing uterine disease.

Glucose tolerance tests (GTT) can be used to measure IR (Lozner et al., 1941; Hayirli et al., 2001). This procedure consists of a bolus IV infusion of 50% glucose, followed by monitoring of serum concentrations of glucose and insulin at regular intervals (Lozner et al., 1941). Insulin resistance is evaluated based on glucose clearance rates or the amount of insulin secreted in response to high levels of glucose. Cows that are defined as not resistant to insulin using this traditional GTT method, recover to pre-infusion concentrations of glucose by 80 min post-infusion, whereas IR cows do not (Matteo et al., 2009). A simplified GTT has been developed, whereby a ratio of glucose concentration 80 min after glucose infusion to that prior to infusion are compared, with a ratio of 1.05 being proposed as a cut point potentially useful for identifying animals at risk of developing clinical disease (Matteo et al., 2009). The ability to measure whole blood glucose concentrations cow-side would be useful in expediting the process of the GTT and determining IR status.

Point-of-care diagnostic tools are increasingly available and affordable. Hand-held glucose and ketone measuring systems are commonly used for monitoring of diabetes in human medicine (Guerci et al., 2005). Several dairy cattle studies have reported use of a variety of different hand-held human electronic glucometers. One study used the AccuCheck Active monitor (Roche Diagnostics, Indianapolis, IN) in peripartum Holstein dairy cows, and reported a
Pearson correlation coefficient of 0.82 between the glucometer and laboratory results, suggesting that the hand-held monitor could be used as an alternative (Galvão et al., 2010). Other studies have used human glucometers to measure variations in glucose concentrations of periparturient dairy cows (Leury et al., 2003; Fall et al., 2008), to measure flow rate of a glucose infusion to evaluate the effects of insulin on growth hormone receptor expression (Rhoads et al., 2004) or of bovine somatropin and insulin on milk production (Molento et al., 2002). None of these studies, however, report any data related to validation or test characteristics. One glucose and ketone hand-held meter (Precision Xtra, Abbott Diabetes Care, Abingdon, UK) has been successfully validated for diagnoses of subclinical ketosis in dairy cows, reporting a Pearson correlation coefficient of 0.95 (Iwersen et al., 2009). The ability of this device to accurately measure glucose in dairy cows was briefly evaluated with an R² value of 0.56 being reported (Oetzel and McGuirk, 2008).

The purpose of this study was to evaluate the diagnostic performance of a hand-held glucometer (Precision Xtra) for cow-side use in dairy cattle. Specific objectives were to 1) assess the accuracy of whole blood glucose measurements from the Precision Xtra glucometer relative to the gold standard of a reference chemical analyzer in a diagnostic laboratory; and 2) assess the suitability of the glucometer to classify cows as insulin resistant with a GTT.

**MATERIALS AND METHODS**

A handheld electronic glucose measuring system (Precision Xtra; Abbott Diabetes Care, Mississauga, ON, Canada) was used in this experiment according to the label descriptions of the manufacturer. The system consisted of a handheld meter and electrochemical test strips. A minimum 0.6 μL blood sample is applied to the sensor after the test strip is inserted into the monitor. Once the blood progresses through the test strip via capillary action, it reacts with
glucose oxidase to form gluconic acid, which then reacts with the potassium ferricyanide to create potassium ferrocyanide. The potassium ferrocyanide then reacts with the metal of the test strip electrodes, creating an electrical current. The current generated is directly proportional to the amount of glucose present in the blood. After 5 s the monitor displays the glucose concentration (mmol/L).

This experiment included 81 peripartum Holstein dairy cows housed at the University of Guelph’s Elora and Ponsonby Dairy Research Centre (Elora, Ontario, Canada). These animals were part of an observational study and the sample size was based on predicted immune function outcomes, which are reported in Chapter 3 of this thesis. Animals were cared for according to the Canadian Council on Animal Care guidelines (2009). Data were collected on enrolled animals from 3 wk prior to due date until 5 wk after calving from November 2010 until October 2011.

Duplicate blood samples (n = 709) were taken from the coccygeal vein and/or artery into vacuum tubes (Vacutainer, Becton Dickson, Franklin Lakes, NJ) with the preservative sodium fluoride potassium oxalate (NaF; the gold standard for glucose analysis as NaF stops enzymatic activity of the glycolytic pathway; Peakman and Elliott, 2008) or without any preservative. After samples were obtained, the tubes were gently inverted 10 times to ensure thorough mixing with the preservative, and subsequently placed in a chilled container. Glucometer readings were taken on the whole blood with no preservative immediately following collection. Whole blood was transferred to the sensor of the glucometer test strip by touching the sensor directly to a drop of blood in the lid of the vacuum tube after collection. The glucose concentrations (mmol/L) displayed on the glucometer were recorded. One meter was used for all of the glucose measurements. Blood was separated through centrifugation (30 min, 1,000 x g). Serum was collected, frozen, and stored in a -20°C freezer until chemical analysis. The serum was sent to
the Animal Health Laboratory (University of Guelph) for determination of glucose concentrations using a commercial reagent kit (GLUC3, Roche Diagnostics, Indianapolis, IN, USA) and auto-chemistry analyzer (cobas c311, Roche Diagnostics). The analytical sensitivity of the glucose assay was 0.1 mmol/L, and the inter- and intra-assay coefficient of variation were 2.9 and 2.4% respectively.

**Glucose Tolerance Tests**

Fifty-nine simplified GTT (Matteo et al., 2009) were conducted 1 wk prior to calving and consisted of an IV bolus infusion of 0.25 mg/kg dextrose (Dextrose 50%, Vétquinol Canada Inc., Lavaltrie, QC) based on standard estimated body weight of 650 kg. Duplicate blood samples were taken immediately before dextrose infusion and at 10, and 80 min after. Animals were classified as insulin resistant if the ratio of blood glucose concentrations at 80 min after dextrose infusion to prior to infusion was ≥ 1.05 (Matteo et al., 2009).

**Statistical Analyses**

Statistical analyses were performed using SAS (SAS version 9.2, SAS Institute Inc., Cary, NC). Glucose concentrations obtained from the serum preserved with NaF and analyzed in the laboratory were considered the gold standard for all analyses. Samples from both routine data collection (at -3, -2, -1, 1, 2, 3, 4, and 5 wk relative to calving) and GTT were used, including 60 observations of circulating glucose at peak concentrations 10 min after the bolus infusion associated with the GTT. Pearson correlation coefficients were calculated. A Passing-Bablok linear regression correlation plot was performed, using proc reg and calculating 95% predictive confidence intervals, comparing the values from the glucometer to the gold standard laboratory measurements (Passing and Bablok, 1983). Using only correlation coefficients may not always be suitable for evaluation of test performance (Bland and Altman, 1986), therefore a Bland-
Altman plot was used to graphically assess the residuals between the Precision Xtra and the gold standard.

Using a GTT ratio of 1.05 (i.e. glucose concentration 80 min after infusion vs. just prior to infusion), the suitability of the Precision Xtra for diagnosing IR was assessed. The GTT ratios calculated from Precision Xtra glucose concentrations were compared to those calculated from the gold standard NaF samples. The sensitivity and specificity of the glucometer GTT 1.05 cut point were calculated. To identify an optimal test threshold for the glucometer relative to the 1.05 gold standard GTT ratio, glucometer GTT ratios were dichotomized. The optimal cut point was identified as the value that produced the highest sum of sensitivity and specificity.

RESULTS

Figure 2.1 depicts a Passing-Bablok correlation plot between the glucose concentrations as measured by the laboratory and the glucometer. The Pearson correlation coefficient was 0.95 ($P < 0.001$). The intercept was not equal to zero ($P < 0.0001$) and the slope was not equal to 1 ($P < 0.0001$). The following equation was determined as a result of linear regression analysis:

\[ \text{laboratory glucose} = 0.86 \times (\text{glucometer glucose} + 0.6) \]

Plotting of the residuals between the glucometer and laboratory readings against their mean revealed that the glucometer readings were on average 0.03 points lower than the laboratory results (Figure 2.2). 96% of the data points fell between the 95% confidence intervals in the Bland-Altman plot (Figure 2.2).

Using 1.05 as the GTT ratio cut point, as defined by Matteo et al. (2009), the ability of the glucometer to detect IR was compared to that of the laboratory glucose readings (Table 2.1). The sensitivity and specificity of the glucometer at predicting insulin sensitivity were 84% and 52% respectively (Kappa = 0.37; Table 2.2). When the glucometer values were adjusted using the equation obtained from the linear regression, the sensitivity and specificity of the
glucometers performance at predicting insulin sensitivity were 81% and 56% respectively (Kappa = 0.37). Different cut points for the glucometer were assessed in relation to the gold standard 1.05 value to identify a GTT ratio for the glucometer that optimized both sensitivity and specificity (Table 2.2). The optimal cut point of 1.15 was identified for the glucometer GTT ratios, producing a sensitivity of 69% and specificity of 85% (Kappa = 0.53) relative to the 1.05 cut point for the gold standard.

**DISCUSSION**

Cows experience a period of IR around the time of calving (Hayirli, 2006; Matteo et al., 2009). Excessive or premature IR may be associated with clinical disease (Hayirli, 2006) and inflammatory status (Tilg and Moschen, 2005; Hotamisligi. 2006). The ability to identify cows with peripartum IR may allow for implementation of measures to mitigate or prevent development of disease. The use of a point-of-care glucometer to diagnose IR would reduce both the cost of testing and time required to obtain results, by avoiding more costly and potentially lengthy traditional laboratory tests. The Precision Xtra glucometer costs $0-40 and each test approximately $0.89, whereas the list price for glucose analysis at the Animal Health Laboratory (University of Guelph) is $8.

Although the Precision Xtra has been validated for use in dairy cows for measuring subclinical ketosis (Iwersen et al., 2009), this is the first study to validate its ability to determine glucose concentrations in dairy cows relative to the gold standard. This hand-held glucometer performed well compared with the gold standard when measuring glucose concentrations. The correlation between the gold standard and glucometer was high, and the evaluation of the residuals revealed an even distribution, with most observations falling within the 95% confidence intervals, especially for values within the normal range for dairy (i.e. not samples
taken at peak glucose concentrations 10 min after GTT dextrose infusion). These results suggest that the Precision Xtra may be a useful point-of-care tool, as it is reliable at detecting glucose concentrations that reflect those determined in the laboratory. Other glucometers have been assessed for performance in dairy cows (Galvão et al., 2010), however there may be practical benefits in the ability to use one monitor, the Precision Xtra, to measure both glucose and ketones in dairy cows.

The Precision Xtra diagnosed IR using a cut point of 1.05 for the GTT ratio, with a sensitivity of 84% and specificity of 52% relative to the gold standard. Although the specificity of this test was poor, due to the nature of IR, sensitivity is a more important test characteristic. Interventions that may be taken to mitigate the negative consequences of IR have not been studied (Grummer, 2008), but management for NEB such as feeding a more palatable peripartum diet that is higher in energy to minimize fat mobilization (Grummer, 1993) may be useful. Strategies such as dietary interventions are not likely to be detrimental to animals that may have falsely tested positive for IR (Grummer, 2008), thus ruling out animals without IR is less important than ensuring all those with IR are identified. Due to the tendency of the Precision Xtra to measure glucose 0.03 points lower than the gold standard, and no improvement in test characteristics after the application of an adjustment equation, a different cut point may be identified to better reflect the 1.05 GTT ratio for the gold standard. The optimum cut point for the GTT as measured by the glucometer was identified as 1.15, which was determined to improve the sum of sensitivity and specificity of the test. More investigation is required to determine whether the 1.05 GTT ratio cut point for laboratory values is meaningful in revealing animals at risk of developing disease, as the current research is limited.
Due to the strong correlation and low variation in residuals between glucose concentrations obtained from whole blood by the Precision Xtra and serum glucose concentrations, the handheld glucometer appears suitable for rapid measurement of glucose including in a glucose tolerance test under field conditions in dairy cattle.
**Table 2.1** Two by two table evaluation of the performance of a hand-held glucometer (Precision Xtra, Abbott Diabetes Care, Mississauga, ON, Canada) in identifying insulin resistance using a glucose tolerance test (GTT; n = 59) compared with the gold standard (serum preserved in sodium fluoride and analyzed with a commercial kit by an auto-chemistry analyzer; Roche Hitachi 912, Roche, Basel, Switzerland). A GTT ratio was calculated as the glucose concentration 80 min after vs. just prior to IV glucose infusion. The cut point of the GTT ratio evaluated was chosen based on previous literature (Matteo et al., 2009), with animals having a GTT ratio ≥ 1.05 being considered insulin resistant. The optimized GTT ratio cut point for the glucometer, based on the gold standard cut point of 1.05, was identified as 1.15.

<table>
<thead>
<tr>
<th>Glucometer Value</th>
<th>Laboratory Value ≥ 1.05</th>
<th>Laboratory Value &lt; 1.05</th>
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<tr>
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</tr>
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</tr>
<tr>
<td>Total</td>
<td>32</td>
<td>27</td>
<td>59</td>
</tr>
</tbody>
</table>
Table 2.2 Test characteristics of diagnosing insulin resistance with glucose tolerance test (GTT; n = 59) ratios. GTT ratios were calculated as the glucose concentration 80 min after IV dextrose infusion relative to just before. Glucometer GTT ratios were investigated against the gold standard (serum with sodium fluoride preservative analyzed with a commercial kit by an auto-chemistry analyzer; Roche Hitachi 912, Roche, Basel, Switzerland) at a cut point of 1.05. Various glucometer cut points were evaluated to gold standard GTT ratio 1.05.

<table>
<thead>
<tr>
<th>Glucometer Cut Point</th>
<th>Se$^1$ (%)</th>
<th>Sp$^2$ (%)</th>
<th>PPV$^3$ (%)</th>
<th>NPV$^4$ (%)</th>
<th>Kappa</th>
</tr>
</thead>
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<td>61</td>
<td>0.41</td>
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</table>

1 Sensitivity
2 Specificity
3 Positive predictive value
4 Negative predictive value

* Highest sum of sensitivity and specificity
Figure 2.1 Passing-Bablok correlation plot for glucose concentrations from a glucometer (Precision Xtra, Abbott Diabetes Care, Mississauga, ON, Canada) against the gold standard: serum preserved in sodium fluoride potassium oxalate and analyzed on an auto-chemistry analyzer (Roche Hitachi 912, Roche, Basel Switzerland). Scatter diagram, including 709 samples from 81 cows, with the regression line (solid central black line), regression confidence limits (outer two solid black lines), and identity line (x = y; dotted line). Values above 6 mmol/L are peak concentrations observed 10 min after dextrose infusion during the glucose tolerance test (GTT), those below 6 mmol/L occurred either during routine sampling, before GTT, or 80 min after GTT IV dextrose infusion. Pearson correlation coefficient = 0.95 (p < 0.001).
Figure 2.2 Bland-Altman plot (709 samples from 81 cows), with the average glucose concentration as measured by the glucometer (Precision Xtra, Abbott Diabetes Care, Mississauga, ON, Canada) and the gold standard serum preserved in sodium fluoride potassium oxalate and analyzed in a laboratory with a commercial reagent kit and auto-chemistry analyzer (Roche Hitachi 912, Roche, Basel Switzerland) on the x-axis, plotted against the difference in glucose concentrations determined by the glucometer and the laboratory on the y-axis. Relative differences in glucose concentrations between the glucometer and laboratory measurements are plotted against the mean concentrations determined by both methods. The mean difference is represented by the solid line, and the 95% confidence limits are depicted by the dashed lines. Values above 6 mmol/L are peak concentrations observed 10 min after dextrose infusion during the glucose tolerance test (GTT), those below 6 mmol/L occurred either during routine sampling, before GTT, or 80 min after GTT IV dextrose infusion.
CHAPTER THREE
ASSOCIATIONS AMONG NEUTROPHIL FUNCTION, METABOLIC INDICATORS, AND REPRODUCTIVE HEALTH IN DAIRY COWS

INTRODUCTION

Insulin resistance (IR) is a phenomenon commonly experienced by dairy cows in the peripartum period (PP; Hayirli, 2006). Insulin resistance is a state in which a physiological level of insulin produces a less than normal biological response (Kahn et al., 1978). Around the time of calving as the energy demands of the late gestation fetus increase and dry matter intake (DMI) no longer meets the metabolic requirements of lactation, consumed glucose is partitioned towards these functions and alternative energy sources must be mobilized to fill the deficit (Goff and Horst, 1997; Hayirli, 2006; Grummer et al., 2008). As glucose is partitioned to the mammary gland, peripheral tissues become resistant to insulin and the body shifts towards fat mobilization to compensate for the negative energy balance (NEB; Vazquez-Añon et al., 1994; Hayirli, 2006). This resistance likely results from elevated NEFA and growth hormone, which have an antagonistic effect on insulin action (Dominci and Turyn, 2002; Kloever and Mooney, 2004). As a result of lipid mobilization, non-esterified fatty acids (NEFA) and β-hydroxybutyric acid (BHBA) increase in circulation (Herbein et al., 1985; Vazquez-Añon et al., 1994). In addition to NEFA and BHBA, many different inflammation mediators are released from fat stores as they are mobilized (Tilg and Moschen, 2005; Hotamisligil, 2006). Excessive or imbalanced circulating concentrations of pro-inflammatory mediators, NEFA, and BHBA may predispose peripartum animals to developing clinical diseases, including those of the reproductive tract.

Uterine disease, such as endometritis, has been associated with factors resulting from fat mobilization. Cows with subclinical ketosis (Reist et al., 2003) or high circulating NEFA are
more likely to develop many different diseases postpartum, including endometritis (Hammon et al., 2006; Dubuc et al., 2010b). Cows in greater NEB, with higher levels of circulating NEFA and BHBA, have reduced immune function, particularly those that go on to develop endometritis or metritis (Hammon et al., 2006; Galvão et al., 2010). The development of endometritis (diagnosed at approximately 1 month postpartum) has been associated with differential expression of inflammatory mediators in the first week after calving (Herath et al., 2009; Sheldon et al., 2009). Pro-inflammatory mediators identified in the etiology of endometritis include interleukins (IL) 1α, 1β, and 6, as well as tumor necrosis factor α (TNFα; Herath et al., 2009), some of which (IL-6 and TNFα) are also associated with lipolysis (Tilg and Moschen, 2005). Cows with metritis (Huzzey et al., 2007; Chan et al., 2010; Dubuc et al., 2010b; Galvão et al., 2010) and endometritis also have greater circulating concentrations of haptoglobin, an acute phase protein involved in immune function, in the first week after calving (Dubuc et al., 2010b; Galvão et al., 2010). In mice, it has been demonstrated that expression of haptoglobin in adipose tissues is greater in obese animals (Chiellini et al., 2002). A deficiency of 25-hydroxyvitamin D (vitamin D), has been associated with IR in humans, particularly those with type 2 diabetes (Borissova et al., 2003; Ford et al., 2005; Forouhi et al., 2008), a metabolic syndrome similar to peripartum IR in dairy cows, with the exception that cows have low circulating glucose concentrations (Lucy, 2004). While some of the direct effects of fat mobilization on immune function in vitro have been elucidated, the impact of NEB and IR on immune cell function in vivo during the PP is not well understood.

Compromised function of the neutrophil, the primary innate immune cell associated with clearing bacterial contamination from the uterus (Hussain, 1989; Gilbert et al., 2007), has been implicated in the development of uterine disease (Cai et al., 1994; Kim et al., 2005), and is
impacted by markers of IR and NEB (Hammon et al., 2006; Galvão et al., 2010). Cows that develop metritis and endometritis have been demonstrated to have reduced neutrophil oxidative burst capacity (Mateus et al., 2002; Hammon et al., 2006). Whether neutrophil phagocytosis contributes to endometritis is unclear as studies have reported either diminished capacity (Kim et al., 2005), or no change in phagocytosis (Kehrli et al., 1989; Hoedmaker, 1992; Cai et al., 1994).

Alteration of neutrophil functions by IR- and NEB-associated metabolites has been demonstrated in in vitro studies. Levels of NEFA commonly experienced in the PP resulted in diminished oxidative burst capacity, although phagocytosis appeared unaffected (Scalia et al., 2006).

Exposure to levels of BHBA similar to those experienced by cows with subclinical ketosis resulted in a reduction of neutrophil oxidative burst function (Hoeben et al., 1997). Due to the neutrophils’ susceptibility to NEFA and BHBA in vitro, as well as a demonstrated reduction in their function in cows with uterine disease, compromised neutrophil function in the PP may connect NEB and development of reproductive tract disease.

The objective of this study was to characterize the associations of IR and markers of adaptation to NEB and lipolysis with neutrophil oxidative burst and phagocytosis function and uterine disease in peripartum dairy cows.

**MATERIALS AND METHODS**

**Animals, Housing, and Management**

Thirty-four primiparous and 47 multiparous (total n = 81, parity 1.6 ± 0.5) Holstein dairy cows housed in tie stall facilities at the University of Guelph Dairy Research Centres (Guelph, ON, Canada) were used in this study. Animals were cared for according to the guidelines of the Canadian Council on Animal Care (2009) and under an Animal Utilization Protocol approved by the University of Guelph Animal Care Committee. Cows were enrolled in the study 3 wk prior to
expected calving and monitored until 5 wk after calving in the period from November 2010 until October 2011. Weeks relative to calving were corrected based on actual calving date. All animals were fed a total-mixed ration (TMR) daily at 0900, 1300, and 1500 h, and cows that had calved were milked daily at 0530 and 1600 h. Data collected during the study are outlined in Appendix 3.1. The sample size for this observational study was based on detection of a 20 (± 20) unit difference in oxidative burst activity between cows with or without a naturally-occurring reproductive disease outcome, which required 16 animals per group.

**Blood Metabolites**

For each week that an animal was enrolled in the trial, blood samples were taken from the coccygeal vein and/or artery into an evacuated sterile tube (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA) without any preservative. Concurrently, 3 vacuum tubes of coccygeal vein and/or artery blood were collected with the anticoagulant acid citrate dextrose (ACD; Vacutainer, Becton Dickinson) for later isolation of neutrophils. To ensure thorough mixing of blood with preservatives, after collection all tubes were inverted 8 times.

Immediately after collection, a handheld electronic glucose and ketone measuring system (Precision Xtra, Abbott Diabetes Care, Mississauga, ON, Canada) was used as described by Iwersen et al. (2009) on the whole blood with no preservative to measure BHBA concentrations on the samples taken during the first 3 wk after calving. Within 3 h of collection, blood in tubes with no preservative was centrifuged to harvest serum, and subsequently frozen and stored at -20°C. Separate aliquots of serum without preservative were stored at -80°C for insulin and IGF-1 analysis.

Serum was sent to the Animal Health Laboratory (University of Guelph) for determination of glucose, NEFA, and haptoglobin concentrations, for each time point, and
BHBA concentrations at 1 wk prior to calving and 4 and 5 wk after, using an auto-chemistry analyzer (cobas 4000 c 311, Roche Diagnostics, Indianapolis, IN, USA). Glucose concentrations were measured using the Roche GLUC3 kit (Roche Diagnostics). The analytical sensitivity of the glucose assay was 0.1 mmol/L, and the inter- and intra-assay coefficient of variation were 2.9 and 2.4% respectively. The NEFA and BHBA concentrations were determined using Randox NEFA and Randox BHBA kits (Randox Laboratories Canada Ltd., Mississauga, ON, Canada). The analytical sensitivity was 0.1 mmol/L for both the NEFA and BHBA assays. The inter- and intra-assay coefficients of variation were 1.0 and 6.3%, respectively for NEFA, and 0.5 and 1.6%, respectively for BHBA. Haptoglobin concentrations were measured using the hemoglobin binding capacity, using a methemoglobin reagent made on-site according to a method described elsewhere (Makimura and Suzuki, 1982; Skinner et al., 1991). The analytical sensitivity of the haptoglobin assay was 0.03 g/L. The inter- and intra-assay coefficients of variation were 5.6 and 3.5%, respectively. Insulin and IGF-1 concentration analysis was carried out using previously described procedures (Chelikani et al., 2004) at the University of Missouri (Animal Sciences Department, University of Missouri, Columbia, MO, USA). The Diagnostic Centre for Population and Animal Health at the University of Michigan (Lansing, MI, USA) determined 25-hydroxyvitamin D concentrations for samples collected 1 wk before parturition. After rapid extraction of vitamin D₂, vitamin D₃, and other hydroxylated metabolites using acetonitrile, serum concentrations of total 25-hydroxyvitamin D were quantified using an equilibrium RIA procedure (DiaSorin, Stillwater MN). The inter- and intra-assay CV for the 25-hydroxyvitamin D quantification were 11% and 10%, respectively.
**Glucose Tolerance Test**

Glucose tolerance tests were completed 1 wk prior to expected calving and consisted of a bolus IV infusion of 0.25 mg/kg dextrose over approximately 2 minutes (Dextrose 50%, Vétquinol Canada Inc., Lavaltrie, QC) based on a standard estimated body weight of 650 kg. Blood samples were obtained just prior to, and 10 and 80 minutes after dextrose infusion, from the coccygeal vessels as previously described.

**Neutrophil Isolation and Function Evaluation**

**Isolation.** Whole blood was collected for neutrophil function analysis into the anticoagulant ACD (8.5 mL of blood into each tube) at 1 and 2 wk prior to expected calving dates and 1, 2, 3, and 5 wk after calving. Neutrophils were isolated from the whole blood with ACD within 3 h of collection. After 25 mL of whole blood was diluted with 10mL of room temperature 1× concentrated phosphate buffered saline (PBS), it was overlaid on 13 mL of room temperature Ficoll-Paque PLUS (General Electric Healthcare Bio-Sciences AB, Uppsala, Sweden), and centrifuged for 30 min at 500 × g. The plasma and buffy coat were removed, and 6 volumes of sterile cold water were added to the remaining cells. To encourage erythrocyte lysis, the suspension was gently inverted for 45 s. To reestablish osmolarity, 3 volumes of 3× concentrated PBS was added to the tubes, followed by a 10 min centrifugation at 250 × g. The supernatant was removed, and washing and centrifugation were repeated until there was no visible hemoglobin in the cell pellet. Cell pellets were resuspended with 500 μL of PBS. Cell concentration and viability were determined by trypan blue exclusion in a hemocytometer chamber. The pelleted neutrophils were diluted to 1 x 10⁶/mL with 1× concentrated PBS with 10% filtered fetal bovine serum (FBS, Invitrogen, Burlington, ON, Canada).
**Oxidative Burst Assay.** Five µM of 2’, 7’–dihydro-dichlorofluorescein-diacetate (H₂DCFDA, Molecular Probes, Eugene, OR, USA) was added to 200 µL of the reconstituted neutrophils in a flow cytometry tube (BD Biosciences, Bedford, MA, USA). The green cellular fluorescence of the H₂DCFDA was measured in a flow cytometer. After oxidization, the non-fluorescent cell-permeable H₂DCFDA is converted within the cells to dichlorofluorescein, a highly fluorescent derivative of H₂DCFDA. The suspension with the neutrophils and H₂DCFDA was then incubated in the dark for 15 min at 37°C with gentle agitation. Two hundred µL of 1× concentrated PBS with 10% FBS (control) or PBS/FBS diluted phorbol myristate acetate (PMA, Sigma, St. Louis, MO, USA) for a total of 15 ng/mL of PMA, was then added, and incubated in darkness for a further 5 min at 37°C with gentle agitation. The addition of PMA was used to stimulate oxidative burst within the neutrophils. The tubes were then placed on ice and protected from light until flow cytometry analysis.

**Phagocytosis Assay.** Activated normal cow serum was made by pooling serum from 40 healthy lactating Holstein cows at the University of Guelph Dairy Research Centres, and incubating it with 100 mg of Zymosan A from *Saccharomyces cerevisiae* (Sigma-Aldrich, St. Louis, MO, USA) per 10 mL of serum at 37°C for 1 h in a rotating rack. The suspension was centrifuged for 15 min at 550 × g, and the supernatant was separated into 2 mL aliquots and stored at -20°C. Two-hundred µL of the reconstituted neutrophils (10⁶) were transferred into flow cytometry tubes. These tubes were incubated for 30 min at 37°C in the dark with 1 × 10⁶ fluorescently labeled 1 µm beads (TransFluo-Spheres® Fluorescent Microspheres, Molecular Probes) and 50 µL of normal cow serum to encourage opsonization of the beads. After the incubation, the cells were washed and re-suspended in flow cytometry buffer and kept on ice in darkness until flow cytometry analysis. Negative controls contained neutrophils that had been
incubated without fluorescent beads. All samples were run in triplicate for both the oxidative burst and phagocytosis assays.

**Flow Cytometry Analysis.** Oxidative burst and phagocytosis were measured on a flow cytometer (FACScan, Becton Dickinson) with Cell Quest software (Becton Dickinson). Forward vs. side scatter cytograms were used to identify the neutrophils (Appendix 3.2), and fluorescence was collected in the neutrophil gate, with 5,000 events for the oxidative burst assay and 20,000 events for phagocytosis. Oxidation of H$_2$DCFDA was measured at 530 nm (FL1) and phagocytosis of fluorescent beads at 645 nm (FL3), on the log scale. Using FlowJo software (Tree Star, Ashland, OR, USA), the shift in the percentage of cells successfully undergoing oxidative burst or phagocytosis was tabulated for each observation. A gate was placed around $\geq$ 96% of the negative control population of neutrophils (i.e. samples with no PMA activation for oxidative burst, and those with no fluorescent beads for phagocytosis) and applied to all observations. The success of oxidative burst or phagocytosis was calculated as the difference in the percentage of cells outside of the negative control gate for positive observations versus those within the gate. Examples of fluorescence histograms obtained through flow cytometry are included in Appendices 3.3 and 3.4 for neutrophil oxidative burst and phagocytosis, respectively. Each sample was assayed in triplicate and the mean value was used for statistical analysis. Cows with 3 or more missing observations for either oxidative burst or phagocytosis were excluded. Some observations were also excluded due to laboratory equipment malfunctions.

**Uterine Health Parameters**

**Vaginoscopy.** Visual evaluation of vaginal discharge was conducted using a disposable vaginal speculum (JorVet Jorgensen Labs, Loveland, CO, USA) at 3 and 5 wk after calving. Discharge was scored based on the rubric outlined by Sheldon et al. (2006). Cows with a score of
3 or 4 on this rubric were classified as having purulent vaginal discharge. The appearance of the cervix and vaginal walls was noted at this time.

**Cervical and Uterine Cytology.** All cows were examined for postpartum endometritis and cervicitis at 5 wk after parturition. Samples were collected from one ring inside the cervix and from the uterine body, with cytobrushes (VWR CanLab, Missauga, ON, Canada). From these samples, cytology slides were created, preserved, and observed for neutrophil populations as previously described by Dubuc et al. (2010a). Cows with endometritis and cervicitis were identified as those with ≥ 6% neutrophils relative to epithelial cells based on previous literature (Dubuc et al., 2010; Deguillaume et al. 2012).

**Statistical Analyses**

All statistical analyses were performed using SAS (Version 9.2, SAS Institute, Cary, NC, USA) with cow as the unit of interest. The difference between samples and negative controls in the mean of the percent shift in cells that successfully performed oxidative burst or phagocytosis was assessed for normality and it was identified that the data were not normal for either function. To normalize the data, the natural logarithm was taken of both the oxidative burst and phagocytosis variables.

The potential associations of continuous variables with oxidative burst and phagocytosis were inspected with scatter plots. All continuous variables were assessed against all four outcomes, endometritis, cervicitis, oxidative burst, and phagocytosis, for overall differences and variation by time, using univariable models and graphical evaluation (mean ± SE). For each week of sampling, continuous variables (i.e. BHBA, NEFA, haptoglobin, GTT ratio for IR evaluation) were dichotomized at various cut points, chosen based on published cut points and the distribution of the present data. At each of these cut points, as well as for endometritis and
cervicitis disease status, the difference in neutrophil oxidative burst and phagocytosis capacity were evaluated over the sampling period by graphing the mean (± SE) at each time point for the cows above or below the cut point, and those with or without disease. Revised quantitative insulin sensitivity check index (RQUICKI), a proposed measure of IR (Holtenius et al., 2007; Kerestes et al., 2009) was calculated based on concentrations of glucose (mmol/L), insulin (ng/mL), and NEFA (mmol/L) using the equation: RQUICKI = 1/[log (glucose concentration) + log (insulin concentration) + log (NEFA concentration)]. Smaller values of RQUICKI imply greater IR. Linear regression was conducted between RQUICKI and indicators of lipolysis, including concentrations of NEFA, BHBA, IR ratios from the GTT, and BCS. Body condition score at time of enrollment was classified as thin (BCS ≤ 2.75), normal (BCS 3.0 – 3.5), and overconditioned (BCS ≥ 3.75). The number of cows classified as thin was only 5, and this category was not significant in the models (p = 0.97), so BCS was recategorized as normal (BCS <3.5) and overconditioned (BCS ≥ 3.75). Parity was categorized into primiparous (parity = 1) and multiparous (parity ≥ 2). The univariable association of each categorical predictor with disease outcomes was assessed with contingency tables and the Chi-square statistic. Variables with P ≤ 0.2 were offered to multivariable models.

For the disease outcomes (endometritis and cervicitis), a logistic regression model (GLIMMIX procedure in SAS) with a binary distribution and logit link function was used. Predictor variables that were only measured once were only considered once to avoid pseudo-replication. For the neutrophil function outcomes (oxidative burst and phagocytosis), a mixed model (MIXED procedure in SAS) was used, with cow as a repeated measure and an autoregressive covariance structure. For each neutrophil function outcome, univariable analyses were conducted using linear regression, with a conservative significance level (p ≤ 0.2).
Predictor variables that met the significance criteria in the univariable evaluation were placed in a multivariable model (either logistic or mixed models, corresponding with the outcomes stated for the univariate evaluation) for backward stepwise analysis. Variables remained in the final model if the $P$-value was $\leq 0.1$, or if their removal changed other coefficients in the final model by $> 20\%$ (i.e. there was confounding). If both a continuous predictor and its dichotomized value at one or more cut points were significant in the univariable analysis, they were tested in separate backwards stepwise multivariable models, and the model with the lowest Akaike information criterion was selected. Collinearity was assessed in instances where $\geq 2$ cut points for the same variable at different time points were significant within the model using a variance inflation factor with a threshold of $\geq 10$ indicating high collinearity. No collinearity was indicated in any of the multivariable models.

**RESULTS**

A total of 81 cows were enrolled in the study. The distribution of clinical and observed diseases, cytological findings, and parity is outlined in Appendix 3.5. The distribution of BHBA, NEFA, insulin, IGF-1, haptoglobin, and glucose concentrations, and oxidative burst and phagocytosis function through the study are described in Appendices 3.6 to 3.13. A summary of glucose, IGF-1, vitamin D, haptoglobin, NEFA, and BHBA is included in Appendix 3.14. The prevalence of endometritis and cervicitis at week 5 postpartum were 34% and 27% respectively. Of cows that had endometritis, 17 also had cervicitis. There were too few ($< 10$) cases of retained placenta, metritis, or purulent vaginal discharge at week 5 for meaningful analysis. The only association between RQUICKI and markers of IR was NEFA ($R^2 = 0.44; P < 0.0001$), all other regressions elicited $R^2$ values $< 0.1$. Cows with NEFA $\geq 0.5$ at 5 wk after calving had greater concentrations of the metabolite throughout the postpartum period than those with NEFA below
0.5 (p < 0.0001; Figure 3.1). No significant relationship was found between NEFA and neutrophil oxidative burst capacity in a linear regression (R^2 = 0.005; p = 0.17; Appendix 3.15).

The outcomes endometritis, cervicitis, oxidative burst, and phagocytosis were assessed in relation to parity, and no significant relationships were identified except for with endometritis (p = 0.1), therefore parity was offered to the endometritis model.

Variables were offered to the models based on significance (p < 0.2) in univariable evaluation, including those variables with significant interactions with time. Predictor variables offered to the endometritis logistic regression model included insulin (p = 0.05; Appendix 3.16), IGF-1 (p = 0.01; Appendix 3.17), parity (p = 0.1), cervicitis (p = 0.001), and neutrophil phagocytosis (p = 0.13; Appendix 3.18). Although BHBA as a continuous variable was not significant (p = 0.8), the following cut points, based on previous literature, were offered to the model: 0.4 mmol/L at 1 wk before calving (p = 0.15), 1.2 mmol/L at 1 wk after calving (p = 0.15), and 1.1 mmol/L at 4 wk after calving (p = 0.15). Haptoglobin was not significant in the univariable analysis (p = 0.4), however at 2 wk after calving, cows with endometritis had higher circulating haptoglobin (p = 0.09; Appendix 3.19), and therefore haptoglobin ≥ 0.7 g/L at 2 wk postpartum was offered to the model. While neutrophil oxidative burst (p = 0.5) was not significant in the endometritis univariable assessment, it was identified that cows with endometritis diagnosed at 5 wk after calving, had reduced oxidative burst function 2 wk after calving (p = 0.02; Appendix 3.20) and subsequently oxidative burst was offered to the model.

The final logistic regression model for endometritis diagnosed at 5 wk after calving is reported in Table 3.1. Cows were more likely to be diagnosed with endometritis if at 1 wk after calving they had circulating concentrations of BHBA ≥ 1.2 mmol/L, increased phagocytosis function, or if they were multiparous animals. Lower levels of IGF-1, neutrophil oxidative burst
function, and having BHBA less than 0.4 mmol/L at 1 wk or 1.2 mmol/L at 4 wk postpartum were also associated with increased odds of endometritis. Cows with cervicitis were more likely to have concurrent endometritis.

For the cervicitis logistic regression model, predictor variables offered included insulin ($p = 0.003$; Appendix 3.21), IGF-1 ($p = 0.11$; Appendix 3.22), glucose ($p = 0.14$; Appendix 3.23), haptoglobin ($p = 0.03$; Appendix 3.24), neutrophil oxidative burst ($p = 0.07$; Appendix 3.25) and phagocytosis ($p = 0.17$; Appendix 3.26), endometritis ($p = 0.001$), IR ratio $\geq 1.1$ ($p = 0.0001$), and BCS at enrolment ($p = 0.02$).

Table 3.2 reports the final logistic regression model for cervicitis diagnosed at 5 wk after calving. Cows were more likely to have cervicitis if they had increased circulating haptoglobin or glucose, a BCS $\leq 3.5$ at 3 wk postpartum, decreased neutrophil phagocytosis capacity, or if they also had endometritis.

Predictor variables offered to the neutrophil oxidative burst linear regression model included IGF-1 ($p = 0.003$), glucose ($p = 0.2$), haptoglobin ($p = 0.02$), week ($p = 0.11$), BCS at enrolment ($p = 0.13$), vitamin D 1 wk prepartum ($p = 0.02$), cervicitis ($p = 0.12$), and IR ratio $\geq 1.15$ ($p = 0.11$).

The final model for the natural log transform of neutrophil oxidative burst capacity is reported in Table 3.3. Oxidative burst was reduced in cows with greater haptoglobin concentrations through the study period or vitamin D measured at 1 wk before calving.

The neutrophil phagocytosis linear regression model was offered the following predictor variables: glucose ($p = 0.2$), haptoglobin ($p = 0.0001$), week ($p = 0.14$), endometritis ($p = 0.16$), and IR ratio $\geq 1.05$ ($p = 0.13$; Figure 3.2). Although overall BHBA was not significant in the univariable analysis, the cut point of 0.5 mmol/L at 5 wk after calving was evaluated ($p = 0.11$).
In addition to offering NEFA as a continuous variable to the phagocytosis model \((p = 0.18)\), the cut point of 0.5 mmol/L at 5 wk after calving was included in a separate model from its continuous counterpart \((p = 0.01; \text{Figure 3.3})\).

Table 3.4 summarizes the model of the natural log transformation of neutrophil phagocytosis function. Phagocytosis was decreased in cows with decreased circulating concentrations of haptoglobin, IR ratio \(\geq 1.05\), or those with NEFA \(\geq 0.5\) mmol/L at 5 wk after calving. Haptoglobin and IGF-1 were identified to have a weak correlation with phagocytosis function, with \(R^2\) values of 0.1 \((p < 0.0001)\) and 0.002 \((p = 0.5)\) respectively. Furthermore, haptoglobin and phagocytosis at 1 wk postpartum had an \(R^2\) value of 0.08 \((p = 0.05; \text{Appendix 3.27})\).

**DISCUSSION**

Both neutrophil phagocytosis and oxidative burst functions were influenced by factors commonly experienced in the PP by dairy cows. The results from this study support previous findings of high haptoglobin being associated with reproductive tract disease and diminished neutrophil oxidative burst function, increased risk of endometritis in cows with high BHBA 1 wk postpartum, and that endometritis and cervicitis are related but different diseases. The results also indicate that neutrophil phagocytosis is negatively impacted in cows that also have persistently high NEFA 5 wk after calving, and those identified with IR through a GTT 1 wk before calving. Diminished phagocytosis function was also associated with increased risk of cervicitis diagnosis at 5 wk postpartum. Additionally, high haptoglobin was negatively associated with neutrophil oxidative burst function. Based on previous literature, it was expected that both NEFA and BHBA, markers of IR and NEB, would have negative impacts on both neutrophil functions and reproductive disease. Additionally, other circulating chemicals known
to be associated with neutrophil function, such as glucose, insulin, and IGF-1, were anticipated to be predictive of either increased or diminished neutrophil function. While some of these relationships were recognized in this study, others were not apparent.

Non-esterified fatty acid concentration, a marker of NEB and IR, has been shown to have a negative impact on neutrophil oxidative burst capacity \textit{in vitro} (Scalia et al., 2006). While previous literature has demonstrated the usefulness of measuring NEFA in very late gestation or early lactation as a predictor of uterine disease (Kaneene et al., 1997; Dubuc et al., 2010b; Opsina et al., 2010; Huzzey et al., 2011), this study did not find high NEFA early in lactation, or prior to calving, to be associated with diminished oxidative burst or phagocytosis neutrophil functions. Hammon et al. (2006) found a negative association between pre-calving NEFA and oxidative burst capacity 1 wk prior to calving, however this relationship only elicited a Pearson correlation coefficient of 0.44, and in the current study a similar analysis produced an $R^2$ of 0.07. Concentrations of NEFA prior to calving may influence neutrophil function, however due to the brevity of cellular exposure to this metabolite at this time, sustained high concentrations of NEFA may have a greater impact.

Neutrophils are more resilient when exposed to high concentrations of NEFA as compared with peripheral blood mononuclear cells (Ster et al., 2012). Unactivated neutrophils are able to metabolize NEFA through oxidation and by storing it as triacylglycerol (Burns et al., 1976; Phillips et al., 1986), possibly allowing them to withstand greater or more sustained levels of NEFA without a reduction in functional capacity. Considering these neutrophil qualities, and that concentrations of NEFA only begin to increase in the final week of gestation, with diminished DMI and increasing mammary gland energy demands initiating lipolysis (Grummer et al., 2004), and that neutrophils remain in circulation for less than 12 h before migrating into
tissues (Tizard, 2009), it is not surprising that prepartum NEFA do not have an inhibitory effect on neutrophil function. It is plausible, however, that sustained high levels of NEFA may negatively impact neutrophil functions. In an in vitro study, neutrophils were incubated for 4 h in serial concentrations of NEFA ranging from 0.0625 to 2 mmol/L, and concentrations from 0.0625 to 0.5 mmol/L were associated with diminished phagocytosis-dependent oxidative burst (Scalia et al., 2006). Cows with NEFA ≥ 0.5 mmol/L at 5 wk postpartum had overall higher NEFA concentrations after calving and a 14% reduction in neutrophil phagocytosis capacity than those with NEFA < 0.5 at this time point. Exposure of neutrophils to sustained levels of NEFA, such as through development and circulation as would be experienced during the 6 wk of lipolysis commonly experienced in the PP, may overwhelm their ability to metabolize NEFA, possibly impacting other aspects of neutrophil function. More research is required to investigate how neutrophils respond to high concentrations of NEFA through the PP, not only in the weeks adjacent to calving, but further into lactation when cows are still in NEB.

Cows with IR, as identified by a GTT, had an 8% reduction in neutrophil phagocytic capacity, as compared to those without IR. Evaluated individually, markers of lipolysis resulting from IR or NEB, based on the results of this study, appear to have limited association with phagocytosis function. An overall measure of IR, however, may be a better indication of how phagocytosis is impacted in the peripartum cow. There have been limited studies investigating the relationships between in vivo NEFA, BHBA, and neutrophil phagocytosis simultaneously in the peripartum dairy cow, with most neutrophil function and metabolite research being conducted in vitro. Due to the complexity of the metabolic syndrome experienced by cows in the PP, it is possible that other factors not measured in the present study may be influencing the relationships between metabolites and neutrophil function. More research is required to
investigate how these markers of NEB interact with each other and other hormones characteristic of the peripartum dairy cow.

Hypovitaminosis D has been associated with increased risk of IR in humans (Borissova et al., 2003; Ford et al., 2005; Forouhi et al., 2008) and compromised neutrophil motility (Lorente et al., 1976). In dairy cows, vitamin D concentrations below 5 ng/mL indicate a deficiency (Horst et al., 1994). None of the cows in this study fell below this vitamin D threshold, therefore adverse effects of hypovitaminosis D on neutrophils, or associations of vitamin D with markers of IR would not be expected, and were not detected. There was a significant but small inverse relationship of serum vitamin D at week -1 with neutrophil oxidative burst activity. The cows included on this trial were only permitted outdoor access for 2 hours a day, and therefore their primary source of vitamin D was provided through feed supplementation. Cows that consume more feed will have a greater intake of vitamin D, reflected in higher circulating concentrations of vitamin D. The small positive relationship that was observed between neutrophil oxidative burst function and vitamin D, therefore, may be reflective of increased DMI. This use of vitamin D as an indicator of DMI cannot be verified however, as feed intake data was not collected. The RQUICKI index, a proposed method for evaluating IR did not prove to be associated with any other measures of IR or NEB in this study, other than eliciting an $R^2$ value of 0.44 with NEFA. Although the assay used to quantify insulin in this study produced different units than others due to a difference in the measure of insulin activity and therefore values cannot be compared directly to previous studies, others have identified that RQUICKI may not be a useful tool in dairy cows for measuring IR (Kerestes et al., 2009).

Increased haptoglobin levels are associated with diminished neutrophil oxidative burst capacity. This phenomenon has been well documented in human medicine. Haptoglobin, either
from peripheral circulation or released from granulocytes within neutrophils (Quaye, 2008) dampen the acute inflammatory response in part by inhibiting oxidative burst (Kim et al., 1995; Rossbacher et al., 1999) directly through receptor-ligand interaction (Oh et al., 1990). Through the cessation of the neutrophil oxidative burst function, haptoglobin assists in mitigating potential damage to surrounding tissues that may occur due to release of excessive reactive oxygen species (Quaye, 2008). Rossbacher et al. (1999) demonstrated that haptoglobin might also have an inhibitory influence on neutrophil phagocytosis in addition to its influence on oxidative burst. Haptoglobin had a strong direct inhibitory effect on neutrophil oxidative burst function, however evidence for its influence on phagocytosis is limited. In the present data, there was an inverse relationship of haptoglobin with neutrophil oxidative burst activity consistent with Rossbacher et al. (1999), but a positive relationship of similar magnitude of haptoglobin with neutrophil phagocytosis function. This result is contradictory to our hypotheses based on previous literature investigating the influence of haptoglobin on neutrophil functions. The apparent relationship between phagocytosis and haptoglobin concentrations in the model may be an artifact of a concurrent increase in phagocytosis capacity and haptoglobin concentrations 1 wk after calving. The increase in phagocytosis capacity increased during the week of calving, which has been described previously (Nagahata et al., 1988; Kehrli et al., 1989; Kim et al., 2005), coincides with the increase in haptoglobin associated with calving (Chan et al., 2010; Huzzey et al., 2011). When the association between week 1 haptoglobin and phagocytosis was assessed specifically, there was no association ($R^2 = 0.08$; Appendix 3.28). Therefore, although haptoglobin appears to be positively related to phagocytosis in the model ($R^2 = 0.11$; Appendix 3.29), this effect is weak and may not truly reflect the data.
High circulating concentrations of haptoglobin in the week after calving were associated with increased risk of cervicitis. Although haptoglobin was not a significant predictor in the endometritis model, in the univariable analysis cows with endometritis had higher circulating levels of haptoglobin after calving. Additionally, neutrophil oxidative burst capacity was reduced by 16% in cows diagnosed with endometritis relative to those without. A greater increase in peripartum concentration of haptoglobin has been identified as a predictor of metritis (Huzzey et al., 2007; Chan et al., 2010; Dubuc et al., 2010b; Galvão et al., 2010) or endometritis (Dubuc et al., 2010b; Galvão et al., 2010). A timely and successful neutrophil response to the uterus is critical in mitigating disease (Gilbert et al., 2007), and there is a substantial influx of neutrophils to the uterus shortly after calving (Hussain and Daniel, 1992; Mateus et al., 2002). Considering that haptoglobin directly inhibits neutrophil oxidative burst capacity (Oh et al., 1990; Kim et al., 1995; Rossbacher et al., 1999), and possibly phagocytosis (Rossbacher et al., 1999), animals that have higher concentrations of haptoglobin may have more reduced neutrophil function, and therefore be at greater risk of subsequent disease, such as endometritis or cervicitis.

Cows were less likely to be diagnosed with endometritis 5 wk after calving if they had a greater circulating concentration of IGF-1. Insulin-like growth factor-1 is important in the enhancement of innate immune cell function. Priming of neutrophils and monocytes with IGF-1 in other species has been demonstrated to enhance superoxide production (Fu et al., 1991; Balteskard et al., 1998). Additionally, exposure of human neutrophils to IGF-1 improved migration, phagocytosis, and oxidative burst capacity (Edwards et al., 1988; Bjerknes and Aarskog, 1995). As IGF-1 receptors have been identified on bovine monocytes (Zhao et al., 1993) and neutrophils (Nielsen et al., 2003), it was hypothesized that IGF-1 might be associated with neutrophil function, but in the present study, despite the association with endometritis, there
was no association detected between circulating IGF-1 and either oxidative burst or phagocytosis capacity of circulating neutrophils

A number of different factors were associated with being diagnosed with either endometritis or cervicitis at 5 wk after calving that did not appear to be directly related to immune function. Multiparous cows were more likely to be diagnosed with endometritis, which is consistent with current literature (Cheong et al., 2011). High BHBA concentrations 1 wk after calving were associated with diagnosis of endometritis. The timing and degree of increased BHBA and its association with endometritis has also been described previously (Reist et al., 2003; Dubuc et al., 2010b). Overconditioned cows were less likely to be diagnosed with cervicitis at 5 wk after calving, however the odds ratios for this relationship is quite low, and therefore not as important in the models as other factors such as high haptoglobin postpartum. High concentrations of BHBA or greater GTT ratios 1 wk prepartum were associated with reduced risk of being diagnosed with endometritis or cervicitis, respectively. Recent literature was used to help determine the cut points for prepartum BHBA (Chapinal et al., 2012) and GTT ratios (Matteo et al., 2009). Although these studies identified cut points for BHBA and GTT ratios 1 wk before calving to be useful in predicting disease, our data do not reflect these findings. In particular, for the GTT ratio, the cut point may be too high to be useful for predicting cervicitis, as 80% of animals were classified as IR by this definition. The cut point for 1 wk prepartum BHBA identified in the current study (≥ 0.4 mmol/L) was slightly lower than previously reported disease-predicting cut points at the same time point (0.6 mmol/L; Chapinal et al., 2012). In addition to fat metabolism, BHBA can also enter circulation byway of the rumen in the form of butyrate, a common volatile fatty acid (Holter et al., 1959). As the 0.4 mmol/L BHBA cut point 1 wk prepartum was substantially lower than those reported in cows with
subclinical ketosis (1.2 – 1.4 mmol/L; LeBlanc, 2010), it is more likely that DMI is a more important factor in circulating BHBA than fat metabolism at this time point.

Results from the present study demonstrated that some markers of NEB and immune function are associated with endometritis, cervicitis, and altered neutrophil function. High haptoglobin 1 wk postpartum was associated with decreased neutrophil oxidative burst function, and increased risk of endometritis and cervicitis. High NEFA 5 wk postpartum and IR, as evaluated by a GTT 1 wk prepartum, negatively impacted neutrophil phagocytosis, and decreased phagocytosis capacity was associated with increased risk of cervicitis. Endometritis was more likely in multiparous animals, those with high BHBA 1 wk postpartum, low IGF-1 concentrations, or that also had cervicitis. Haptoglobin and some markers of NEB appear to be important in both inhibited neutrophil function and development of uterine disease. Further investigation is required to further understand the role of the peripartum IR and NEB metabolic phenomenon, as a whole, on neutrophil function and subsequent uterine disease.
Table 3.1 Final logistic regression model for endometritis diagnosed at week 5 in 81 Holstein dairy cows. Endometritis was diagnosed with uterine cytology samples, and was defined as samples that contained ≥ 6% neutrophils in the total cell population.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Class</th>
<th>% Cows¹</th>
<th>Coefficient</th>
<th>SE</th>
<th>Odds Ratio</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td></td>
<td>0.23</td>
<td>0.81</td>
<td></td>
<td>0.78</td>
<td>0.05</td>
<td>0.078</td>
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<tr>
<td>Insulin-like growth factor ¹</td>
<td>Per 1 ng/mL increase</td>
<td>-0.01</td>
<td>0.005</td>
<td>0.99</td>
<td>0.98 – 1.0</td>
<td>0.05</td>
<td>0.007</td>
</tr>
<tr>
<td>Neutrophil oxidative burst</td>
<td>Per 1 unit increase</td>
<td>-0.01</td>
<td>0.008</td>
<td>0.99</td>
<td>0.97 – 1.0</td>
<td>0.08</td>
<td>0.003</td>
</tr>
<tr>
<td>Neutrophil phagocytosis</td>
<td>Per 1 unit increase</td>
<td>0.05</td>
<td>0.02</td>
<td>1.1</td>
<td>1.0 – 1.1</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Parity</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>≥ 2</td>
<td>Referent</td>
<td>0.86</td>
<td>0.42</td>
<td>2.4</td>
<td>1.0 – 5.5</td>
<td>0.04</td>
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<td>Cervicitis</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>No</td>
<td>Referent</td>
<td>73</td>
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<td></td>
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<tr>
<td>Yes</td>
<td>Referent</td>
<td>27</td>
<td>2.06</td>
<td>7.8</td>
<td>3.5 – 17.7</td>
<td>&lt; 0.0001</td>
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<tr>
<td>Week -1 BHBA ²</td>
<td>Referent</td>
<td>62.2</td>
<td></td>
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<td></td>
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<tr>
<td>≥ 0.4</td>
<td>Referent</td>
<td>37.8</td>
<td>-0.97</td>
<td>0.4</td>
<td>0.2 – 0.8</td>
<td>0.02</td>
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<tr>
<td>Week 1 BHBA ²</td>
<td>Referent</td>
<td>90.0</td>
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<td></td>
<td></td>
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<tr>
<td>≥ 1.2</td>
<td>Referent</td>
<td>10.0</td>
<td>1.79</td>
<td>6.0</td>
<td>2.1 – 17.4</td>
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<tr>
<td>Week 4 BHBA ²</td>
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<td>63.6</td>
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<tr>
<td>≥ 1.1</td>
<td>Referent</td>
<td>36.4</td>
<td>-1.60</td>
<td>0.2</td>
<td>0.09 – 0.4</td>
<td>&lt; 0.0001</td>
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</table>

¹ % cows within predictor
² 95% confidence interval
³ Measured weekly from week -3 to week + 5 relative to calving
⁴ Measured weeks 2 and 1 before calving and 1, 2, 3, and 5 after
⁵ β-hydroxybutyric acid
Table 3.2 Final logistic regression model for cervicitis diagnosed at week 5 in 81 Holstein dairy cows. Cervicitis was diagnosed with cervical cytology samples, and was defined as samples that contained ≥ 6% neutrophils in the total cell population.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Class</th>
<th>% Cows</th>
<th>Coefficient</th>
<th>SE</th>
<th>Odds Ratio</th>
<th>95% CI</th>
<th>P</th>
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</thead>
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<td>1.49</td>
<td>0.002</td>
<td></td>
<td></td>
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<tr>
<td>Insulin$^3$</td>
<td>Per 1 ng/mL increase</td>
<td>34</td>
<td>0.04</td>
<td>0.02</td>
<td>1.0</td>
<td>1.0 – 1.1</td>
<td>0.04</td>
</tr>
<tr>
<td>Glucose$^3$</td>
<td>Per 1 mmol/L increase</td>
<td>36</td>
<td>0.49</td>
<td>0.28</td>
<td>1.6</td>
<td>0.9 – 2.9</td>
<td>0.08</td>
</tr>
<tr>
<td>Haptoglobin$^3$</td>
<td>Per 1 g/L increase</td>
<td>34</td>
<td>1.63</td>
<td>0.63</td>
<td>5.1</td>
<td>1.5 – 17.6</td>
<td>0.01</td>
</tr>
<tr>
<td>Neutrophil phagocytosis$^4$</td>
<td>Per 1 unit increase</td>
<td>34</td>
<td>-0.07</td>
<td>0.03</td>
<td>0.93</td>
<td>0.87 – 0.99</td>
<td>0.02</td>
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<td>Endometritis</td>
<td></td>
<td>66</td>
<td>Referent</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Yes</td>
<td>34</td>
<td>1.63</td>
<td>0.32</td>
<td>5.1</td>
<td>2.7 – 9.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>≤ 3.5</td>
<td>64</td>
<td>Referent</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥ 3.75</td>
<td>36</td>
<td>-1.70</td>
<td>0.37</td>
<td>0.2</td>
<td>0.09 – 0.4</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

1 % cows within predictor
2 95% confidence intervals
3 Measured weekly from week -3 to week +5 relative to calving
4 Measured weeks 2 and 1 before calving and 1, 2, 3, and 5 after
5 Body condition score
Table 3.3 Final model for the natural logarithm transformation of neutrophil oxidative burst function, accounting for repeated measurements at weeks -2, -1, 1, 2, 3 and 5 relative to calving in 70\textsuperscript{1} cows (11 cows were excluded if more than 3 samples were missing due to laboratory equipment malfunction). Oxidative burst was measured as the percentage of cells successfully activated by phorbol myristate acetate, as evaluated using flow cytometry.

<table>
<thead>
<tr>
<th>Predictors</th>
<th>Class</th>
<th>Coefficient</th>
<th>SE</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td></td>
<td>1.75</td>
<td>0.42</td>
<td>0.0001</td>
</tr>
<tr>
<td>Haptoglobin\textsuperscript{1}</td>
<td>per 1 g/L increase</td>
<td>-0.68</td>
<td>0.34</td>
<td>0.05</td>
</tr>
<tr>
<td>25-hydroxyvitamin D\textsuperscript{2}</td>
<td>per 1 ng/mL increase</td>
<td>-0.01</td>
<td>0.005</td>
<td>0.03</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Measured weekly from 3 wk before to 5 wk after calving  
\textsuperscript{2}Measured 1 week prior to calving
Table 3.4 Final model for the natural logarithm transformation of neutrophil phagocytosis function, accounting for repeated measurements at weeks -2, -1, 1, 2, 3 and 5 relative to calving in 51 cows (30 cows were excluded if more than 3 samples were missing due to laboratory equipment malfunction). Phagocytosis was measured as the percentage of neutrophils that successfully phagocytosed at least one fluorescent bead, as evaluated using flow cytometry.

<table>
<thead>
<tr>
<th>Predictors</th>
<th>Class</th>
<th>% Cows$^1$</th>
<th>Coefficient</th>
<th>SE</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td></td>
<td>-2.40</td>
<td>0.09</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>Haptoglobin$^2$</td>
<td>per 1 g/L increase</td>
<td></td>
<td>0.79</td>
<td>0.18</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>IR$^3$ Ratio</td>
<td>≤ 1.05</td>
<td>67.7</td>
<td>Referent</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥ 1.05</td>
<td>32.3</td>
<td>-0.21</td>
<td>0.10</td>
<td>0.05</td>
</tr>
<tr>
<td>Week 5 NEFA$^4$</td>
<td>≤ 0.5</td>
<td>80.4</td>
<td>Referent</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥ 0.5</td>
<td>19.6</td>
<td>-0.36</td>
<td>0.11</td>
<td>0.003</td>
</tr>
</tbody>
</table>

$^1$ % cows within predictor

$^2$ Measured weekly from 3 wk before to 5 wk after calving

$^3$ Insulin resistance ratio calculated from glucose tolerance tests as the circulating glucose concentration 80 minutes after intravenous dextrose infusion relative to the concentration just prior to infusion

$^4$ Non-esterified fatty acid
Figure 3.1 Mean (± SE) serum concentrations of non-esterified fatty acids (NEFA) for 81 Holstein dairy cows, dichotomized by NEFA ≥ 0.5 at 5 wk postpartum (p < 0.0001), with 27% (n = 22) of animals above the threshold.
Figure 3.2 Mean (± SE) weekly phagocytosis activity for 56 Holstein dairy cows, stratified by a glucose tolerance test (GTT) ratio of 1.05, administered at week -1. Cows with a GTT ratio ≥ 1.05 (n = 33) may be considered insulin resistant. Phagocytosis was measured as the percent of neutrophils that successfully consumed a fluorescent bead, as evaluated using flow cytometry.
Figure 3.3 Mean (± SE) phagocytosis activity for 56 Holstein dairy cows, stratified by week 5 serum non-esterified fatty acids (NEFA) concentrations of 0.5 mmol/L, with 23% of the cows (n = 13) being above the threshold. Phagocytosis was measured as the percent of neutrophils that successfully consumed a fluorescent bead, as evaluated using flow cytometry.
CHAPTER FOUR

GENERAL CONCLUSIONS

Conclusions

The overall goal of the experiments reported in this thesis was to contribute to the understanding of how markers of insulin resistance (IR) and negative energy balance (NEB) in the peripartum period (PP) influence neutrophil function and reproductive disease in peripartum dairy cows. In particular, neutrophil oxidative burst and phagocytosis functions were evaluated in relation to endometritis and cervicitis.

The first objective of this project was to validate a hand-held glucose meter for cow-side use, ultimately for assessment of IR. The glucometer used (Precision Xtra, Abbott Diabetes Care) was identified as a reliable alternative for measuring whole blood glucose concentrations in peripartum dairy cows, as compared with measurements by an auto-chemistry analyzer in a laboratory. Simplified glucose tolerance tests (GTT) were conducted, and the ratio of glucose concentrations 80 min after a bolus IV infusion of dextrose relative to just prior to infusion, was used to identify cows at risk of IR, based on previous research (Matteo et al., 2009). Linear regressions between GTT as a continuous measurement against various different markers of lipolysis (i.e. NEFA, BHBA, and glucose concentrations) did not reveal any significant relationships. Using the GTT ratio cut point of 1.05, based on glucose concentrations measured in the laboratory as the gold standard, the performance of the glucometer to identify cows with IR was evaluated. It was determined that a GTT ratio cut point of 1.15 for the glucometer should be used to reflect the 1.05 GTT ratio for laboratory values. When the GTT ratio was used as a predictor of reproductive disease or neutrophil function, the 1.05 ratio was found to be associated with an 8% decrease in neutrophil phagocytosis capacity relative to those below the cut point.
Despite the performance of Precision Xtra in rapidly and accurately determining glucose concentrations, the merit of the 1.05 gold standard GTT ratio in identifying animals at risk of developing postpartum disease remains unclear, and evaluation against a traditional GTT in a more extensive field trial would be beneficial in exploring the ratios predictive capacity.

The second objective was to characterize the associations of peripartum IR and markers of NEB with neutrophil oxidative burst and phagocytosis function, and disease of the reproductive tract. Endometritis and cervicitis were associated with several metabolic markers of IR and NEB, indicating a previously supported link between immune function and uterine disease in peripartum cows (Hammon et al., 2006; Galvão et al., 2010). Neutrophil phagocytosis capacity was reduced by 14% in cows with non-esterified fatty acid (NEFA) ≥ 0.5 mmol/L at wk 5 postpartum relative to those with NEFA < 0.5 mmol/L (Figure 3.3). It was revealed that these cows, with high NEFA at 5 wk after calving had greater concentrations of NEFA throughout the postpartum period as compared with animals that had returned to normal levels by this time point (Figure 3.1). Cows with IR, as evaluated by a GTT ratio of 1.05 at 1 wk before calving, had an 8% reduction in overall neutrophil phagocytosis relative to cows that did not have IR (Figure 3.2). Although the GTT in this study was limited in its ability predict disease outcomes, it was useful in identifying animals with reduced neutrophil phagocytosis capacity. Additionally, reduced phagocytosis was associated with the development of cervicitis, and reduced oxidative burst with development of endometritis. These findings further support a possible link between peripartum metabolic imbalance and development of uterine disease through the diminished function of neutrophils.

Haptoglobin, an indicator of inflammation, in the early PP was associated with development of reproductive disease and diminished neutrophil function. Cows with high
circulating concentrations of haptoglobin had greater odds of developing endometritis or cervicitis. High haptoglobin was also indicative of inhibited neutrophil oxidative burst capacity. In addition to the metabolic abatement of neutrophil function that contributes to development of reproductive diseases, inflammation of the uterus and cervix, as reflected by high levels of haptoglobin, also appears to be an important factor in the incidence of uterine disease in peripartum cows.

Overall, the results of this project, in part support the theory that IR in the PP and its associated metabolites can impact neutrophil function, thereby contributing to an increase in the odds of developing reproductive disease. Although this study did not support the involvement of all makers of lipolysis in both diminished neutrophil oxidative burst and phagocytosis function, as in previous literature, some associations were identified including between high concentrations of NEFA at week 5 postpartum and decreased phagocytosis performance. Factors involved in fat metabolism, including BHBA, insulin, and glucose were associated with development of reproductive disease, suggesting that these compounds may influence other components of immune function, resulting in increased susceptibility to disease. As some of the risk factors for developing endometritis and cervicitis were different than those that influenced neutrophil oxidative burst and phagocytosis function, other components are likely involved between IR and development of reproductive disease.

**Future Research**

Despite the contributions of this study to the understanding of how IR in the PP influences neutrophil function and subsequent development of reproductive tract disease, many questions remain unanswered.
Firstly, the use of a GTT ratio for determination of IR and as a predictor of disease requires further investigation. The actual proportion of cows that are IR during the PP is not known, so it is unclear how to use information gleaned from GTT to evaluate neutrophil function or identify animals at increased risk of developing disease. Using the ratio cut point of 1.05 described by Matteo et al. (2009) for the simplified GTT, 55% of peripartum animals were identified as IR. Evaluation of IR could potentially be improved through a number of different methods. A higher cut point for the simplified GTT could help identify animals that are in a greater depth of IR and may be at greater risk of developing disease postpartum. Additionally, adding additional information concerning the insulin response to glucose challenge, such as measuring circulating concentrations of insulin during the GTT, may be useful. The creation of a meaningful cut point would require validation against more traditional measures of IR, such as an area under the curve analysis of a full GTT. A larger scale study that evaluates the cut points’ predictive ability against various metabolically-associated transition diseases would also be helpful in determining its usefulness as a research or clinical tool.

While there have been numerous in vitro studies evaluating the influence of different concentrations of NEFA on neutrophil functionality, few consider phagocytosis capacity or length of exposure time. Results from this study indicate that NEFA concentrations of 0.5 mmol/L or greater have a negative impact on neutrophil phagocytosis capacity. To further explore this relationship, it would be useful to investigate how exposure time of neutrophils to levels of NEFA commonly experienced by dairy cows in the PP affect phagocytosis performance.

Although this study supports that markers of IR and NEB, such as NEFA and measuring IR through a GTT, can negatively impact different aspects of neutrophil function, and that the
same markers are associated with increased odds of developing reproductive tract disease, the results also suggest that there are other factors besides the neutrophil involved in the relationship between IR and uterine disease. Metabolically associated factors, such as BHBA at 1 wk postpartum, glucose, and insulin, were important in the development of endometritis and cervicitis that were not significant in diminished neutrophil oxidative burst and phagocytosis function. This difference in predictors indicates that other factors may be involved. It is possible that the combined effects of IR may have either synergistic or antagonistic consequences that are not obvious when metabolites are considered individually. Diminished function of other immune cells may also be involved. Numerous in vitro studies have investigated the impact of different markers of NEB on neutrophil functions individually, but more research is required to elucidate the full impact of IR on immune function in vivo. Due to the inhibitory effect on neutrophil oxidative burst capacity by haptoglobin, evaluation of the influence of IR on other immune components important at this time, such as monocytes, may also provide further insight into how PP metabolism contributes to reproductive disease.
REFERENCES


Canadian Council on Animal Care. 2009. CCAC guidelines on: The care and use of farm animals in research, teaching and testing. Canadian Council on Animal Care, Ottawa, ON, Canada.


**APPENDICES**

**Appendix 3.1** Outline of the data that were collected through the duration of animal enrollment on the study.

<table>
<thead>
<tr>
<th>Collected Data</th>
<th>Week Relative to Calving</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-3</td>
</tr>
<tr>
<td>Glucose</td>
<td>✓</td>
</tr>
<tr>
<td>β-hydroxybutyric acid</td>
<td>✓</td>
</tr>
<tr>
<td>Non-Esterified Fatty Acids</td>
<td>✓</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>✓</td>
</tr>
<tr>
<td>Insulin</td>
<td>✓</td>
</tr>
<tr>
<td>Insulin-Like Growth Factor –1</td>
<td>✓</td>
</tr>
<tr>
<td>Glucose Tolerance Test</td>
<td>✓</td>
</tr>
<tr>
<td>25-Hydroxyvitamin D</td>
<td>✓</td>
</tr>
<tr>
<td>Body Condition Score</td>
<td>✓</td>
</tr>
<tr>
<td>Uterine and Cervical Cytology</td>
<td>✓</td>
</tr>
<tr>
<td>Vaginoscopy</td>
<td>✓</td>
</tr>
<tr>
<td>Neutrophil Oxidative Burst and Phagocytosis Assays</td>
<td>✓</td>
</tr>
</tbody>
</table>
Appendix 3.2 A representative example of flow cytometry output with a rectangular gate around the neutrophil population (n = 5000 cells). The cells within the gate represent 95% of the events measured by the flow cytometer.
Appendix 3.3 A representative example of flow cytometry output for the neutrophil oxidative burst function assay (n = 5000 cells). The black histogram represents the fluorescence of unactivated neutrophils, the negative controls, and the grey histogram represents the cells activated with phorbol myristate acetate. The x-axis is the log fluorescence of the cells.
Appendix 3.4 A representative example of flow cytometry output for the neutrophil phagocytosis function assay (n = 20,000 cells). The histogram represents the cells that phagocytosed ≥ 1 1-μm fluorescent bead (TransFluo-Spheres® Fluorescent Microspheres, Molecular Probes). The x-axis is the log fluorescence of the cells.
Appendix 3.5 Summary of observed diseases in 81 Holstein dairy cows were followed from 3 weeks prior to calving until 5 weeks after calving. Endometritis and cervicitis were diagnosed with cytology in all cows at week 5 postpartum as ≥ 6% neutrophils in cell population. Purulent vaginal discharge at week 3 and 5 was muco-purulent or purulent discharge based on vaginoscopy. Retained placenta, metritis, ketosis, left displaced abomasum, hypocalcaemia, and mastitis were diagnosed based on clinical signs by the herd veterinarian.

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>% of cows</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endometritis</td>
<td>27</td>
<td>34</td>
</tr>
<tr>
<td>Cervicitis</td>
<td>22</td>
<td>27</td>
</tr>
<tr>
<td>Retained placenta</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Metritis</td>
<td>3</td>
<td>3.7</td>
</tr>
<tr>
<td>Ketosis</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Left displaced abomasum</td>
<td>5</td>
<td>6.2</td>
</tr>
<tr>
<td>Hypocalcaemia</td>
<td>5</td>
<td>6.2</td>
</tr>
<tr>
<td>Mastitis</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>Purulent vaginal discharge at 3 wk after calving</td>
<td>41</td>
<td>51</td>
</tr>
<tr>
<td>Purulent vaginal discharge at 5 wk after calving</td>
<td>6</td>
<td>7.4</td>
</tr>
</tbody>
</table>
Appendix 3.6 Mean (± SE) concentrations of serum β-hydroxybutyric acid (BHBA) for 81 cows sampled weekly. For weeks 1, 2, 3, 4, and 5 after calving, the percentage of animals with BHBA $\geq 1.2$ was 10, 22, 26, 34, and 29%, respectively. Values at weeks -1, 4, and 5 relative to calving were measured from serum by the Animal Health Laboratory at the University of Guelph (Guelph, ON, Canada) using a commercial kit and an auto-chemistry analyzer (Roche Hitachi 912, Roche, Basel Switzerland). Values at weeks 1, 2, and 3 relative to calving were measured using a hand-held ketone monitor (Precision Xtra, Abbott Diabetes Care, Mississauga, ON, Canada).
Appendix 3.7 Mean (± SE) concentrations of serum non-esterified fatty acids (NEFA) for 81 Holstein dairy cows sampled weekly.
Appendix 3.8 Mean (± SE) concentrations of serum insulin for 81 Holstein dairy cows sampled weekly.
Appendix 3.9 Mean (± SE) concentrations of serum insulin-like growth factor 1 (IGF-1) for 81 Holstein dairy cows sampled weekly.
Appendix 3.10 Mean (± SE) concentrations of serum haptoglobin for 81 Holstein dairy cows sampled weekly.
Appendix 3.11 Mean (± SE) concentrations of serum glucose for 81 Holstein dairy cows sampled weekly.
Appendix 3.12 Mean (± SE) oxidative burst activity for 70 Holstein dairy cows sampled weekly. Oxidative burst was measured as the percentage of cells activated by phorbol myristate acetate, as evaluated using flow cytometry.
Appendix 3.13 Mean (± SE) phagocytosis for 56 Holstein dairy cows. Phagocytosis was measured as the percent of neutrophils that phagocytosed ≥ 1 fluorescent bead(s), as evaluated using flow cytometry.
Appendix 3.14 Overview of the different blood parameters as measured in 81 Holstein dairy cows. Glucose, insulin-like growth factor-1 (IGF-1), haptoglobin, and non-esterified fatty acids (NEFA) were measured from serum each week from 3 wk prepartum to 5 wk postpartum. 25-hydroxyvitamin D was measured once 1 wk before calving from serum. β-hydroxybutyric acid (BHBA) was measured at weeks -1, 4, and 5 relative to calving from serum by an auto-chemistry analyzer, and at weeks 1, 2, and 3 from whole blood by a hand-held meter.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Minimum</th>
<th>Median</th>
<th>Maximum</th>
<th>Standard Deviation</th>
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</thead>
<tbody>
<tr>
<td>Glucose (mmol/L)</td>
<td>1.1</td>
<td>3.2</td>
<td>6.9</td>
<td>0.6</td>
</tr>
<tr>
<td>IGF-1 (ng/mL)</td>
<td>14.0</td>
<td>77.2</td>
<td>234.6</td>
<td>38.7</td>
</tr>
<tr>
<td>Vitamin D (nmol/L)</td>
<td>53.0</td>
<td>83.5</td>
<td>123.0</td>
<td>15.8</td>
</tr>
<tr>
<td>Haptoglobin (g/L)</td>
<td>0.01</td>
<td>0.1</td>
<td>1.7</td>
<td>0.2</td>
</tr>
<tr>
<td>NEFA (mmol/L)</td>
<td>0</td>
<td>0.3</td>
<td>2.4</td>
<td>0.3</td>
</tr>
<tr>
<td>BHBA (mmol/L)</td>
<td>0.2</td>
<td>0.7</td>
<td>4.5</td>
<td>0.7</td>
</tr>
</tbody>
</table>
Appendix 3.15 Relationship ($R^2 = 0.005; p = 0.17$) of serum non-esterified fatty acid (NEFA) with the percentage of neutrophils that were successfully activated by phorbol myristate acetate, for 70 Holstein dairy cows. The black line represents the linear regression relationship, with 95% confidence intervals (grey lines).
Appendix 3.16 Mean (± SE) serum concentrations of insulin for 81 Holstein dairy cows sampled weekly, stratified by endometritis status. Endometritis (n = 27) was diagnosed with cytology at 5 wk postpartum as ≥ 6% neutrophils in cell population.
Appendix 3.17 Mean (± SE) serum concentrations of insulin-like growth factor-1 (IGF-1) for 81 Holstein dairy cows sampled weekly, stratified by endometritis status. Endometritis (n = 27) was diagnosed with cytology at 5 wk postpartum as ≥ 6% neutrophils in cell population.
Appendix 3.18 Mean (± SE) weekly phagocytosis activity for 56 Holstein dairy cows sampled weekly, stratified by endometritis status. Endometritis was diagnosed with cytology in all cows at week 5 postpartum as ≥ 6% neutrophils in the cell population. The proportion of animals with endometritis was 32%. Phagocytosis was measured as the percent of neutrophils that successfully phagocytosed ≥ 1 fluorescent bead(s), as evaluated using flow cytometry.
Appendix 3.19 Mean (± SE) serum concentrations of haptoglobin for 81 Holstein dairy cows sampled weekly, stratified by endometritis status. Endometritis (n = 27) was diagnosed with cytology at 5 wk postpartum as ≥ 6% neutrophils in cell population.
Appendix 3.20 Mean (± SE) oxidative burst activity for 70 Holstein dairy cows sampled weekly, stratified by endometritis status. Oxidative burst was measured as the percentage of cells activated by phorbol myristate acetate, as evaluated using flow cytometry. Endometritis (n = 24) was diagnosed with cytology at 5 wk postpartum as ≥ 6% neutrophils in cell population.
Appendix 3.21 Mean (± SE) serum concentrations of insulin for 81 Holstein dairy cows sampled weekly, stratified by cervicitis status. Cervicitis (n = 22) was diagnosed with cytology at 5 wk postpartum as ≥ 6% neutrophils in cell population.
Appendix 3.22 Mean (± SE) serum concentrations of insulin-like growth factor-1 for 81 Holstein dairy cows sampled weekly, stratified by cervicitis status. Cervicitis (n = 22) was diagnosed with cytology at 5 wk postpartum as ≥ 6% neutrophils in cell population.
Appendix 3.23 Mean (± SE) serum concentrations of glucose for 81 Holstein dairy cows sampled weekly, stratified by cervicitis status. Cervicitis (n = 22) was diagnosed with cytology at 5 wk postpartum as ≥ 6% neutrophils in cell population.
Appendix 3.24 Mean (± SE) serum concentrations of haptoglobin for 81 Holstein dairy cows sampled weekly, stratified by cervicitis status. Cervicitis (n = 22) was diagnosed with cytology at 5 wk postpartum as ≥ 6% neutrophils in cell population.
Appendix 3.25 Mean (± SE) oxidative burst activity for 70 Holstein dairy cows sampled weekly, stratified by cervicitis status. Oxidative burst was measured as the percentage of cells activated by phorbol myristate acetate, as evaluated using flow cytometry. Cervicitis (n = 20) was diagnosed with cytology at 5 wk postpartum as ≥ 6% neutrophils in cell population.
Appendix 3.26 Mean (± SE) weekly phagocytosis activity for 56 Holstein dairy cows, stratified by cervicitis status. Cervicitis was diagnosed with cytology in all cows at week 5 postpartum as ≥ 6% neutrophils in the cell population. The proportion of animals with cervicitis was 23%. Phagocytosis was measured as the percent of neutrophils that successfully consumed ≥ 1 fluorescent bead(s), as evaluated using flow cytometry.
Appendix 3.27 Relationship (R² = 0.08; p = 0.05) of serum haptoglobin concentrations with the percentage of neutrophils that successfully phagocytosed ≥ 1 fluorescent bead(s), in week 1 after parturition, for 49 Holstein dairy cows. The black line represents the linear regression relationship, with 95% confidence intervals (grey lines).
Appendix 3.28 Relationship (R² = 0.11; p < 0.0001) of serum haptoglobin concentrations with the percentage of neutrophils that successfully phagocytosed ≥ 1 fluorescent bead(s), for 56 Holstein dairy cows. The black line represents the linear regression relationship, with 95% confidence intervals (grey lines).
Appendix 3.29 Relationship (R² = 0.002; p = 0.5) of serum insulin-like growth factor-1 (IGF-1) with the percentage of neutrophils that successfully phagocytosed ≥ 1 fluorescent bead(s), for 56 Holstein dairy cows. The black line represents the linear regression relationship, with 95% confidence intervals (grey lines).
Appendix 3.30 Relationship ($R^2 = 0.03; p = 0.001$) of serum haptoglobin concentrations with the percentage of neutrophils that were successfully activated by phorbol myristate acetate, for 70 Holstein dairy cows. The black line represents the linear regression relationship, with 95% confidence intervals (grey lines).
Appendix 3.31 Relationship (R2 = 0.01; p = 0.02) of serum insulin-like growth factor-1 (IGF-1) with the percentage of neutrophils that were successfully activated by phorbol myristate acetate, for 70 Holstein dairy cows. The black line represents the linear regression relationship, with 95% confidence intervals (grey lines).
Appendix 3.32 Box and whisker plots of 25-hydroxyvitamin D. IR ratio is the insulin resistance ratio calculated from glucose tolerance tests at 1 wk prior to calving, as the circulating glucose concentration 80 minutes after intravenous dextrose infusion relative to the concentration just prior to infusion. Endometritis and cervicitis was diagnosed with cytology samples from the uterine body and cervix respectively, and was defined as samples that contained ≥ 6% neutrophils in the total cell population.