Association of post-parturient endometritis with serum metabolites, cortisol and neutrophil function over an extended sampling period in dairy cows

by

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Dedication

This dissertation is dedicated to my friends and family who provided constant support and encouragement, and the running community of Saskatoon who led me on all sorts of memorable urban adventures. Finally, to Arlo, and to new beginnings.

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List of Abbreviations

APC	Allophycocyanin
ATP	Adenosine triphosphate
BCS	Body condition score
BHB	β-hydroxybutyrate
CBC	Complete blood count
CD	Cluster of differentiation
DAMP	Damage associated molecular patterns
DHR-123	Dihydrorhodamine-123
DIM	Days in milk
DMI	Dry matter intake
FITC	Fluorescein isothiocyanate
hL	Hectoliter
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor-1
IL-10	Interleukin-10
MFI	Median fluorescence intensity
NADPH	Nicotinamide-adenine dinucleotide phosphate
NEFA	Non-esterified fatty acids
NET	Neutrophil extracellular trap
NK	Natural killer
PAMP	Pathogen associated molecular patterns
PMA	Phorbol 12-myristate 13-acetate
ROS	Reactive oxygen species
SD	Standard deviation

TcR	T-cell receptor
TGF-β	Transforming growth factor-β
TNF-α	Tumor necrosis factor-α
VWP	Voluntary wait period

Abstract

Background: In dairy cows, the development of endometritis following parturition can adversely affect reproduction, leading to subfertility. The metabolic profile, serum cortisol and peripheral blood neutrophil phagocytic ability of cows that develop endometritis have yet to be fully characterized over an extended period.

Objectives: To determine the temporal changes in serum metabolites, serum cortisol and neutrophil phagocytic ability during the extended pre- and post-partum period and relate this to the development of endometritis in dairy cows.

Methods: Blood samples were collected from 40 dairy cows (Canadian Holstein) five weeks before the expected calving date and then repeated biweekly until seven weeks postpartum (eight samples/animal). Serum glucose, calcium, beta-hydroxybutyrate (BHB), non-esterified fatty acids (NEFA), urea and cortisol concentrations were measured. Peripheral blood neutrophil phagocytic ability was evaluated using flow cytometry. Cows with endometritis (> 18 % neutrophils/100 cells) were identified at five weeks postpartum using a cytological evaluation of uterine cytosmears collected using the modified cytobrush technique.

Results: Twelve out of 40 cows (30 %) were diagnosed with endometritis around five weeks postpartum. Compared to non-endometritic cows, endometritic cows had higher glucose concentrations at parturition ($P < 0.01$) and seven weeks postpartum ($P = 0.02$), higher calcium concentrations one week prior to parturition ($P = 0.02$) and at parturition ($P = 0.02$), and higher BHB concentrations one week following parturition ($P = 0.03$). Furthermore, endometritic cows had lower peripheral neutrophil counts ($P < 0.01$) with a lower neutrophil phagocytic ability ($P = 0.05$) at one week postpartum than their non-endometritic counterparts. The NEFA, urea, or cortisol concentrations did not differ between the two groups over the sampling period.

Conclusions: Based on the current data, the development of endometritis was associated with higher serum concentrations of total calcium one week prior to parturition, higher serum glucose and total calcium at parturition, and higher BHB concentrations, lower peripheral blood neutrophil

count and lower phagocytic ability one week following parturition. Monitoring of these key metabolites may aid in the early identification of at-risk cows.

1 General Introduction and Literature Review

1.1 Background

The dairy industry is one of the most important agricultural sectors in Canada, with 1.4 million dairy cows and heifers found on 10,095 dairy farms nationwide.[1] Each year, these farms produce 93.4 million hL of milk and contribute approximately CA$19.9 billion to Canada's gross domestic product.[1] To meet the ever-increasing demand for dairy products both domestically and internationally, cows have been selected for high milk production and can produce on average 10,702 kg of milk for each lactation (305 days).[1] However, achieving these production targets requires careful management of the health and nutrition of every cow.

The dairy production cycle relies heavily on a cow's ability to become pregnant, maintain the pregnancy and calve on time. Canadian dairy producers target a 13 - 14-month interval between two successive calvings (termed the inter-calving interval) to sustain their herds' average milk production and meet their allotted milk quota. The inter-calving interval includes the lactation period and the duration when the cows are not being milked (dry period, 45-60 days). The duration between the last three weeks of the dry period (close-up dry period) and three weeks of subsequent lactation, often referred to as the transition period, has been identified as the most challenging time for dairy cows. This periparturient period includes demanding physiological events, including the rapid phase of fetal growth, calving, initiation of the next lactation and finally uterine involution. As a result, dairy cows undergo a complex shift in their nutritional, metabolic and immunologic demands during this time. Consequently, transition dairy cows must be carefully managed as they are more susceptible to metabolic disorders, mastitis and uterine diseases, particularly those with high milk production.[2]

The most common reasons for involuntary culling on dairy farms in North America are reproductive failure (16.8 % of all cows culled), mastitis (10.6 % of all cows culled) and lameness (6.88 % of all cows culled).[3] Uterine disease is the leading cause of reproductive failure as it increases the number of artificial inseminations required per pregnancy, delays the onset of ovarian cyclicity and reduces the overall pregnancy rate.[3] Of all uterine diseases, endometritis

(inflammation limited to the endometrial lining) is the most prevalent and affects between 15 to 53 % of all dairy cows in North America.[3] Therefore, strategies to promote endometrial health are of utmost importance to dairy producers.

Following calving, cows are traditionally allowed a voluntary wait period (VWP) lasting between 45 and 60 days before their first breeding. During the VWP, cows not only experience increasing metabolic stress associated with peak lactation four to seven weeks following calving but also need to resume ovarian cyclicity and complete uterine involution to be ready for their next pregnancy.[4] Within days of parturition, the high concentrations of circulating steroid hormones present during pregnancy will return to basal values, followed by an increase in plasma follicle-stimulating hormone concentration around seven days postpartum. This will prompt the development of ovarian follicles, with subsequent waves of follicular development every seven to ten days. In postpartum cows, the ovulation of a dominant follicle would indicate a return to ovarian cyclicity.[5] Following calving, the bovine genital tract, in particular the uterus, responds to the presence of cellular debris and microbes by initiating an active inflammatory response characterized by an influx of immune cells (mainly neutrophils) into the uterine mucosa and uterine lumen.[6] During the first two weeks postpartum, areas of the uterine epithelium that take part in placentation (caruncles) slough off completely, thus making cows more susceptible to infection during this period. Coincident with the waning of uterine inflammation, re-epithelization of caruncular regions of the uterus is completed by the 5th week postpartum, thereby reinstating the epithelial barrier. However, the completion of uterine involution does not occur for another 7-10 days.[6,7]

As mentioned above, uterine involution is a complex inflammatory process that usually takes 35-45 days to complete in cattle and allows the uterus to return to its pre-gravid state.[7] However, perturbation of this repair mechanism through metabolic or uterine disease can disrupt the normal reparative process.[8] Common uterine diseases encountered in the postpartum period include metritis, clinical and subclinical endometritis, and pyometra.[9] Metritis occurs within 21 days of parturition, although is most commonly encountered within 10 days, and is characterized by an enlarged, fluid-filled uterus with a fetid odor and inflammation affecting all tissue layers of the uterus[9]. Clinical signs can range from being inapparent to systemic toxemia and shock.[10] Clinical endometritis is defined as inflammation limited to the endometrium ≥ 21 days postpartum, often with a mucopurulent or purulent discharge noted in the uterus and vagina.[10] Subclinical

endometritis is diagnosed when there are no clinical signs of uterine inflammation, but the proportion of neutrophils seen on cytobrush or endometrial flush samples exceeds the threshold associated with decreased reproductive performance, which is usually between 5 % and 18 % depending on the time of sampling.[11] If endometritis is present after ovulation and during diestrus, the persistent inflammatory state can inhibit normal uterine production of prostaglandins leading to failure of the corpus luteum to regress.[12] Luteal levels of progesterone would maintain a closed cervix, allowing pus to accumulate in the uterine lumen (pyometra). While these cows do not tend to show overt clinical signs, they become subfertile or infertile.[12]

Endometritis is the most consequential uterine disease in terms of the prevalence and impact on reproductive efficiency in postpartum dairy cows. The prevalence of endometritis varies across dairy herds, with 5-35 % of dairy cows reportedly affected.[7,13] Along with the cost of treatment, endometritis causes poor conception rates[14] and embryonic losses through the generation of a hostile environment for the developing embryo[15] and perturbation of luteal tissue function.[16] In a case-controlled study of 1,865 cows in Canada, it was found that the development of clinical endometritis increased the time to the first insemination by five days, increased the median days open by 28 days, reduced conception rates at first insemination by eight percent and affected cows were 1.7 times more likely to be culled for reproductive failure than cows without clinical endometritis.[17] Studies elsewhere have indicated an increase in calving to conception interval in postpartum dairy cows diagnosed with endometritis when compared to non-endometritic cows.[18] Treatment of endometritis is mainly reliant on the use of antimicrobials; however, their use comes with the concern for antimicrobial resistance and economic losses from the withdrawal of milk for sale during treatment.[10]

Although the development of endometritis is likely multifactorial, a key observation is that all cows suffer from altered immunological function during the periparturient period.[2] However, the pathophysiology surrounding peripheral immunosuppression and its roles in normal uterine involution and the development of postpartum endometritis is not fully elucidated. The following sections will provide a brief overview of the current understanding of peripartum physiology and pathophysiology, including uterine immune function and status, metabolic perturbations and neutrophil function.

1.2 The normal postpartum uterus

1.2.1 *Microbial contamination and clearance*

Following parturition, cows will experience microbial contamination of the uterus due to the breakdown of genital tract physical barriers and a vacuum effect created by uterine contractions/relaxations. Therefore, the microbiota of the bovine postpartum uterus is influenced by environmental, fecal and vaginal microbes during the first few weeks postpartum.[7] During the first week postpartum, Gram-negative bacteria predominate and are replaced by Gram-positive bacteria as the predominant population during the second and third week postpartum.[19] The majority of cows clear bacterial contaminants by the end of the fourth week postpartum.[19] Commonly cultured bacteria that are isolated and identified as non-specific uterine pathogens include *Escherichia coli, Trueperella pyogenes, Fusobacterium necrophorum* and *Prevotella melaninogenicus*.[20–24] Interestingly, similar bacterial species are encountered in the uteri of cows that clear the contamination temporally versus those that sustain the inflammation.[20,21]

It is currently unclear whether specific bacteria, an increased abundance of bacteria, certain combinations of bacteria or the failure of host tolerance and immunological function lead to the development of uterine disease.[10] It is interesting to note that certain virulence factors are only produced by the bacterial isolates from cows with endometritis.[24,25] Such isolates are thought to suppress nonspecific immunity through mechanisms such as inhibition of intracellular metabolism, blocking of cell surface receptors, inhibition of chemotaxis and phagocytic cell adhesion and promoting the production of pro-inflammatory cytokines.[26]

1.2.2 *Uterine defense mechanisms*

1.2.2.1 Physical barriers and innate immunity

A number of innate immunological defense mechanisms exist within the female genital tract and uterine mucosa to defend against microbial infection. Physical barriers including vulvar sealing, the cervix, cervico-vaginal mucus secretion and the uterine epithelium are the first line of defense against microbial entry and colonization.[7] Aside from acting as a physical barrier, mucosal epithelial cells also play an active role in uterine immunity through the secretion of anti-microbial peptides and proteins,[27] and the production of cytokines[28] which act as potent chemoattractants and promote leukocyte maturation.[7] The recognition of pathogen-associated molecular patterns

(PAMPs) and damage-associated molecular patterns (DAMPs) by epithelial pattern recognition receptors can also incite an inflammatory response.[27]

Leukocytes involved in the innate immune response play a vital role in the bovine uterine immune response immediately following calving. After normal parturition there is marked neutrophil recruitment within the first week, with neutrophils comprising up to 68 % of cells obtained from endometrial cytology.[29] By the time uterine involution is nearing completion (four weeks postpartum), the proportion of neutrophils obtained from endometrial cytology drops to approximately 15 %.[26] Neutrophils are the most important immune cell involved in the acute inflammatory response postpartum, and contribute to the clearance of microbial and cellular debris through migration directed by chemotaxis, phagocytosis and the production of reactive oxygen species through oxidative burst processes. Macrophages also play an important role in the innate immune response through phagocytosis, antigen presentation and the regulation of uterine inflammation.[30] Natural killer (NK) cells have also been identified in the uterus; however, their functional role during the postpartum period remains unclear.[31]

1.2.2.2 Adaptive immunity

Isolated lymphoid aggregates have been identified throughout the bovine genital tract, and while they have only been studied in limited detail, they are thought to contain B- and T-lymphocytes as seen associated with other mucosal surfaces.[31] B-lymphocytes are clearly important in the bovine genital tract immune response due to the presence of immunoglobulin (Ig) A and IgG in cervico-vaginal mucus, and IgA, IgG and IgE in the uterus.[32,33] These immunoglobulins are primarily thought to facilitate microbial phagocytosis by neutrophils and macrophages through opsonization. However, it is currently unclear how these immunoglobulins are transported across the uterine epithelial cell barrier, with proposed mechanisms including passive diffusion, active receptor-mediated transport and secretion.[7]

Both cluster of differentiation (CD) 4 expressing T-lymphocytes (T-helper cells) and CD8 expressing T-lymphocytes (cytotoxic T-cells) are present in uterine tissue, particularly in perivascular areas. Occasional T-lymphocytes are also found in the uterine glandular epithelium and within the uterine lumen. Both the CD4+ and CD8+ cells may express either the αβT-cell receptor (TcR) or the γδTcR.[31] While the precise role of T-lymphocytes in the postpartum uterine immune response remains unclear; it is thought that lymphocyte populations expressing the γδTcR

play an immunomodulatory role through the expression of anti-inflammatory (TGFβ, IL10) and pro-inflammatory (TNFα, IFNγ) cytokines.[34]

1.3 Peripartum metabolic changes

High-producing dairy cows face complex metabolic events during the periparturient period, often resulting in significant metabolic stress.[35] The nutrient requirements of the fetus peak three weeks prepartum, yet dry matter intake by the cow typically decreases by 10-30%.[36] Furthermore, within three weeks of the onset of lactation, the energy requirements for increased milk yield, milk fat, lactose and protein production will rise to approximately three times their resting metabolic rate requirements.[37] Modern dairy cattle have been genetically selected for high milk production; however, they are unable to consume enough feed to meet the metabolic demands of lactation, leading to a state of negative energy balance.[35] To overcome this, body tissues are catabolized to supply metabolic energy. This state of negative energy balance is reflected by decreased blood glucose, glutamine and insulin-like growth factor-1 (IGF-1) concentrations in postpartum cows.[37] An increased demand for energy derived from lipolysis will simultaneously result in increased serum ketone body (β-hydroxybutyrate (BHB)) and non-esterified fatty acid (NEFA) concentrations.[36] In addition, cows will frequently experience a brief but substantial period of hypocalcemia at the time of calving.[38]

Increased metabolic stress during the periparturient period has been proposed as a significant contributor to the transient immunosuppression seen in dairy cows.[37] Furthermore, metabolic stress during early lactation has been shown to be associated with an increased incidence of postpartum disease, particularly uterine disease[39–41] and mastitis.[42] However, the mechanisms linking metabolic derangements, immune function and postpartum disease development are only partially understood.[38]

1.4 Peripartum neutrophil function

1.4.1 *Peripheral neutrophil population*

As neutrophils play a pivotal role in the early immune response against pathogens encountered during parturition, recent research has focused on the factors leading to the derangement of neutrophil function during the periparturient period. Previous studies have shown that circulating neutrophil counts will increase at the time of calving, followed by a sharp reduction

one week postpartum and a slow return to normality over the following three weeks.[43,44] Normal physiologic events may explain this pattern during this period. At calving, a rise in cortisol is thought to both downregulate neutrophil apoptosis and decrease the ability of circulating neutrophils to migrate into tissues, often leading to neutrophilia.[45] Following parturition, the presence of tissue damage, local inflammation and bacterial contamination drive circulating neutrophils to undergo migration towards the mammary gland[46] and uterus,[29] with a consequent decrease in circulating neutrophil counts.[44] As the state of local and systemic inflammation associated with parturition resolves, circulating neutrophil counts return to baseline values.[45]

Once in the tissues, neutrophils can interact with and remove bacteria and/or damaged cells using various mechanisms. Bacterial phagocytosis by the neutrophils is facilitated by cell surface receptors for antibodies, pathogen-associated molecular patterns (PAMPs) and complement proteins.[47] Once ingested by the neutrophil, microbes are killed by oxygen-dependent and oxygen-independent mechanisms.[45] The most well-characterized oxygen-dependent mechanism is the nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase-mediated respiratory burst to synthesize reactive oxygen species (ROS) that damage bacteria.[45] Neutrophils also contain cytoplasmic granules, which house many pro-inflammatory and antimicrobial proteins, including lactoferrin, matrix metalloproteinase 9, gelatinase and myeloperoxidase.[38] Activated neutrophils will either release these proteins into the surrounding environment by exocytosis or into the phagosomes (containing ingested microbes) by granule-phagosome fusion to kill pathogens.[38] Finally, neutrophils may also form neutrophil extracellular traps (NETs) consisting of extruded chromatin, histones and cationic antimicrobial proteins to ensnare and kill microbes.[45]

Several studies have assessed peripheral neutrophil functionality in early postpartum cows with or without periparturient diseases. Initial work by Kehrli *et al.*[48] demonstrated impairment of neutrophil function during the week following calving in the eight primiparous cows included in their study. The most pronounced changes in function were related to the oxidative burst activity of neutrophils. More recent work on a larger dataset from dairy cows during the transition period has shown that calving day is associated with altered gene expression in peripheral neutrophils indicating a potential shift towards an immature phenotype.[49] Subsequent work has demonstrated a strong association between impairment of one or more aspects of neutrophil function and the occurrence of retained placenta, endometritis, metritis, purulent vaginal discharge and mastitis.[42,50–53] Impairment of neutrophil killing function through oxidative burst processes is the

most consistent finding in post-parturient cows that develop disease when compared to their healthy counterparts; however, neutrophil migration capacity and phagocytic ability appear to be variably affected.[38] Importantly, these demonstrated changes in neutrophil function precede the development of clinically detectable disease[52] and, in some cases, even precede parturition.[50,51]

1.4.2 *Uterine neutrophil population*

Immediately following parturition, there is a cytokine-mediated influx of neutrophils into the uterine lumen, which persists for the first 24 days in milk.[29] In normal cows, the number of neutrophils in the uterus will steadily decline until the fourth week postpartum when uterine involution is nearly complete. At this stage neutrophils should only represent approximately 15 % of all cells recovered on endometrial cytology (further discussed in section 1.6).[25,26] However, the persistence of higher neutrophil proportions beyond the point of normal uterine involution indicates ongoing inflammation and endometritis.[25] Some studies have suggested that although neutrophils may be present in increased numbers in uteri of cows with endometritis, their phagocytic ability is decreased when compared to neutrophils isolated from non-endometritic cows.[25,44] However, it is not known whether the loss of phagocytic activity directly contributes to bacterial persistence or the bacteria themselves alter neutrophil function to cause endometritis. It is also possible that the state of negative energy balance experienced by almost all cows postpartum leads to decreased neutrophil glycogen stores, thereby leading to reduced neutrophil function.[54] However, other studies have demonstrated reduced neutrophil function beyond the period of negative energy balance (typically three weeks postpartum).[26,44] From these observations, it's likely that the development of impaired neutrophil function is multifactorial.

1.4.3 *Metabolic factors affecting neutrophil function*

Not only do postpartum cows face markedly increased energy requirements due to the onset of lactation, but the process of mounting an inflammatory response such as that observed during uterine involution is highly dependent on the available energy. For example, it is estimated that the glucose requirement for an acute immune response in an adult dairy cow approaches one kilogram of glucose over a 12 h period.[55] The physiologic mechanisms designed to partition nutrients to the mammary gland induce a state of peripheral insulin resistance, thereby limiting glucose availability to other tissues. Glucose appears to be the primary energy source for activated bovine neutrophils while in circulation; however, once in the tissues, neutrophils must rely on

intracellular stores of glycogen to maintain their energy supply.[38,55] Limiting glucose availability appears to negatively impact neutrophil function, in particular oxidative burst processes. Lower oxidative burst capacity in the circulating neutrophils has been observed in the cows that proceeded to develop metritis within one week of parturition or endometritis within four weeks of parturition.[52] One variable that might explain this finding was that circulating neutrophils taken from cows in the lowest quartile for feed intake in the periparturient period had approximately 50 % of the oxidative capacity of those from cows in the top quartile for feed intake. This effect appeared to last from one week before parturition to three weeks following parturition. Furthermore, it has been demonstrated that cows with decreased intracellular glycogen stores in circulating neutrophils at the time of parturition had a greater likelihood of developing metritis three to seven days later.[41]

Negative energy balance will also lead to an increased reliance on fatty acid oxidation as a source of energy, resulting in the increased production of damaging reactive oxygen species.[56] If there is a concurrent deficiency of antioxidants such as sulfur-containing amino acids and/or selenium, intracellular mechanisms used by neutrophils to protect them from ROS produced as part of their killing functions will also be compromised.[38,57] Recent studies have shown that oral administration of antioxidants such as choline, L-carnitine and methionine to Holstein cattle improved selected production indices and peripheral neutrophil oxidative capability and decreased the expression of selected genes involved in inflammation.[56,58]

Elevated serum NEFA and ketone concentrations manifest as a maladaptive response to the metabolic demands of lactation and other physiological processes during the postpartum period.[59] These metabolites are associated with a heightened risk of several metabolic and infectious diseases, including retained fetal membranes, metritis, endometritis, prolonged anovulation and decreased milk yield.[60] The precise mechanisms by which higher serum NEFA and ketone concentrations affect neutrophil function remains incompletely understood; however, the presence of ketonemia and elevated NEFA concentrations implies decreased glucose availability for neutrophil metabolism.[38] Furthermore, it is also possible that BHB and NEFA may directly affect innate immune function. Exposing circulating neutrophils from mid-lactation cows to increasing concentrations of NEFA, but not BHB, *in vitro* has been shown to cause a dose-dependent reduction in oxidative burst function.[52,61] In a similar study utilizing neutrophils from cows with naturally occurring differences in blood BHB concentrations, neutrophil chemotaxis

was found to be reduced in cows with blood BHB concentrations > 1.6 mmol/L.[62] However, *in vitro* culture has indicated that it is not the BHB alone, rather a combination of BHB with other ketones, including acetoacetate and/or acetone, that inhibits neutrophil chemotaxis.[62] When taken together, these studies suggest a state of negative energy balance has both direct and indirect effects on neutrophil function and that further characterization of the mechanisms involved is warranted.

1.4.4 *The role of serum total calcium in neutrophil function*

Hypocalcemia is the most common metabolic disorder among periparturient dairy cows with an estimated incidence of 7.7 %,[63] and has been associated with an increased risk of endometritis,[63,64] metritis,[65] displaced abomasum[66] and decreased milk production.[67] Calcium is essential for neutrophil activation and is an integral part of intracellular signal transduction.[68] Therefore, hypocalcemia around calving time may be one of the factors that contribute to neutrophil dysfunction. In one study, neutrophils collected from cows with clinical hypocalcemia had lower intracellular calcium concentrations and impaired phagocytic ability when compared to neutrophils from their normocalcemic counterparts.[69] Similarly, Martinez *et al*.[70] found that neutrophils from cows with a blood total calcium concentration < 2.15 mmol/L (classified as subclinical hypocalcemia) during the first three days postpartum had impaired phagocytic and oxidative burst capacity. Moreover, at least two-thirds of the metritic cows in that study were associated with a blood total calcium concentration < 2.15 mmol/L during the first three days postpartum.[70] Interestingly, it appears that the duration of hypocalcemia in the four days following parturition may be more predictive of disease risk than the nadir concentration in the first 24 h following parturition.[65] In a separate study, cows diagnosed with hypocalcemia within three days of calving were two times more likely to develop clinical endometritis at 21 days in milk.[64] However, when a group of low-parity periparturient cows were supplemented with injectable calcium soon after calving, no difference in neutrophil oxidative burst or phagocytic ability was detected when compared to non-supplemented cows.[71] It should be noted that the cows in this study were clinically healthy, and the possibility remains that differences may arise when cows affected by postpartum diseases are included. Clearly, further investigation into the role hypocalcemia plays in the development of uterine diseases is warranted.

1.4.5 *Hormonal factors affecting neutrophil function*

It is currently postulated that the combined effects of stressors including parturition, nutritional deficiencies, the onset of lactation and movement to the milking herd can exacerbate the degree of immunosuppression through increased concentrations of circulating epinephrine, norepinephrine and cortisol.[72] As a result, there is increased production of anti-inflammatory cytokines, decreased production of pro-inflammatory cytokines, suppression of leukocyte activity and interference with immunoglobulin and complement activity.[2] In particular, reduced neutrophil surveillance activity has been associated with excess circulating cortisol.[73] Cortisol has been observed to down-regulate the expression of the cell surface molecules L-selectin and CD18, which are essential for cell adhesion and intracellular signaling.[73] Evidence also suggests that fluctuations in 17β-estradiol and progesterone during the periparturient period may also have a negative effect on the neutrophil function, as *in vivo* administration of 17b-estradiol, but not progesterone, was found to be associated with decreased neutrophil chemotaxis and decreased survival following migration.[74] However, other studies have provided conflicting results. Hussain *et al*.[75] identified that the basal levels of serum progesterone and increased estrogen concentrations during estrus were associated with an increase in the phagocytic ability of blood neutrophils. Moreover, in an experiment utilizing neutrophils from ovariectomized cows, no reduction in oxidative burst capacity was noted after administration of 17β-estradiol, estrone or progesterone.[76] When this body of research is taken together, it is highly likely that a combination of metabolic and hormonal factors during the periparturient period interact to create the state of immunosuppression that is commonly seen.

1.5 Parity and body condition score as predisposing risk factors

Identifying risk factors for postpartum disease that are both distinguishable and manageable by the farmer is crucial to maintaining the health and productivity of a dairy cow herd. Two important risk factors identified to date are parity and prepartum body condition score (BCS), both of which are primarily related to the risk of significant negative energy balance postpartum.[77]

Parity has been shown to influence a cow's ability to clear postpartum bacterial infections. In theory, primiparous cows have had limited exposure to pathogens when compared to their multiparous counterparts, thereby slowing their adaptive immune response and increasing their risk of infection.[78] However, as discussed above, the role of the innate immune response is more important in the immediate postpartum period than that of the adaptive immune response. Indeed

Mehrzad *et al*. found neutrophils from primiparous cows to have a greater oxidative burst ability than those from multiparous cows.[79] Despite partitioning of available nutrients towards their growth, it is postulated that primiparous cows are at decreased risk of profound negative energy balance due to lower lactational demands[48] and their ability to maintain dry matter intake even when unwell,[80] thereby leading to improved neutrophil function. Multiparous cows have been shown to experience a greater negative energy balance, leading to increased serum concentrations of BHB and NEFA.[81] As discussed previously, this likely leads to a more significant impact on neutrophil function in these cows and a greater propensity for uterine disease in multiparous cows versus primipara.[82]

High body condition score (BCS > 4 on a 5-point scale) at the time of parturition can also lead to increased mobilization of the products of lipid oxidation in the postpartum period, including NEFA and BHB.[59] These cows are at risk of lower serum glucose concentrations, increased incidence of reproductive disorders and decreased reproductive performance following parturition.[83] A recent study also indicates that pre-calving BCS may alter the inflammatory state of neutrophils themselves. Cows with an ideal prepartum BCS of 4 on a 10-point scale showed significant increases in neutrophil gene expression of antimicrobial peptides and the anti-inflammatory cytokine interleukin-10 when compared to cows with a BCS of 5.[84] Furthermore, the change in BCS over the five weeks following parturition has also been shown to be significant when assessing for the risk of postpartum disease.[85] A loss of ≥ 0.5 BCS points (on a 5-point scale) during this time is associated with a greater degree of negative energy balance and increased risk of infectious disease.[86,87] Uterine infections specifically have been associated with both pre- and post-parturient BCS loss.[88] However, other studies have also identified a greater risk of metritis in cows that calve when already in a thin body condition.[85,89] When taken together, these observations suggest that the association between the risk of infectious disease and BCS is non-linear with extremes in body condition posing the greatest risk.

1.6 Diagnosis of endometritis

Several methods are available to detect endometrial inflammation in cows, including ultrasonography, bacterial culture, histologic examination, and endometrial cytology using either the cytobrush technique or small volume uterine lavage.[90,91] Of these methods, the endometrial cytology is considered the reference method for detection of subclinical endometritis due to the

superior quality of the samples and repeatability.[91] Regarding the collection of a uterine sample for cytology, the cytobrush technique is a more simple and rapid technique when compared to small volume uterine lavage.[92] It is recommended that a minimum of 300 cells are counted when assessing a cytological smear for the highest reproducibility.[93] The number of neutrophils present is then expressed as a proportion of all cells counted, including epithelial cells, macrophages, lymphocytes and others.[8] However, establishing a cutoff value for the diagnosis of endometritis is complicated because the proportion of neutrophils in the uterus declines over time.[8] The founding study by Kasimanickam *et al*.[92] established a threshold for the diagnosis of endometritis at ≥ 18 % neutrophils for samples collected between 20 and 33 days postpartum and ≥ 10 % for samples collected 34 to 47 days postpartum. In their study, cows with neutrophil percentages above these values showed a decreased incidence of pregnancy during the current lactation.[92] Later studies, such as that by Madoz *et al*.[94], have used a general threshold of 5 % neutrophils for all cows sampled between 21 and 62 days postpartum. A possible reason for the discrepancy in cutoff values between studies is that reproductive performance was used as an indicator for the effects of subclinical endometritis, which is itself influenced by several other factors including the use of synchronization programs, length of the voluntary withholding period and the detection of estrus.[8] Further studies are therefore required to reach a consensus on the optimal threshold for the cytologic diagnosis of subclinical endometritis.

1.7 Research objectives and hypotheses

Currently there are inconsistent results in the literature regarding the association of cortisol and serum metabolites with the development of endometritis in postpartum dairy cows. Furthermore, it is unknown when peripheral neutrophil function is altered (pre- or post-calving period) in the cows that eventually develop endometritis. The overall research objective was to document the temporal changes in select serum metabolites, cortisol, and peripheral neutrophil function during the extended pre- and post-calving period and to determine their relationship with the development of endometritis in postpartum dairy cows. This study was unique due to the comprehensive approach to assess neutrophil function using clinical and laboratory methodologies in conjunction with the metabolic/production status in a cohort of dairy cows. A comprehensive longitudinal study such as this allows for the evaluation of factors leading to the development of endometritis at five weeks post-calving.

Considering that all cows experience a certain degree of immunosuppression during the periparturient period[2,83,95] and the higher susceptibility of dairy cows to metabolic disorders,[2,36] we intended to test the overall hypothesis that increased production/metabolic demands would lead to greater immunosuppression during the periparturient period. This study builds on the current understanding of the mechanisms of immunosuppression by using an extended sampling window when compared to previous studies, and comparing an array of hematologic, metabolic, and neutrophil functionality parameters within a set cohort of dairy cows.

2 Serum metabolite and cortisol concentrations during the periparturient period and their relationship with postpartum endometritis in dairy cows

2.1 Introduction

During the transition period, dairy cows face considerable metabolic challenges associated with a decrease in dry matter intake (DMI) and a concurrent increase in energy demand for lactation.[59] Sub-optimal herd management during this time can lead to an increased risk of metabolic derangements, postpartum uterine disease and poor fertility. Understanding which metabolites to measure, and at what time, to identify cows at risk of endometritis would allow the development of herd health strategies to optimize health and performance.

Several blood metabolites have been used to monitor metabolic changes during the transition period and predict the likelihood of postpartum disease.[83,96] Serum glucose, β-hydroxybutyrate (BHB) and non-esterified fatty acid (NEFA) concentrations have all been used to assess the ability of the cow to adapt to a state of negative energy balance following parturition.[83] Specifically, an increased risk of endometritis, low milk yield, prolonged anovulatory period and early culling was found to be associated with serum or blood BHB ≥ 1.2 mmol/L in the first two weeks following calving.[60] Serum NEFA > 0.3 mmol/L in the first two weeks prior to calving has been associated with increased risk of retained placenta, metritis, poor reproductive performance and early culling.[60] Less commonly, urea has also been used to assess negative energy balance, with low urea concentrations thought to reflect a reduction in DMI and/or inability of the liver to convert ammonia to urea efficiently.[97]

Subclinical hypocalcemia is also common among periparturient dairy cows and has been associated with increased incidence of postpartum uterine disease, milk production losses and increased risk of culling.[66,96] Specifically, a blood total calcium of < 2.15 mmol/L within the first three days postpartum has been identified as a risk factor for impaired immune cell function and the development of metritis.[70]

It is also proposed that the physiological and social stressors encountered during the periparturient period can exacerbate the state of immunosuppression through increased

concentrations of circulating epinephrine, norepinephrine and cortisol.[72] In particular, excess circulating cortisol has been associated with reduced neutrophil surveillance activity through down-regulation of the cell surface adhesion molecules L-selectin and CD18.[73]

Monitoring for changes in body condition score (BCS) has become an essential part of herd management, as it provides a quick visual assessment of overall energy balance and risk of postpartum disease.[77] The loss of body condition following parturition is common in most dairy cows in response to partitioning nutrients towards milk production. However, excessive loss (greater than 0.5 points on a 1 – 5 point scale) has been associated with a greater degree of negative energy balance, higher risk of ketosis and reduced reproductive performance.[39]

As discussed previously, endometritis is the most consequential uterine disease in terms of its prevalence and impact on reproductive efficiency in postpartum dairy cows. Among the most widely accepted risk factors for the development of endometritis are problems encountered during calving (i.e. retained placenta and dystocia) and metritis.[17] However, there is a paucity of information regarding the metabolic and hormonal risk factors for the development of endometritis during the periparturient period.[18,98] We hypothesized that cows which develop endometritis experience greater disturbances in metabolic and hormonal indicators of stress during the periparturient period when compared to non-endometritic cows. Therefore, the objectives of this study were (a) to determine the temporal changes in indicators of metabolic status (glucose, BHB, NEFA, urea and total calcium) and cortisol during the periparturient period and their association with the development of endometritis; and (b) to assess the relationship of BCS change during the five weeks following parturition with the above indicators of metabolic status, and the subsequent development of endometritis.

2.2 Methods

2.2.1 *Animals*

Dairy cows were maintained in group housing pens at the Rayner Dairy Research and Teaching Facility, University of Saskatchewan, Saskatoon, SK, Canada. At the time of this study, the herd consisted of 115 Holstein milking cows with an average milk yield of 43.2 kg per cow per day. Experimental protocols were approved by the University Committee on Animal Care and Supply (AUP# 20190063) and research was conducted in accordance with the Guidelines of the Canadian Council on Animal Care. Any clinical disease that arose in the research subjects was

identified and treated by trained farm personnel. Any animals removed from the herd were excluded from the study.

2.2.2 *Experimental design and animal sampling*

Forty-two cows were enrolled in this observational cohort study conducted over two sampling periods, May to December 2019 and April to September 2020. The cows to be sampled were identified five weeks before their predicted calving date using computerized health records (Dairy Comp 305, Lactanet, ON, Canada). Each cow enrolled in the study was sampled as the cow reached the appropriate number of days pre- or postpartum. Blood sampling was performed biweekly, with an additional sample taken at the time of calving as outlined in Table 2.1. Each sample was collected at the same time in the morning, prior to feeding, to account for daily fluctuations in cortisol and metabolite concentrations. Blood samples were collected from the coccygeal vein using BD Vacutainer serum collection tubes (Cat # 367820, BD, Franklin Lakes, NJ, USA) for metabolite and cortisol samples, BD Vacutainer K_2EDTA collection tubes (Cat # 367844, BD, Franklin Lakes, NJ, USA) for CBC samples and BD Vacutainer Lithium Heparin collection tubes (Cat # 367880, BD, Franklin Lakes, NJ, USA) for flow cytometry samples. All collection tubes were placed on ice immediately following sampling for transport to the laboratory. Collection tubes for serum were left at room temperature for 20-30 minutes prior to centrifugation at 3,000 x g for 10 min (Medifuge, Baxter Canlab). The serum was then transferred to microcentrifuge tubes (Cat # 89004-298, VWR, Mississauga, ON, Canada) and stored at -80°C until further processing. Endometrial cytobrush samples were obtained for cytological analysis at five weeks postpartum using the procedure detailed below (Section 2.2.5). Cow age, lactation number and treatment for any metabolic or infectious disease were also recorded. An outline of the data collected at each timepoint is detailed in Table 2.1.

2.2.3 *Body condition scoring*

Cow body condition score was recorded at calving and five weeks postpartum by the author (AC). Cows were assigned a score between 1 and 5, with quarter-point divisions, using the visual guidelines developed by Edmonson *et al.*[99] Cows were classified as either maintaining body condition if their change in BCS over the five week period was between +0.25 and -0.25, or as losing body condition if their change in BCS over the 5 week period was -0.5 or -0.75.

2.2.4 *Serum metabolite and cortisol measurement*

All serum samples were submitted to Prairie Diagnostic Services Inc. (Saskatoon, SK, Canada). All serum metabolites were measured using a Cobas c 311 analyzer (Roche Diagnostics, IN, USA) according to the guidelines provided by the manufacturer. Total calcium and glucose concentrations were determined using a two-point end assay, urea and BHB concentrations were determined using a kinetic enzymatic assay, and NEFA concentrations were determined using a spectrophotometric assay. The intra-assay and inter-assay coefficients of variation were less than 5% for each assay. Serum cortisol concentrations were measured on an IMMULITE 1000 (Siemens Healthcare, ON, Canada) analyzer using a solid-phase, competitive chemiluminescent enzyme immunoassay. The intra-assay and inter-assay coefficients of variation were 7.8 % and 7.7 %, respectively.

2.2.5 *Endometrial sampling and cytologic assessment*

A previously reported cytobrush technique[100] was used to obtain endometrial samples at five weeks postpartum. Briefly, a cytobrush device was designed using a commercially available 0.5 mL universal artificial insemination (AI) gun with the stylet modified to enable a shortened cytobrush (Cat # 4201-CB-8B, Fischer Scientific, Ottawa, ON, Canada) handle to be swaged onto the end of the stylet. Cows were restrained in a headlock stanchion to restrict back-forth and side-side movement. The technique used to obtain endometrial cytological samples was similar to what has been previously reported.[100,101] For each cow, the cytobrush sample was rolled onto two glass microscope slides for cytological evaluation. These slides were allowed to air dry for 2-3 hours prior to staining with modified Wright Giemsa's stain using an automated slide stainer (Hema Tek slide stainer, IL, USA). All slides were evaluated by the author (AC) by recording 100 cells per quadrant (400 cells total) to obtain a differential cell count. Cell types recorded included individualized epithelial cells, neutrophils, lymphocytes, macrophages, mast cells, eosinophils and plasma cells. Other notable features (epithelial cell clumping, mucus, damaged and smeared cells) were recorded but not included in the differential cell count. Previous studies have indicated that cytological samples containing ≥ 18% neutrophils (100 x neutrophils / (neutrophils + other nucleated cells)) are indicative of endometritis at five weeks postpartum;[8,101,102] therefore, cows in this study were classed as endometritic on cytology if there were ≥ 18 % neutrophils present, or non-endometritic if there were < 18 % neutrophils present. One cow was sampled six weeks

postpartum. In this cow, a designation of endometritis was made if there were ≥ 10 % neutrophils present on cytology.[8]

Table 2.1 Outline of the data collected from each animal throughout the duration of the study

Data Collected	Week Relative to Calving							
	-4.5	-2.5	-1	0	1	3	5	7
CBC	✓	✓	✓	✓	✓	✓	✓	✓
BCS				✓			✓	
Weight				✓			✓	
Uterine cytology							✓	
Glucose		✓	✓	✓	✓	✓	✓	✓
Urea				✓	✓	✓	✓	✓
Calcium		✓	✓	✓	✓	✓	✓	✓
BHBA			✓	✓	✓	✓	✓	
NEFA			✓	✓	✓	✓	✓	✓
Cortisol	✓	✓	✓	✓	✓	✓	✓	

2.2.6 *Statistical analysis*

All statistical analyses were performed using Stata (Version 17.0, College Station, TX, USA). The data were assessed for normality using the Shapiro Wilk test. Cows were assigned to one of two groups based on the presence or absence of subclinical endometritis at five weeks postpartum. For each continuous variable (serum glucose, total calcium, BHB, NEFA, urea and cortisol concentrations), the Wilcoxon Rank Sum test was used to ascertain which timepoints were significantly different between the two groups. Association between the loss of ≥ 0.5 BCS points from the day of calving to five weeks postpartum and the development of endometritis was assessed using the Fisher's Exact test. P-values < 0.05 were considered significant.

2.3 Results

2.3.1 *Study population*

Individual animal age, parity and observed diseases are outlined in Table 2.2. The cows had a median age of 49 months (range 23 to 114 months) and median parity of 3 (range 1 to 6).

Table 2.2 Cows included in the study with age, parity and recorded co-morbidities.

Cow ID	Age (months)	Parity	Comorbidities
1	114	6	H
2	101	5	SE, H
3	96	6	RFM, H
4	96	5	
5	91	6	SE
6	83	5	Ma
7	83	5	Tw
8	79	4	Tw, RFM, SE
9	70	3	
10	69	3	Tw, RFM, SE, H
11	68	4	Ke
12	66	3	SE
13	66	4	
14	63	3	SE
15	62	4	Ke
16	60	4	Tw, RFM, Met, SE
17	60	4	Ma
18	55	3	
19	53	3	
20	50	3	Ma
21	50	3	
22	48	3	
23	48	3	
24	48	2	
25	47	3	
26	46	2	
27	46	2	RFM, SE
28	43	2	SE
29	43	2	
30	41	2	
31	41	2	
32	41	2	
33	38	2	
34	38	2	Ma
35	37	2	

Table 2.2 (Continued)

36	**37**	**1**	**SE**
37	34	2	RFM
38	26	1	
39	26	1	SE
40	23	1	SE

H, hypocalcemia; SE, subclinical endometritis; RFM, retained fetal membranes; Ma, mastitis; Tw, twins; Ke, clinical ketosis.

2.3.2 *Uterine cytology*

Cytobrush sampling yielded diagnostic smears for 40 cows included in the study. Uterine samples could not be obtained for two cows due to narrow openings at 2nd or 3rd cervical ring. Of the 40 smears examined, 12 (30 %) were diagnosed with endometritis (≥ 18% neutrophils). As expected, the average proportion of neutrophils in the uterine samples from endometritic cows was higher ($P < 0.01$) at 31.8 % (range 19 – 73 %) compared to 1.3 % (range 0.25 – 6.5 %) in non-endometritic cows.

2.3.3 *BCS changes*

Cows lost on average 0.25 BCS points in the five weeks postpartum (ranging from a loss of 0.75 points to a gain of 0.25 points), and 17 of 40 cows (42.5 %) lost ≥ 0.5 BCS points during this period. No significant association between a loss of ≥ 0.5 BCS points and the development of endometritis was found ($P = 0.40$). A comparison of BCS change between endometritic (n = 12) and non-endometritic (n = 28) cows is shown in Figure 2.1.

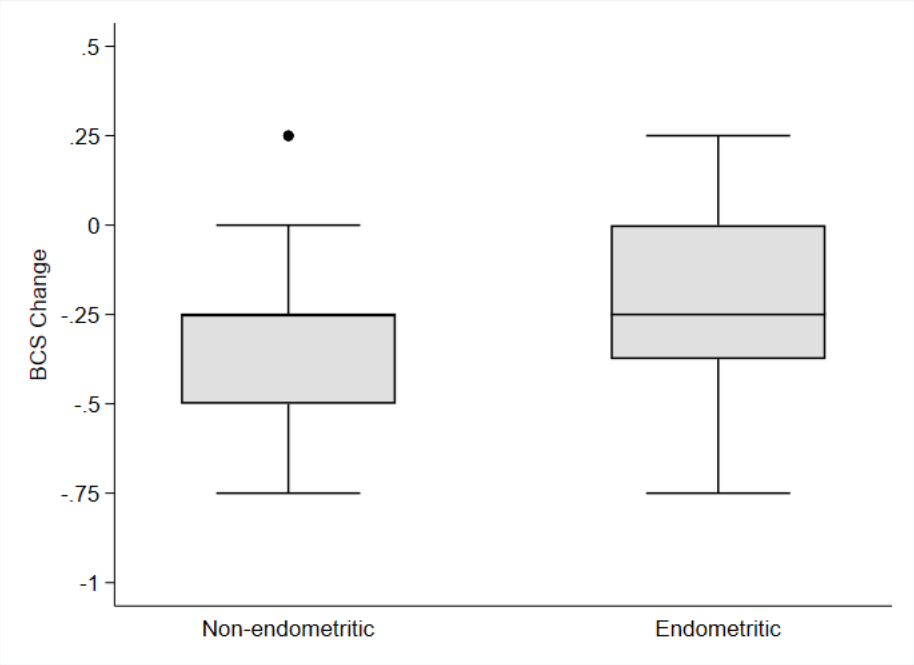


Figure 2.1 Body condition score change (median and interquartile range) between calving and five weeks postpartum in non-endometritic and endometritic cows. No significant differences were observed between the two groups ($P = 0.40$).

2.3.4 *Serum metabolites*

2.3.4.1 Calcium

Temporal changes in the median serum total calcium concentrations over time for all cows are shown in Figure 2.2. Overall, a median decrease in the total serum calcium concentration was observed at calving compared to other timepoints. When stratified by the presence of endometritis, endometritic cows had significant median increase in serum total calcium concentrations one week prior to parturition ($P = 0.02$) and at calving ($P = 0.02$) than non-endometritic cows (Figure 2.3 A).

When stratified by BCS maintenance or loss, cows that lost body condition had significant median decrease in blood total calcium concentrations at one week prior to calving ($P = 0.02$) than those that maintained body condition (Figure 2.3 B).

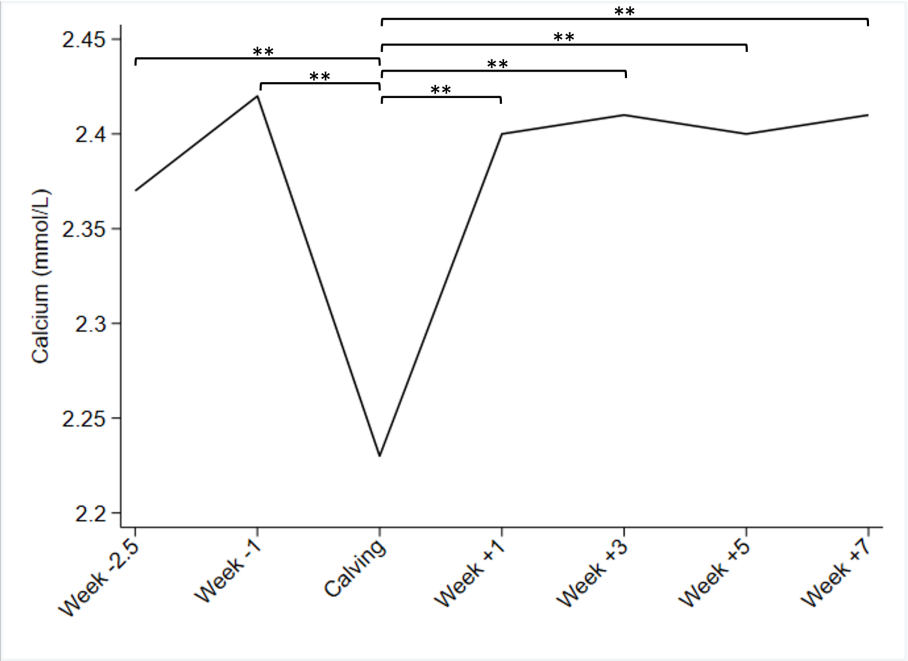
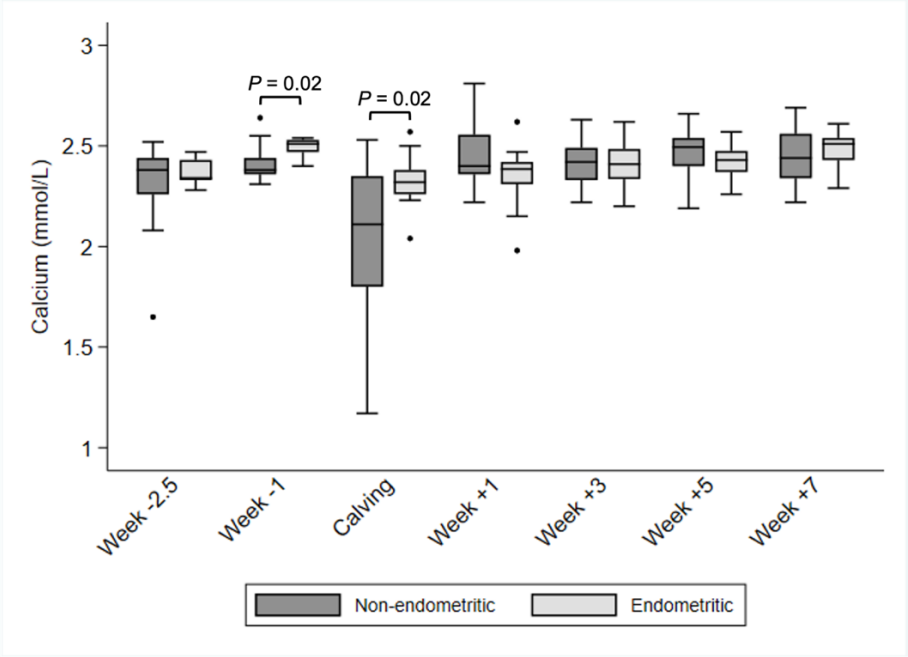


Figure 2.2 Median serum total calcium concentration of all cows (n = 40) during the study period. The median calcium concentration at calving was compared to every other timepoint. Bars represent statistically significant differences between timepoints; ** $P < 0.01$.

A



B

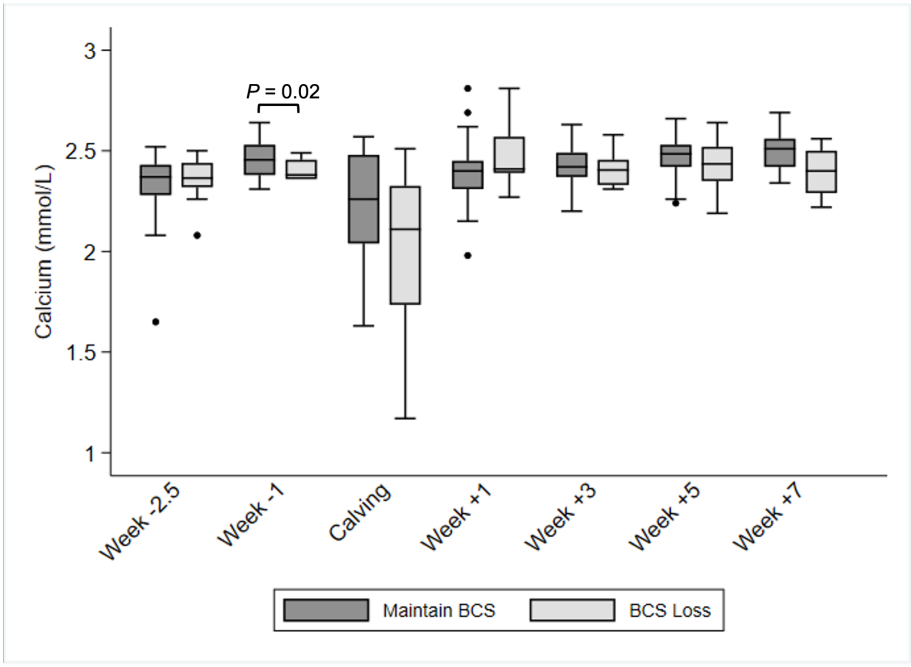


Figure 2.3 Serum total calcium concentrations (median and interquartile range) at each timepoint grouped by (A) the presence or absence of endometritis at five weeks postpartum, and (B) the maintenance or loss of ≥ 0.5 BCS points in the 5 weeks following calving.

2.3.4.2 Glucose

Temporal changes in the median serum glucose concentrations over time for all cows are shown in Figure 2.4. Endometritic cows had significant median increases in glucose concentrations at calving ($P < 0.02$) and seven weeks postpartum ($P = 0.02$) compared to non-endometritic cows (Figure 2.5 A). When stratified by BCS maintenance or loss, cows that lost body condition had median decrease in blood glucose concentrations at one week prior to calving ($P = 0.01$) than those that maintained body condition (Figure 2.5 B).

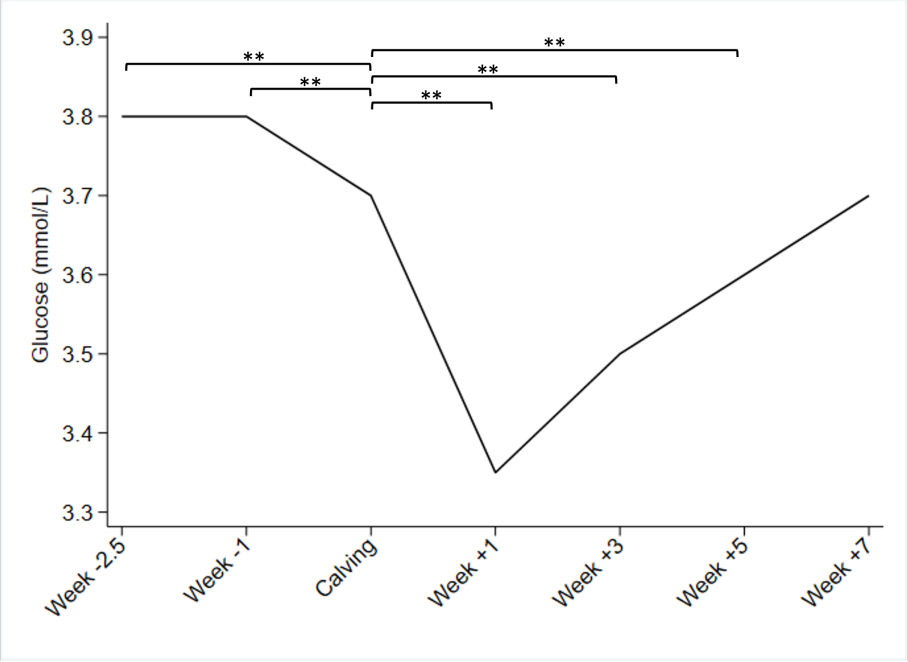
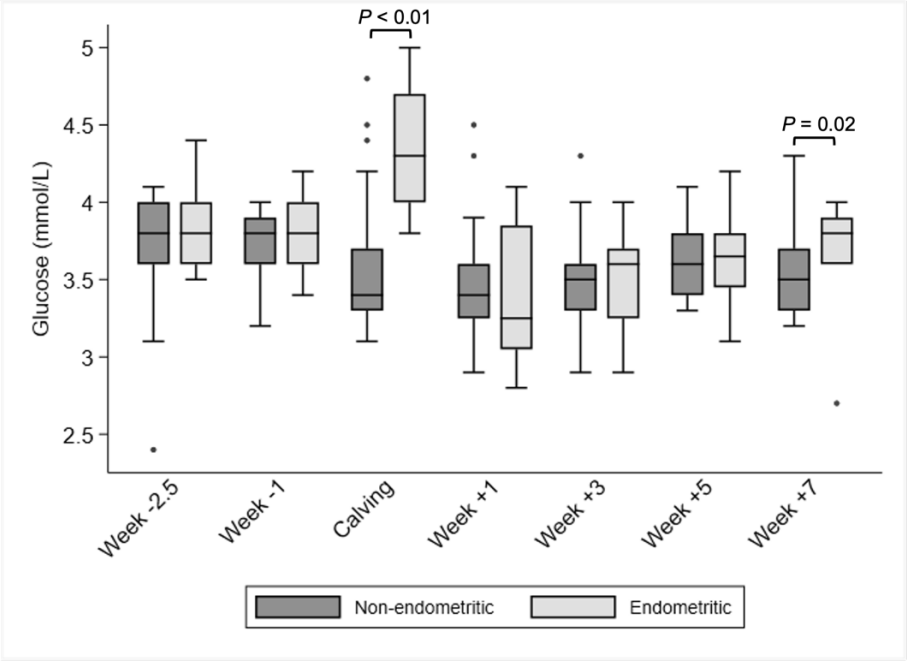


Figure 2.4 Median serum glucose concentration of all cows (n = 40) during the study period. The median glucose concentration at calving was compared to every other timepoint. Bars represent statistically significant differences between timepoints; ** $P < 0.01$.

A



B

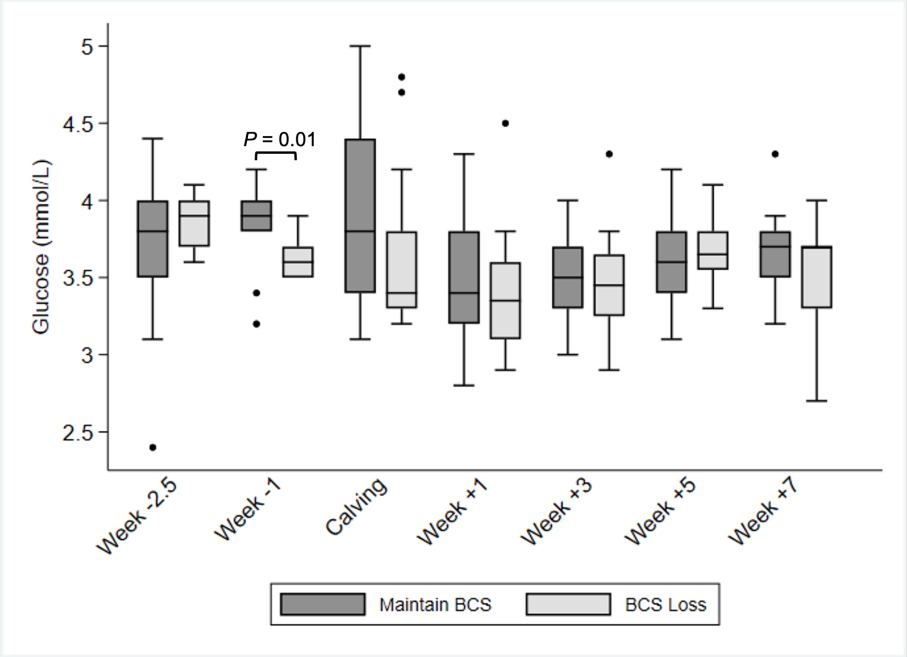


Figure 2.5 Serum glucose concentrations (median and interquartile range) at each timepoint grouped by (A) the presence or absence of subclinical endometritis at 5 weeks postpartum, and (B) the maintenance or loss of ≥ 0.5 BCS points in the 5 weeks following calving.

2.3.4.3 BHB

Temporal changes in the median serum BHB concentrations over time for all cows are shown in Figure 2.6.

Endometritic cows had significantly higher serum BHB concentrations at one week postpartum (P = 0.03) than non-endometritic cows (Figure 2.7 A). When stratified by BCS maintenance or loss, cows that lost body condition had higher serum BHB concentrations at one week postpartum (P = 0.04) than those that maintained body condition (Figure 2.7 B).

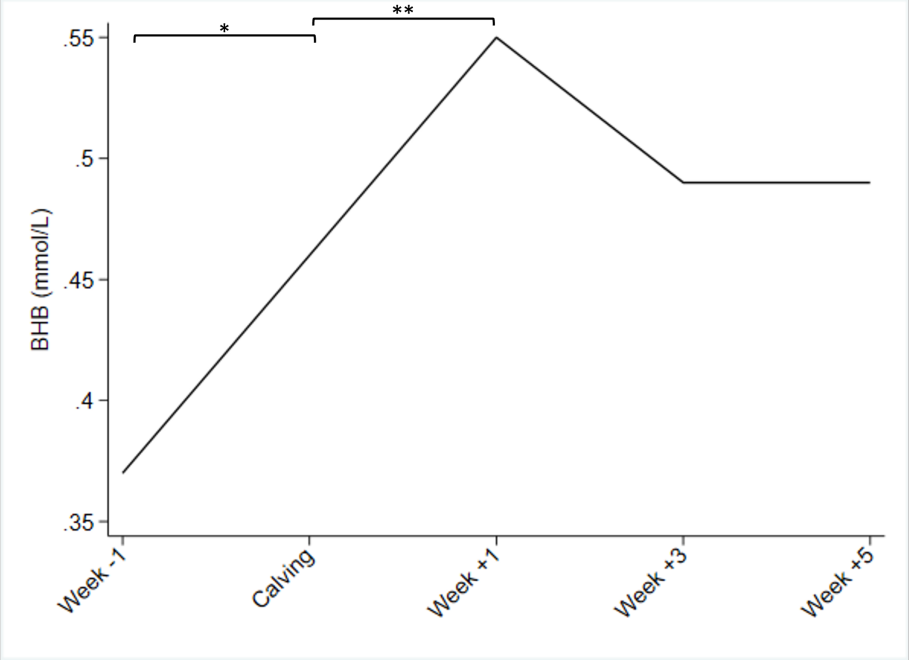
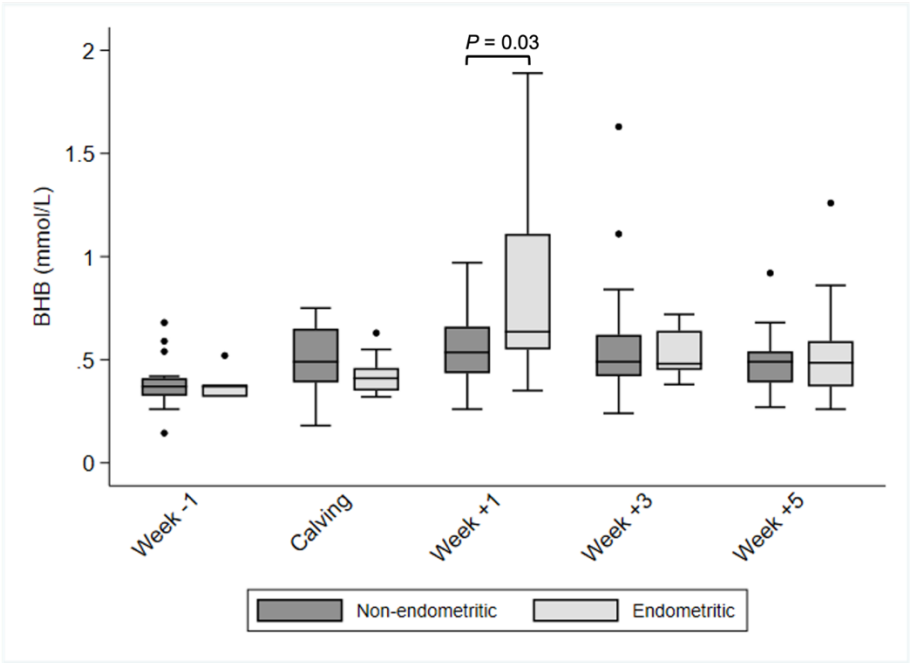


Figure 2.6 Median serum BHB concentration of all cows (n = 40) during the study period. The median BHB concentration at calving was compared to every other timepoint. Bars represent statistically significant differences between timepoints; * $P < 0.05$; ** $P < 0.01$.

A



B

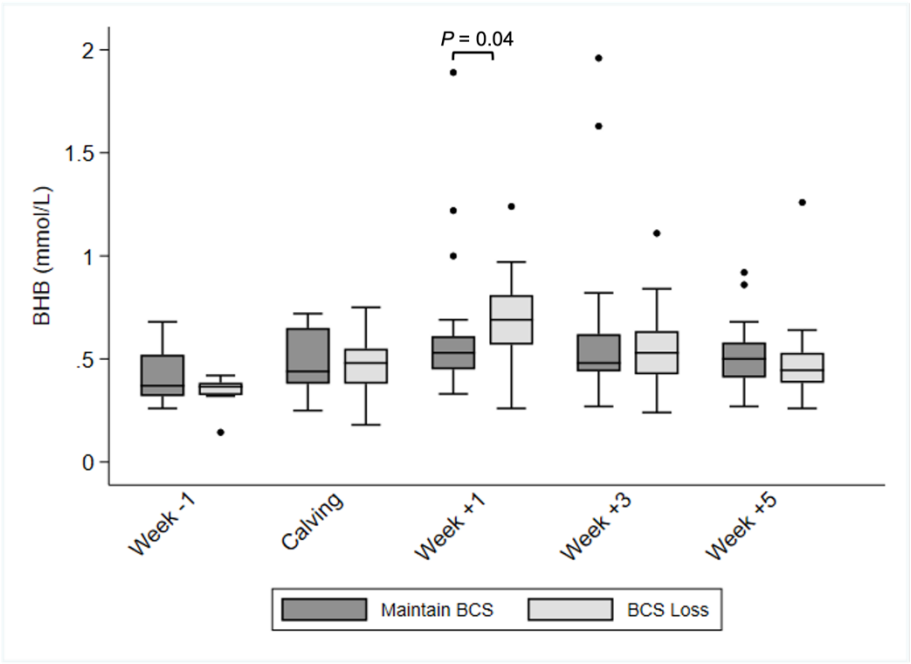


Figure 2.7 Serum BHB concentrations (median and interquartile range) at each timepoint grouped by (A) the presence or absence of subclinical endometritis at 5 weeks postpartum, and (B) the maintenance or loss of ≥ 0.5 BCS points in the 5 weeks following calving.

2.3.4.4 NEFA

Temporal changes in the median serum NEFA concentrations over time for all cows are shown in Figure 2.8.

No statistically significant differences were found for serum NEFA concentrations at any timepoint when stratified by the presence of endometritis or change in BCS (Figure 2.9).

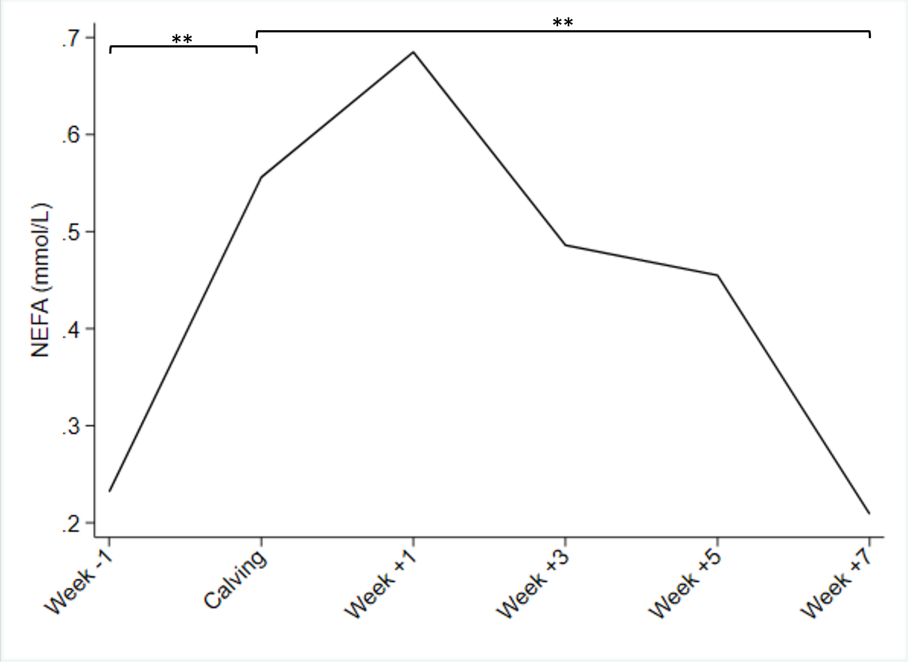
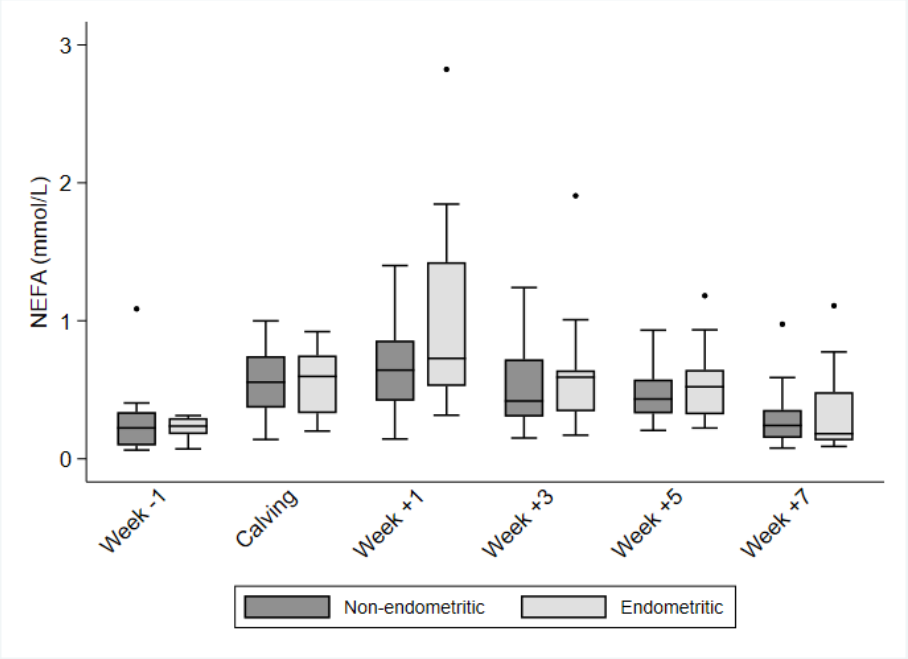


Figure 2.8 Median serum NEFA concentration of all cows (n = 40) during the study period. The median NEFA concentration at calving was compared to every other timepoint. Bars represent statistically significant differences between timepoints; ** $P < 0.01$.

A



B

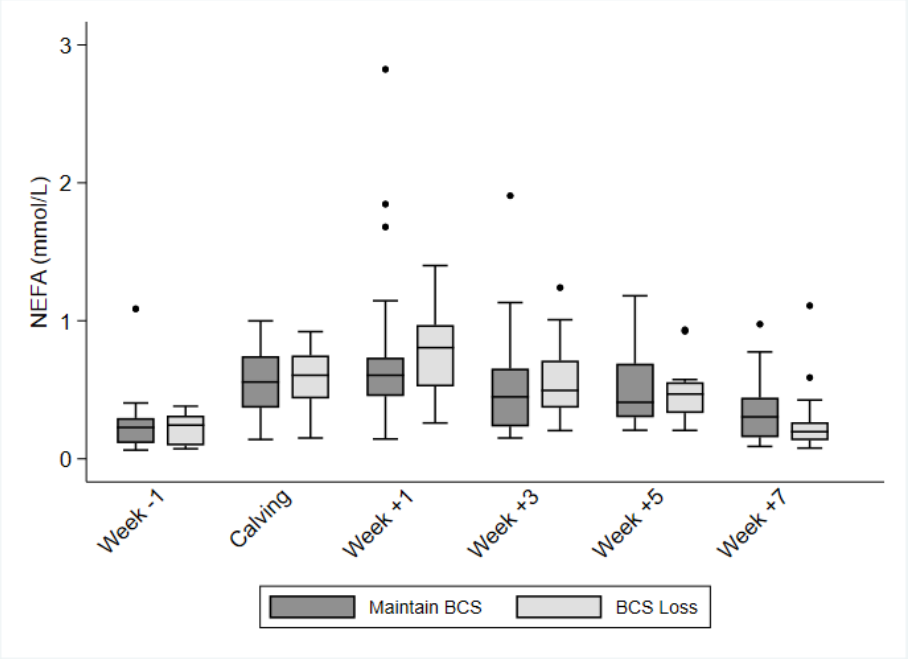


Figure 2.9 Serum NEFA concentrations (median and interquartile range) at each timepoint grouped by (A) the presence or absence of subclinical endometritis at 5 weeks postpartum, and (B) the maintenance or loss of ≥ 0.5 BCS points in the 5 weeks following calving.

2.3.4.5 Urea

Temporal changes in the median serum urea concentrations over time for all cows are shown in Figure 2.10.

No statistically significant differences were found for serum urea concentrations at any timepoint when stratified by the presence of endometritis or change in BCS (Figure 2.11).

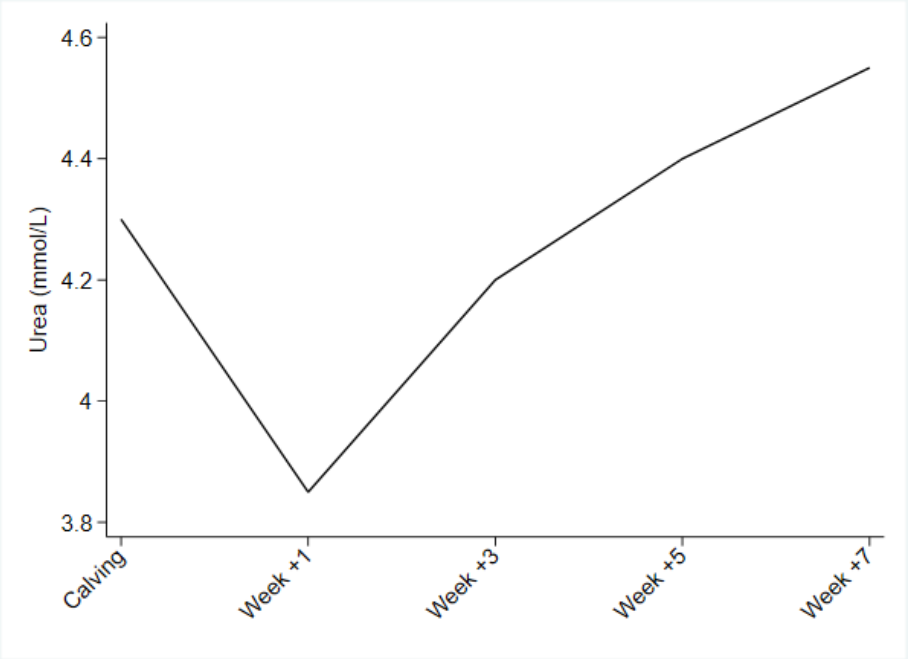
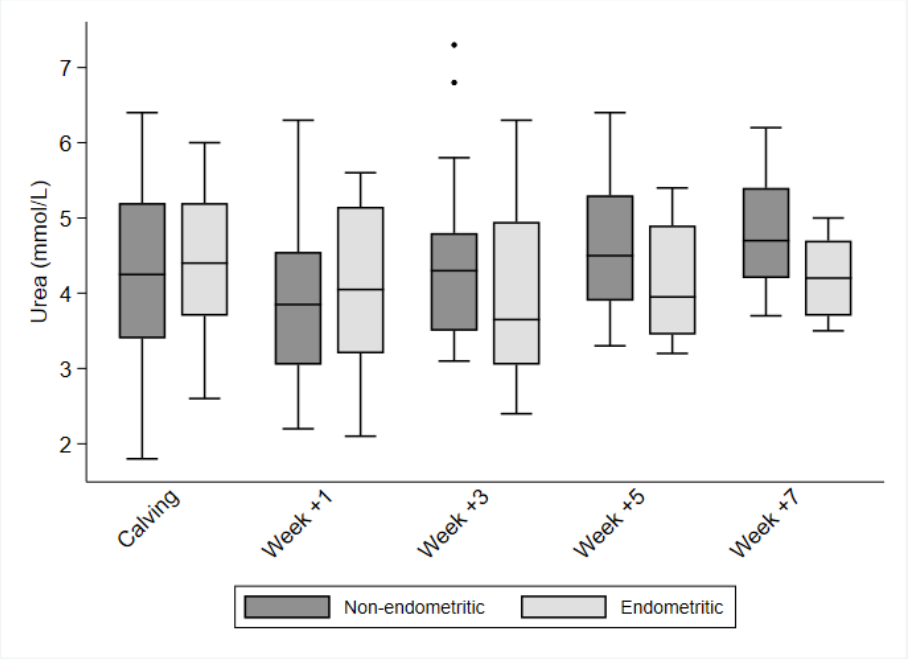


Figure 2.10 Median serum urea concentration of all cows (n = 40) during the study period. No statistically significant differences were found between the median urea concentration at calving and at other timepoints.

A



B

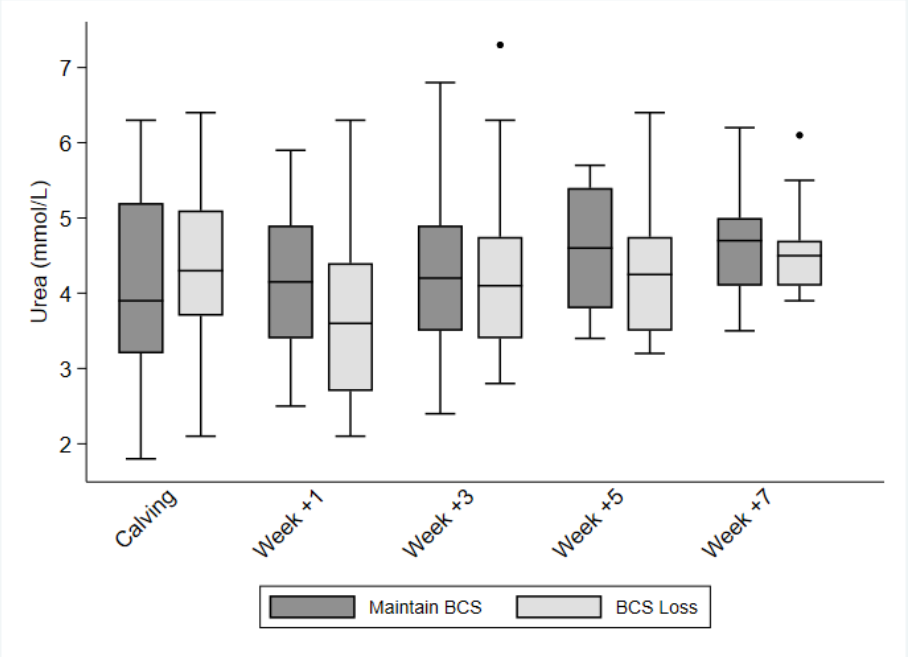


Figure 2.11 Serum urea concentrations (median and interquartile range) at each timepoint grouped by (A) the presence or absence of subclinical endometritis at 5 weeks postpartum, and (B) the maintenance or loss of ≥ 0.5 BCS points in the 5 weeks following calving.

2.3.5 *Serum cortisol*

An overview of serum cortisol concentrations throughout the sampling period is shown in Figure 2.12. Overall, a median increase in the serum cortisol concentration was observed at calving compared to other timepoints. No statistically significant differences were found for serum cortisol concentrations at any timepoint when stratified by the presence of endometritis or change in BCS (Figure 2.13).

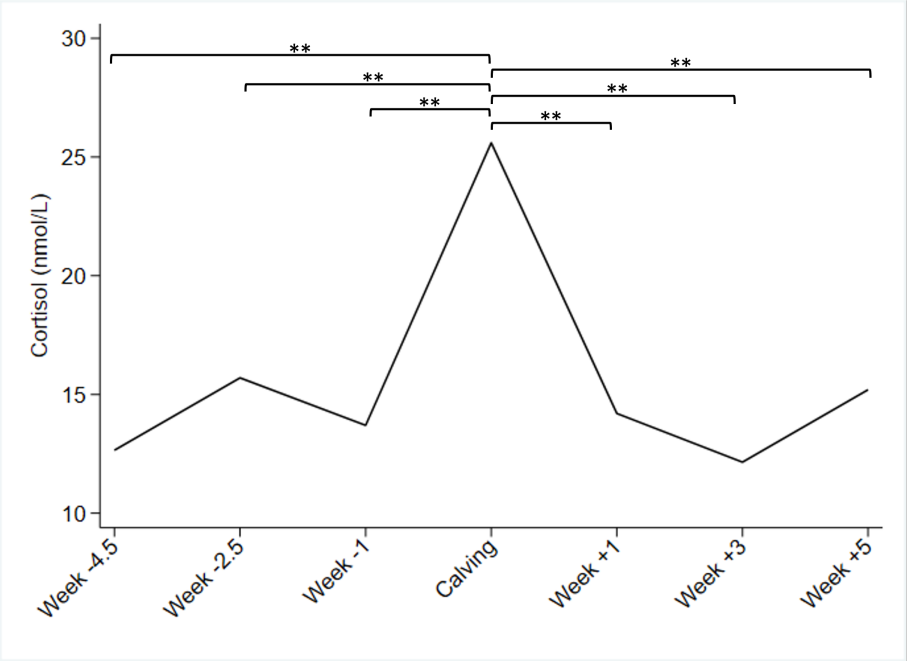
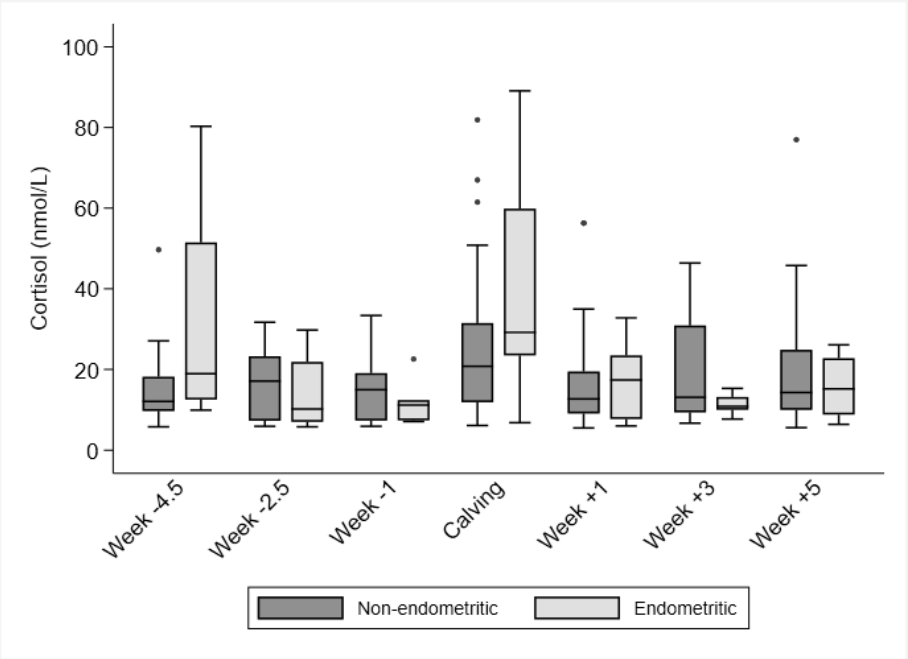


Figure 2.12 Median serum cortisol concentration of all cows (n = 40) during the study period. The median cortisol concentration at calving was compared to every other timepoint. Bars represent statistically significant differences between timepoints; ** $P < 0.01$.

A



B

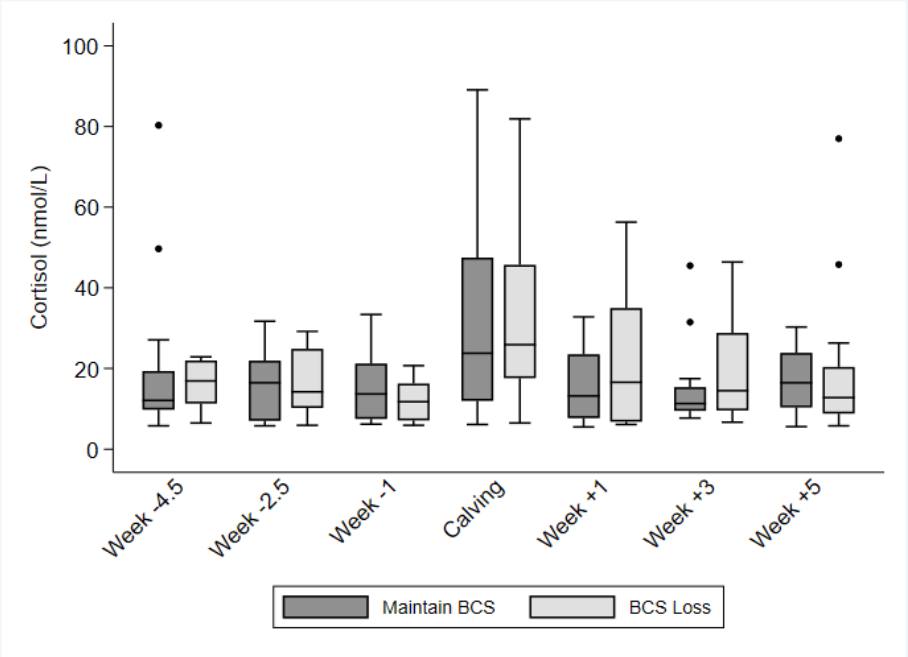


Figure 2.13 Serum cortisol concentrations (median and interquartile range) at each timepoint grouped by (A) the presence or absence of subclinical endometritis at 5 weeks postpartum, and (B) the maintenance or loss of ≥ 0.5 BCS points in the 5 weeks following calving.

2.4 Discussion

This study described the temporal changes in serum metabolites and cortisol concentrations during the periparturient period in a cohort of dairy cows. We identified significant differences in serum total calcium, glucose and BHB concentrations in cows with and without subclinical endometritis. An increase in serum total calcium concentrations at one-week pre-calving, glucose and total calcium concentrations on the day of calving and BHB concentrations one-week following calving were observed in cows that developed endometritis at five weeks postpartum when compared to their non-endometritic counterparts. No statistically significant differences were observed in serum urea or NEFA concentrations between these groups. In this study no statistically significant association was found between BCS loss and the development of endometritis.

Maintaining appropriate serum calcium concentrations during the periparturient period is essential for preserving optimal nerve and muscle tone,[97] and immune cell function.[70] Our results demonstrated increased serum total calcium at one week prior to calving and the day of calving in cows that were later identified with endometritis. This finding has not been previously reported; however, several studies instead report an association between hypocalcemia and metritis. In one such study, cows classified as being at high risk for the development of metritis (having at least one of dystocia, twins or retained placenta) which were diagnosed with subclinical hypocalcemia (at least one serum total Ca measurement of ≤ 2.15 mmol/L between 1 and 3 DIM) were at greater risk of developing metritis than cows that were able to maintain their serum calcium above this value.[70] Another study also found that cows diagnosed with metritis had lower serum total calcium concentrations in the four weeks following parturition than unaffected cows.[97] However, recent data suggest that the pattern and duration of hypocalcemia in the four days following calving is more predictive of disease (ketosis, metritis or displaced abomasum) risk than a transient episode of hypocalcemia in the first 24 hours following calving.[65] It is possible that a period of hypocalcemia was masked in our study population by administration of calcium supplements by farm staff or differences in dietary calcium intake. Furthermore, as our study assessed calcium on the day of calving and then again one week later, it is also possible that cows that went on to develop either transient or persistent hypocalcemia during the first 7 DIM were missed due to our study design. A more frequent sampling might have identified significant fluctuations of calcium in this herd.

Mounting an immune response and partitioning nutrients toward milk production following calving requires large amounts of energy in the form of glucose.[38] Traditionally, it was thought that energy restriction around the time of calving could lead to decreased leukocyte function and increased risk for postpartum infection.[38,55] However, in our study endometritic cows displayed higher serum glucose concentrations than non-endometritic cows at the time of calving and at seven weeks postpartum. Of these two timepoints, only elevations in serum glucose at the time of calving are significant for predicting the risk of endometritis. Our findings are concordant with a study by Galvao *et al*., which showed that cows that developed postpartum uterine disease had greater blood glucose concentrations at the time of calving than their clinically normal counterparts.[41] Moreover, a study by Bicalho *et al.* found that plasma glucose at three DIM was the best predictor for the development of metritis and endometritis, where cows with higher glucose concentrations had 6.6 times higher odds of being diagnosed with metritis and 3.5 times higher odds of developing clinical endometritis when compared with cows with lower glucose concentrations.[103] It is currently unknown whether the high glucose concentrations seen around the time of calving are directly responsible for an increased risk of uterine disease. Although neutrophils are highly dependent on the uptake of extracellular glucose and intracellular glycogen stores to provide energy for chemotaxis and microbial killing, studies have shown that increased glucose concentrations could impair neutrophil adherence and dysfunction.[104,105] However, rather than having a direct effect, the statistically higher glucose concentrations in the endometritic group may instead indicate that these cows experienced more pronounced physiologic stressors at the time of calving. Parturition causes inflammation and an associated transient increase of cortisol and acute phase protein synthesis, leading to hyperglycemia through induction of peripheral insulin resistance and the promotion of hepatic glycogenolysis and gluconeogenesis.[103] It is plausible that higher glucose concentrations in the immediate periparturient period signify a greater degree of physiologic stress and the presence of other known risk factors for the development of endometritis (retained fetal membranes, metritis).[103] Although an association between serum cortisol and the development of endometritis was not found in this study, it is possible the small sample size prevented the identification of significant differences between groups. Clearly, further research is required to investigate the role of increased glucose concentrations and the subsequent development of endometritis.

A normal physiologic response to the increased metabolic demands of lactation is the production of BHB and NEFA, which act as an alternative fuel source for tissues, including the brain and heart.[60] Excessive elevations of serum BHB and/or NEFA concentrations indicate a pronounced state of negative energy balance and have been variably associated with an increased risk of metabolic and infectious disease during the transition period.[38] Our study showed higher serum BHB in endometritic cows at one week postpartum when compared to non-endometritic cows. A previous study demonstrated higher serum concentrations of BHB at one week following calving in cows that developed puerperal metritis compared to clinically normal cows.[97] In line with these findings, it appears that cows with elevated BHB concentrations in the first 3 to 7 DIM are also at greater risk of early removal from the herd, poor fertility and low milk yield.[35] Elevated BHB within the first week of calving is most commonly associated with excessive fat mobilization following the onset of lactation, rather than the inability of the liver to meet the need for gluconeogenesis.[60] Therefore, cows with larger fat stores and high BCS at the time of calving are at increased risk for ketonemia early in lactation. This notion is also reflected in our results where BHB concentrations were found to be significantly elevated at one week postpartum in cows that lost from 0.5 to 0.75 BCS points during the first five weeks of lactation. The precise mechanism behind an elevated BHB and the increased risk of postpartum diseases such as endometritis has not been fully characterized. One study identified decreased NET formation and bactericidal activity of bovine neutrophils exposed *in vitro* to increasing BHB concentrations.[106] Other studies utilizing neutrophils and serum from periparturient cows have also identified an association between elevated BHB and the impairment of neutrophil function.[52,61] Given the crucial role of neutrophils in endometrial health and microbial defenses in the days following calving,[107] it is plausible that early elevations in BHB may place the cow at risk to develop endometritis.

Interestingly, no association between a BCS loss of ≥ 0.5 points and the later development of endometritis was apparent in this study. Excessive loss of body condition is often associated with a more severe state of negative energy balance and an increased risk of disorders such as ketosis.[83] Indicators of a state of negative energy balance were seen in the BCS loss group in the current study with these cows displaying lower glucose concentrations one week prior to calving and higher BHB concentrations one week following calving than cows that maintained their body condition. Two factors likely contributed to this discrepancy; first, the sample size may have been too small to detect significant differences between groups. Second, although the assessment of

BCS was carried out consistently by the author (AC), it is still a subjective measurement and therefore brings with it a degree of variability that may have obscured statistically significant results. A study involving a larger sample size, particularly of cows affected by endometritis, is required to further characterize the relationship between BCS loss and the development of postpartum uterine disease.

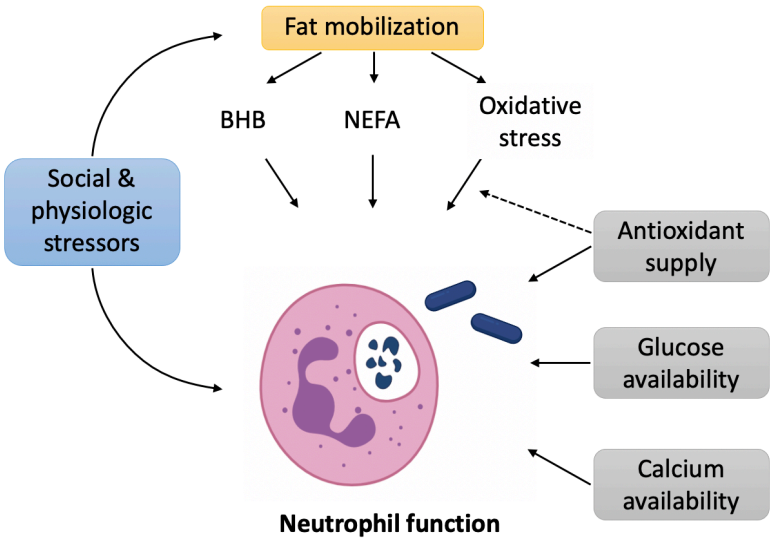
A particular limitation of this study was that feed intake of the individual cows was not recorded due to the farm management system in place. All of the metabolites measured in this study can be influenced by the amount of feed intake;[108] for example, lower intake can lead to lower serum calcium, lower urea, lower glucose and a greater degree of negative energy balance resulting in higher BHB and/or NEFA.[108] To account for this, individual animal feed intake could be measured in future studies using an automatic feeding system.

Another limitation of this study was the inclusion of cows of varying parity. Multiparous cows are typically faced with a higher metabolic demand than primiparous cows due to greater milk yields.[109] This elevated metabolic stress likely translates to altered energy and lipid metabolism, reduced neutrophil function and an increased risk of postpartum disease.[109] Ideally, equal representation of primiparous and multiparous cows should be included in any future investigations to account for these differences.

In conclusion, our results indicate that the development of endometritis is associated with higher serum concentrations of total calcium one week prior to parturition, higher glucose and total calcium concentrations at the time of parturition, and higher BHB concentrations one week postpartum. This information may be useful in understanding early risk factors for the development of endometritis in dairy cows and aid in the development of treatment and preventative regimes.

Transition

Chapter 2 described the changes in serum metabolite and cortisol concentrations during the periparturient period and established potential associations between serum glucose, total calcium and BHB around the time of parturition and the development of endometritis. One mechanism by which perturbations in these metabolites may link to the development of endometritis is through their influence on neutrophil function.[38] Neutrophils play a pivotal role in the early immune response to bacterial contaminants in the postpartum uterus, and the progression and eventual resolution of inflammation.[45] Neutrophil function is affected by a variety of factors, including the metabolic and hormonal factors investigated in Chapter 2, as well as genetic and environmental variables (see Transition Figure 1 below).[38] However, the timing and degree to which neutrophil function is impaired during the periparturient period is only partially understood and is, therefore, investigated further in the following chapter.



Transition Figure 1: Factors that influence the function of neutrophils in dairy cows. This scheme is simplified as there are likely interactions among these known factors and others not depicted here such as the cow's genetics. Feed intake lags milk production in early lactation, so most dairy cows will

mobilize fat stores. Nutritional formulation and feeding management determine the potential supply of immune system inputs, with additional variability introduced by social group and environmental stressors. BHB, β-hydroxybutyrate; NEFA, Non-esterified fatty acids. Figure adapted from LeBlanc.[38]

3 Neutrophil function during the periparturient period and its relationship to endometritis in postpartum dairy cows

3.1 Introduction

Following parturition there is a rapid influx of leukocytes, primarily neutrophils, into the uterine lumen to aid in eliminating bacteria and repairing the endometrial epithelium.[7] For neutrophils to perform this function well, they must have the ability to migrate into tissues, ingest bacteria and have efficient bactericidal functions such as the oxidative burst.[45] Previous studies have identified neutrophil dysfunctionality in the immediate periparturient period and have suggested this is involved in the pathogenesis of postpartum uterine disease.[51,52,110] However, few studies have investigated neutrophil functionality over an extended period to identify possible early perturbations in immune function.

Normal changes in the blood leukocyte profile over the periparturient period include a leukocytosis due to increased neutrophil counts in the days leading up to calving, followed by a decrease in the days following calving due to neutrophil migration to peripheral tissues.[44] However, there is a paucity of information on the effect of endometritis on the blood leukocyte profile in dairy cows.

We hypothesized that endometritic cows experience greater decreases in neutrophil function during the periparturient period. Therefore, the objectives of this study were to (a) determine blood neutrophil function (phagocytic ability and oxidative burst) and overall leukocyte profile over the periparturient period in relation to endometritis development; and (b) assess the effect of BCS change during the five weeks following parturition on blood neutrophil function and leukocyte profile. Understanding which aspects of neutrophil function are altered, and at what time, may aid in the understanding of the pathophysiology of endometritis. Furthermore, understanding the role that fluctuations in body condition play in affecting neutrophil function would also allow the development of heard health strategies to optimize health and performance.

3.2 Methods

3.2.1 *Animals*

Animal details and sampling protocols are described in Chapter 2 of this thesis.

3.2.2 *Complete blood cell count*

Samples collected for CBC analysis were submitted to Prairie Diagnostic Services Inc. (Saskatoon, SK, Canada). Leukocyte differential counts were obtained using an ADVIA 2120 (Siemens Healthcare Diagnostics, NY, USA) analyzer and verified on blood smear review by trained laboratory staff. The manual count was used for statistical analysis.

3.2.3 *Neutrophil function evaluation*

3.2.3.1 Phagocytosis assay

In vitro phagocytic ability of neutrophils was determined using a previously described method[111] with minor modifications. Briefly, the addition of fluorescein isothiocyanate (FITC)-labeled *Staphylococcus aureus* to whole blood allows for the quantification of neutrophils containing ingested bacteria based upon the generation of a green fluorescence signal. 25 µL of stabilized FITC-labelled *S. aureus* suspension (4 x 10^5 bacteria/µL, Cat # 352058, Fisher Scientific, Ottawa, ON, Canada) was added to 2 of 3 aliquots of 100 µL of heparinized whole blood. Of the two aliquots with added bacteria, one aliquot acted as the experimental sample while the second aliquot was used as the isotype control. The aliquot without added bacteria acted as the negative control. All tubes were incubated at 37 ºC for 20 min, with vortex agitation performed every 5 minutes during the incubation. Phagocytosis was stopped by placing all tubes on ice. To eliminate fluorescence associated with non-phagocytosed bacteria, 100 µL of quenching solution (750 µl of 0.4 % trypan blue solution (Cat # 15250-061, Fisher Scientific, Ottawa, ON, Canada) plus 11.25 ml of 0.1 M citrate buffer pH 4 (Cat # AC258580010, Fisher Scientific, Ottawa, ON, Canada)) were added to each tube and incubated on ice for one minute. The cells were washed twice with 4 mL phagocytosis assay buffer (PBS + 1 % fetal bovine serum) and pelleted by centrifugation at 3500 rpm (SERO-FUGE 2002 series, Becton Dickinson) for 5 minutes. Cells were resuspended in 2 mL RBC lysis solution (10 x RBC Lysis Buffer (Cat # 420301, BioLegend, San Diego, CA, USA) diluted 1:10 in MΩH_2O) and incubated at 37 ºC for 5 min. After

centrifugation for 10 min at 3500 rpm, the pellets were resuspended in 100 µl phagocytosis assay buffer and kept on ice. 50 µL of primary antibody (anti-bovine granulocyte monoclonal antibody, (Cat # WS0609B-100, Kingfisher Biotech, Saint Paul, MN, USA) diluted 1:50 in phagocytosis assay buffer) was added to each tube except the isotype control, and all tubes were then incubated on ice for 15 minutes. Cells were washed twice with 400 µL phagocytosis assay buffer, prior to addition of 100 µL secondary antibody (Allophycocyanin AffiniPure F(ab')$_2$ Fragment Donkey Anti-Mouse IgG, (Cat # 715-136-151, Jackson ImmunoResearch Laboratories, PA, USA)) diluted 1:100 in phagocytosis assay buffer) to all tubes, including the isotype control. After incubation and washing as described for the previous addition of primary antibody, cells were resuspended in 200 µL phagocytosis assay buffer and filtered prior to flow cytometry.

3.2.3.2 Oxidative burst assay

In vitro oxidative burst ability of neutrophils was determined using a method based on the Neutrophil/Monocyte Respiratory Burst Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) with minor modifications. This assay allows the detection of reactive oxygen species formed by the membrane-bound NADPH oxidase upon stimulation with the Protein kinase C ligand phorbol 12-myristate 13-acetate (PMA). The addition of fluorogenic dihydrorhodamine 123 (DHR123) allows the detection of ROS through its oxidation to the membrane-adherent and fluorescent rhodamine 123. 10 µL of DHR123 (0.5 µg/mL) was added to 3 separate 100 µL aliquots of heparinized whole blood in test tubes (Fisher Scientific, Ottawa, ON, Canada), which were then incubated at 37 ºC for 15 min. Following incubation, 25 µL of PMA (200 nmol/L) was added to 2 of the 3 aliquots. Of the 2 aliquots with added PMA, one aliquot acted as the experimental sample while the second aliquot acted as the isotype control. The aliquot without added PMA acted as the negative control. All tubes were incubated at 37 ºC for 15 min and then placed on ice. Lysing of RBCs and addition of primary and secondary antibodies was performed as described for the phagocytosis assay. Finally, each pellet was resuspended in 500 µL oxidative burst assay buffer (RMPI 1640 base medium (Fisher Scientific, Ottawa, ON, Canada) with 1 µmol/mL calcium chloride (Cayman Chemical, Ann Arbor, MI, USA) and 10 mg/mL bovine serum albumin (Cayman Chemical, Ann Arbor, MI, USA)) and filtered prior to flow cytometry.

3.2.4 *Flow cytometry*

Flow cytometry data were acquired using the CytoFLEX Flow cytometer (Beckman Coulter Canada Inc., Mississauga, ON, Canada) and analyzed using FLOWJO software (Tree Star, Inc., Ashland, OR, USA). For both assays, blood leukocytes were first plotted based on forward vs. side scatter properties. Neutrophils were then identified using the anti-bovine neutrophil – APC vs. side scatter plot, and data acquisition was continued until a total of 15,000 neutrophils were counted for the phagocytosis assay, or 15,000 leukocytes were counted for the oxidative burst assay. The percentage of neutrophils was calculated by gating on the total leukocyte population. For both the phagocytosis and oxidative burst assays, neutrophils identified based on the anti-bovine neutrophil – APC vs. side scatter plot were gated to plot anti-bovine neutrophil – APC vs. FITC. Both the percentage and median fluorescence intensity (MFI) of neutrophils undergoing oxidative burst or containing FITC-labelled *S. aureus* were recorded. The fluorescence intensity was proportional to the degree of oxidation or phagocytosis displayed per neutrophil. Fluorescence was acquired on a log scale and the difference between the MFI of positive cells and the MFI of the control samples was calculated. Examples of the gating strategy used can be seen in Supplemental Figures 1 and 2.

3.2.5 *Statistical Analysis*

All statistical analyses were performed using Stata (Version 17.0, College Station, TX, USA). The data was assessed for normality using the Shapiro-Wilk test. Cows were assigned to one of two study groups based on the presence or absence of subclinical endometritis at five weeks postpartum. For each continuous variable (individual leukocyte counts, phagocytosis percentage, phagocytosis MFI, oxidative burst percentage, oxidative burst MFI), the Wilcoxon Rank Sum test was used to ascertain which timepoints were significantly different between the two groups. P-values < 0.05 were considered significant.

3.3 Results

3.3.1 *Peripheral leukocyte profile*

3.3.1.1 Neutrophils

Temporal changes in the median blood neutrophil count over time for all cows are shown in Figure 3.1.

Endometritic cows had significantly lower segmented neutrophil counts one week following parturition ($P < 0.01$) than non-endometritic cows (Figure 3.2 A). When stratified by BCS maintenance or loss, cows that lost body condition had significantly lower segmented neutrophil counts at 2.5 weeks prior to calving ($P = 0.04$) than those that maintained body condition (Figure 3.2 B).

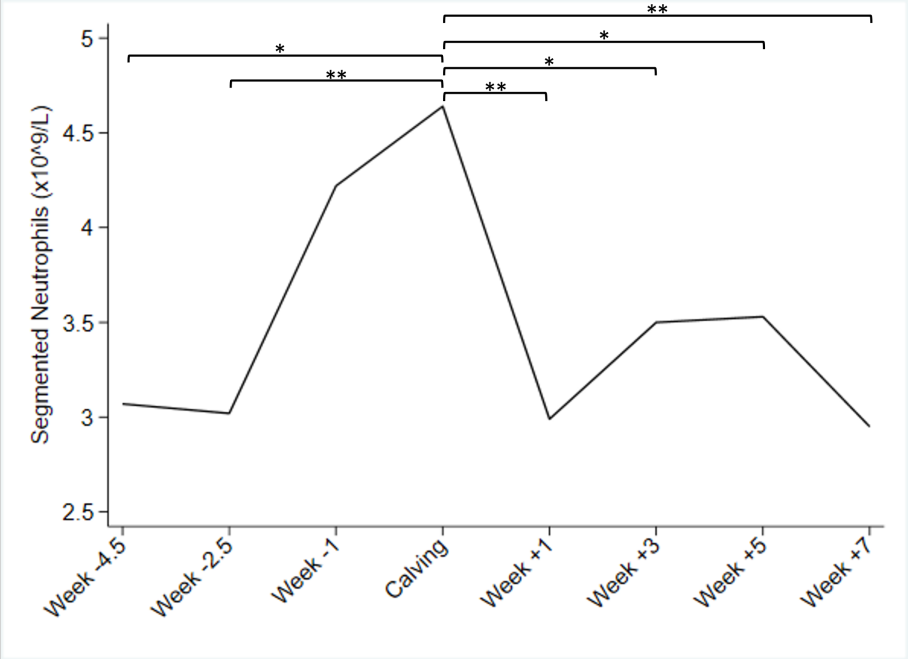
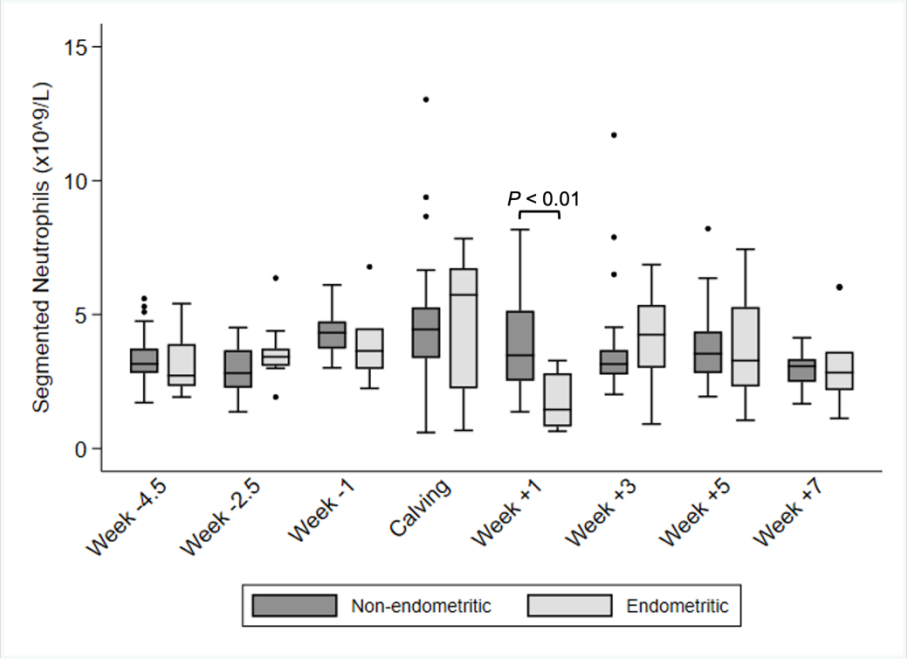


Figure 3.1 Median segmented neutrophil count of all cows (n = 40) during the study period. The median segmented neutrophil count at calving was compared to every other timepoint. Bars represent statistically significant differences between timepoints; * P < 0.05; ** P < 0.01.

A



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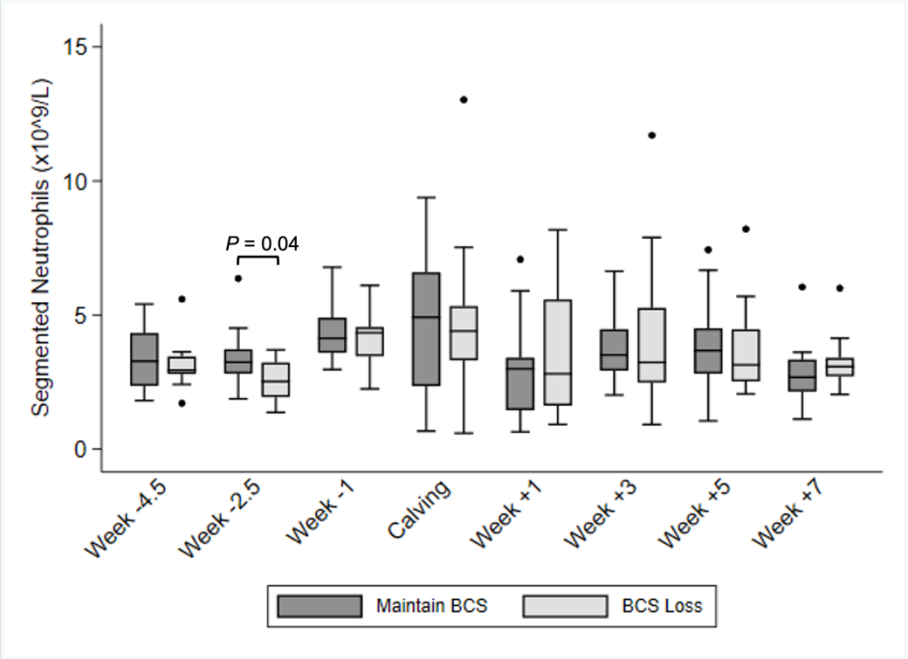


Figure 3.2 Blood segmented neutrophil count (median and interquartile range) at each timepoint grouped by (A) the presence or absence of subclinical endometritis at 5 weeks postpartum, and (B) the maintenance or loss of ≥ 0.5 BCS points in the 5 weeks following calving.

3.3.1.2 Lymphocytes

Temporal changes in the median blood lymphocyte count over time for all cows are shown in Figure 3.3.

Endometritic cows had significantly lower lymphocyte counts at 2.5 weeks prior to calving ($P = 0.02$) than non-endometritic cows (Figure 3.4 A). When stratified by BCS maintenance or loss, cows that lost body condition had lower lymphocyte counts at 5 weeks following parturition ($P < 0.01$) than those that maintained body condition (Figure 3.4 B).

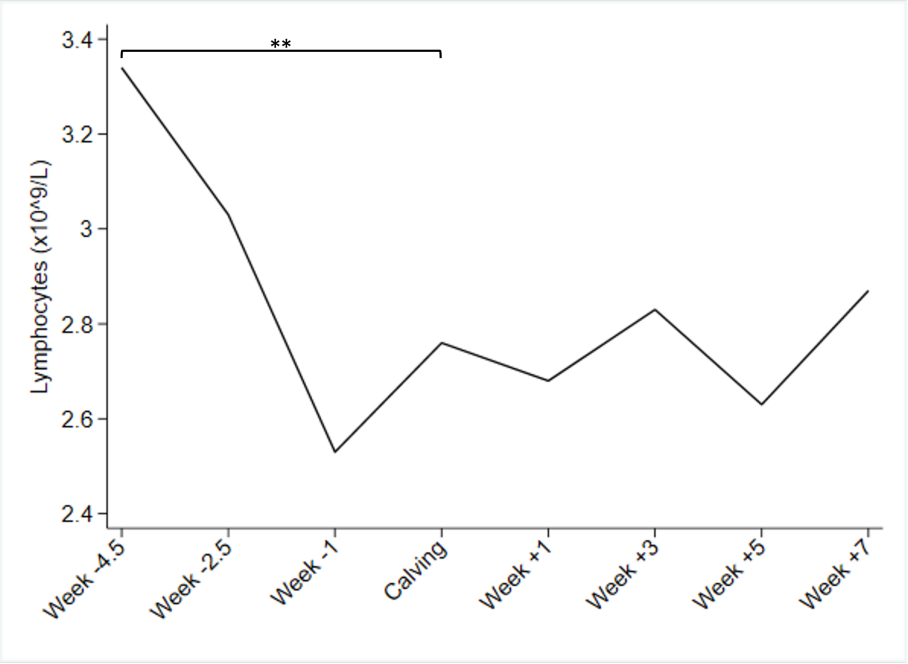
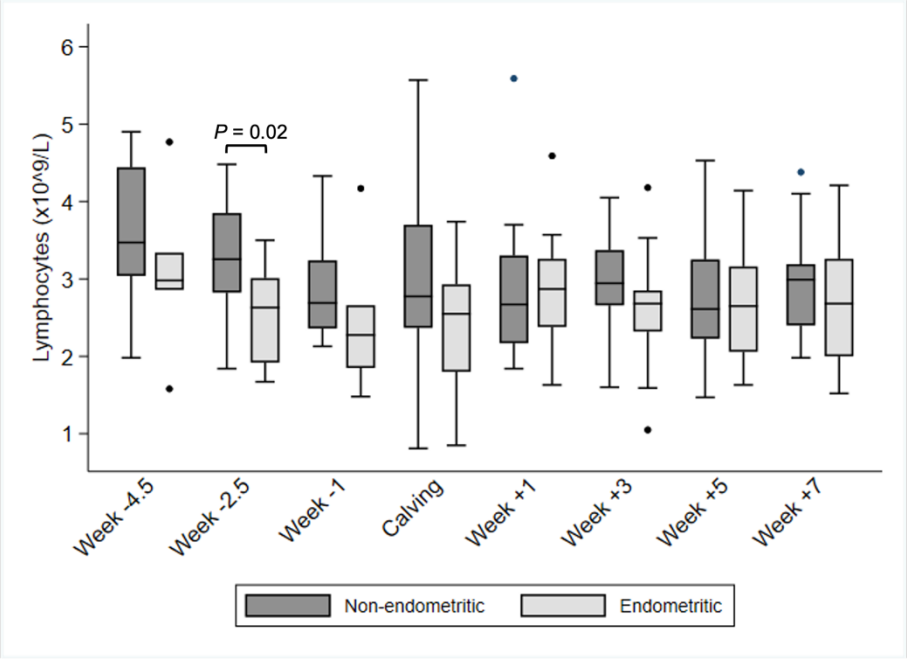


Figure 3.3 Median lymphocyte count of all cows (n = 40) during the study period. The median lymphocyte count at calving was compared to every other timepoint. Bar represents statistically significant differences between timepoints; ** $P < 0.01$.

A



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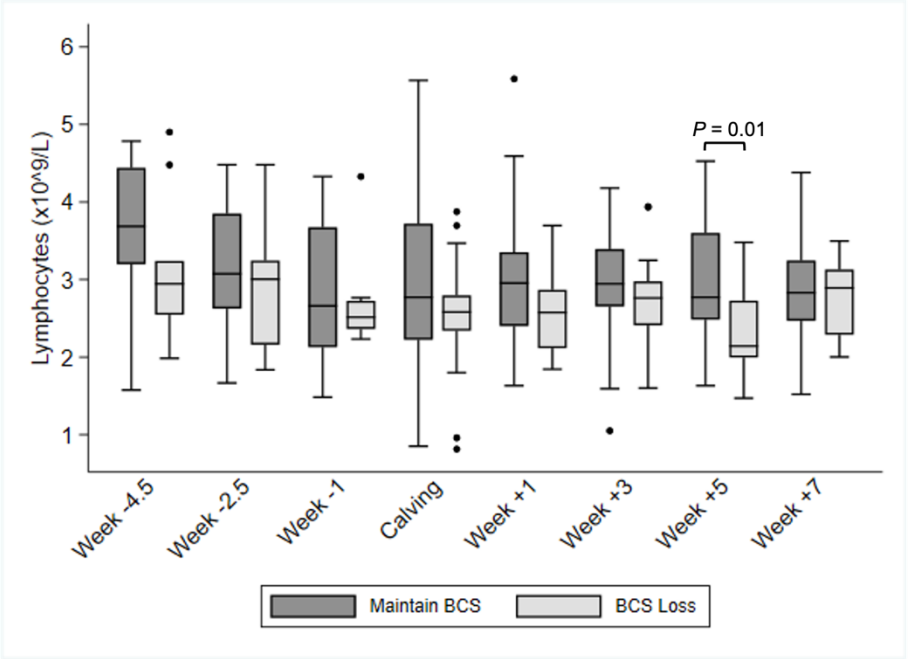


Figure 3.4 Blood lymphocyte counts (median and interquartile range) at each timepoint grouped by (A) the presence or absence of subclinical endometritis at 5 weeks postpartum, and (B) the maintenance or loss of ≥ 0.5 BCS points in the 5 weeks following calving.

3.3.1.3 Monocytes

Temporal changes in the median blood monocyte count over time for all cows are shown in Figure 3.5.

No statistically significant differences were found for monocyte counts at any timepoint when stratified by the presence of endometritis or change in BCS (Figure 3.6).

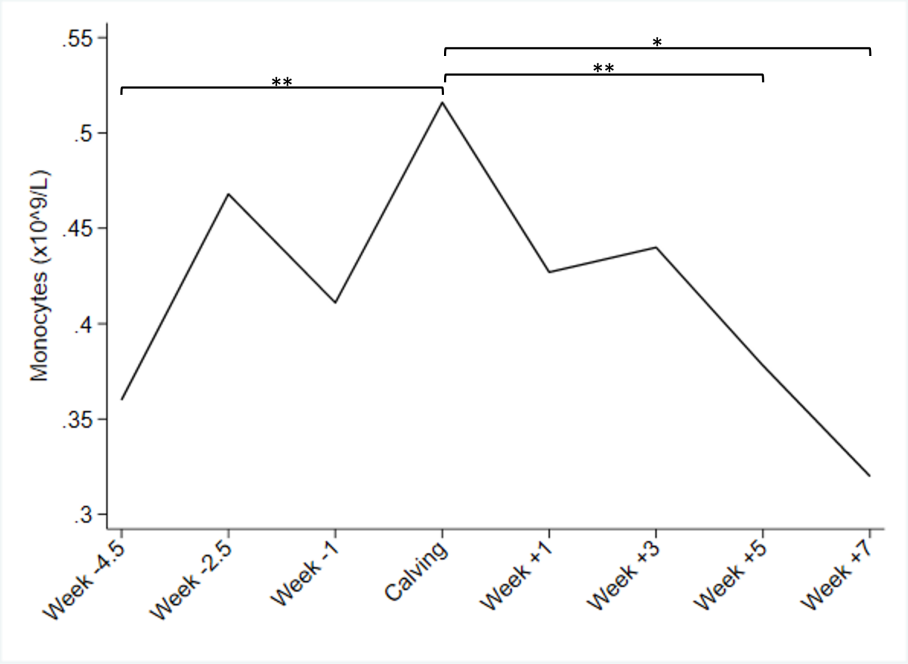
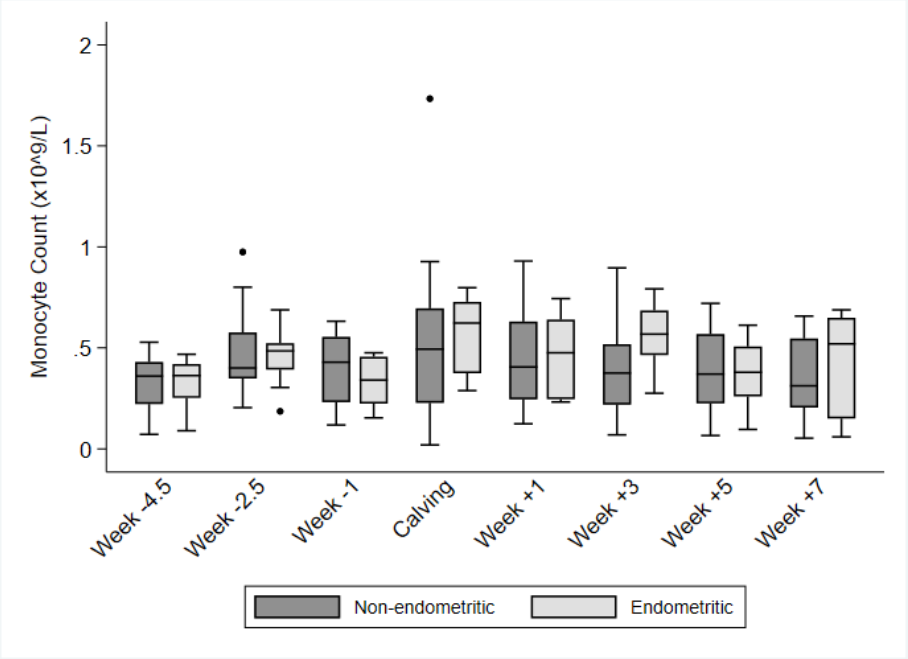


Figure 3.5 Median monocyte count of all cows (n = 40) during the study period. The median monocyte count at calving was compared to every other timepoint. Bars represent statistically significant differences between timepoints; * $P < 0.05$; ** $P < 0.01$.

A



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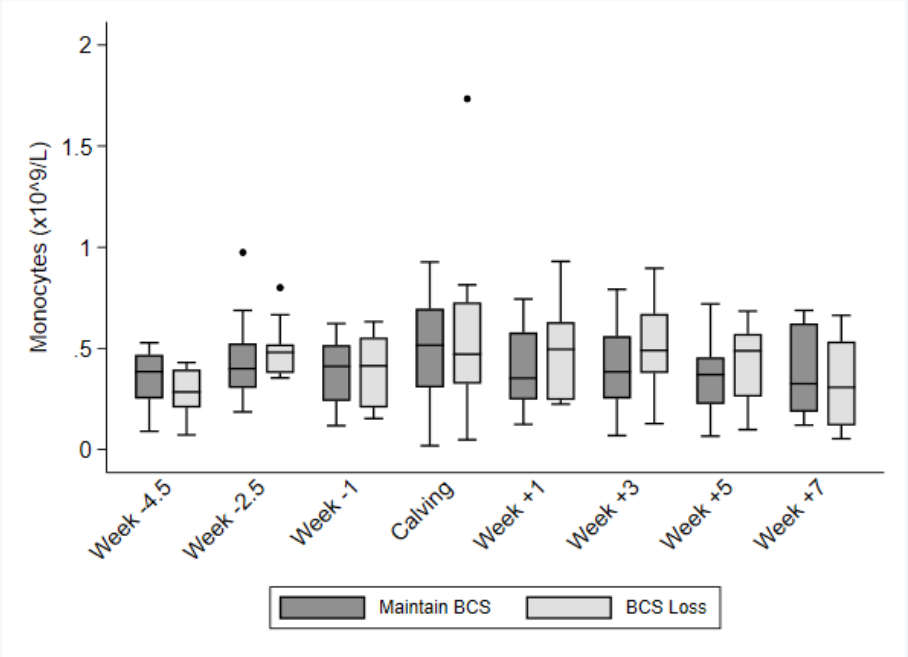


Figure 3.6 Blood monocyte counts (median and interquartile range) at each timepoint grouped by (A) the presence or absence of subclinical endometritis at 5 weeks postpartum, and (B) the maintenance or loss of ≥ 0.5 BCS points in the 5 weeks following calving.

3.3.2 *Neutrophil Phagocytosis Assay*

Temporal changes in the median percentage of neutrophils containing phagocytosed bacteria and MFI over time for all cows are shown in Figures 3.7 and 3.9.

Endometritic cows had a significantly lower percentage of neutrophils containing phagocytosed labeled bacteria at one week following parturition ($P = 0.05$) than non-endometritic cows (Figure 3.8 A). Endometritic cows also had a significantly lower MFI value at 5 weeks following parturition ($P < 0.01$) than non-endometritic cows (Figure 3.10 A). No significant differences were found between the percentages of neutrophils containing phagocytosed labeled bacteria or MFI values when stratified by BCS maintenance or loss at any timepoint (Figures 3.8 B and 3.10 B).

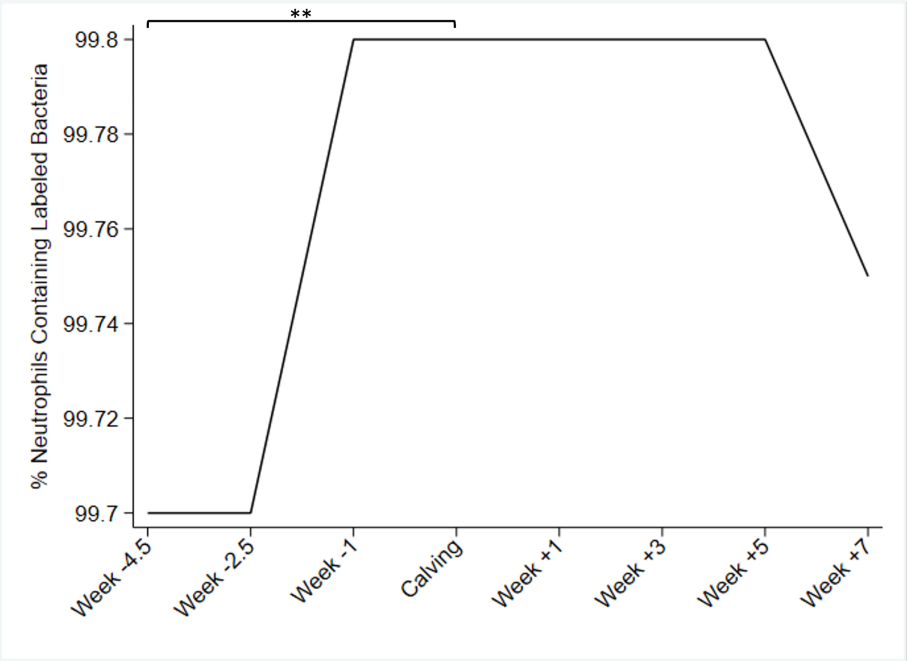
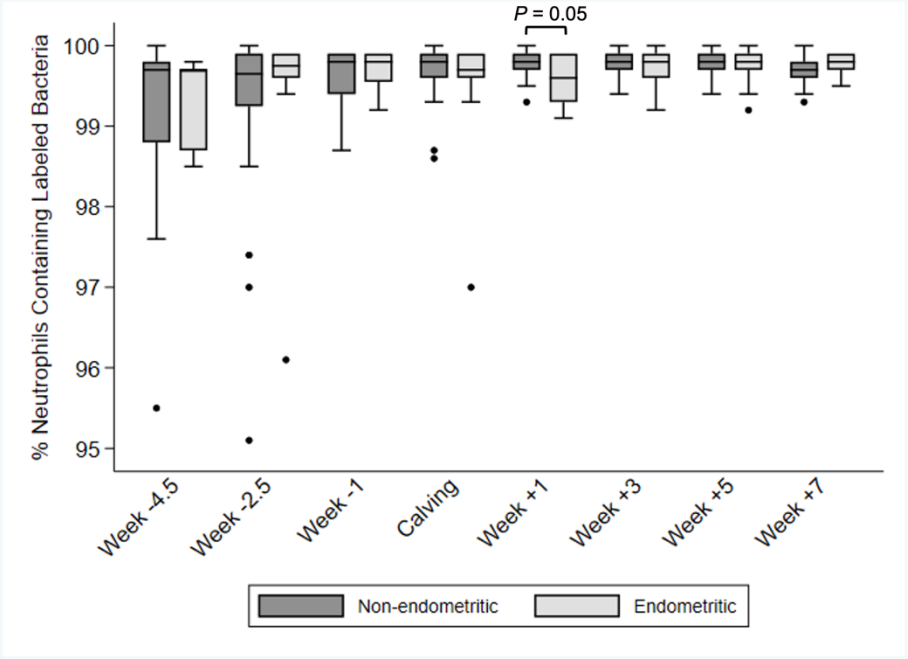


Figure 3.7 Median percentage of neutrophils containing labeled bacteria from all cows (n = 40) during the study period. The median percentage of neutrophils containing labeled bacteria at calving was compared to every other timepoint. Bar represents statistically significant differences between timepoints; ** $P < 0.01$.

A



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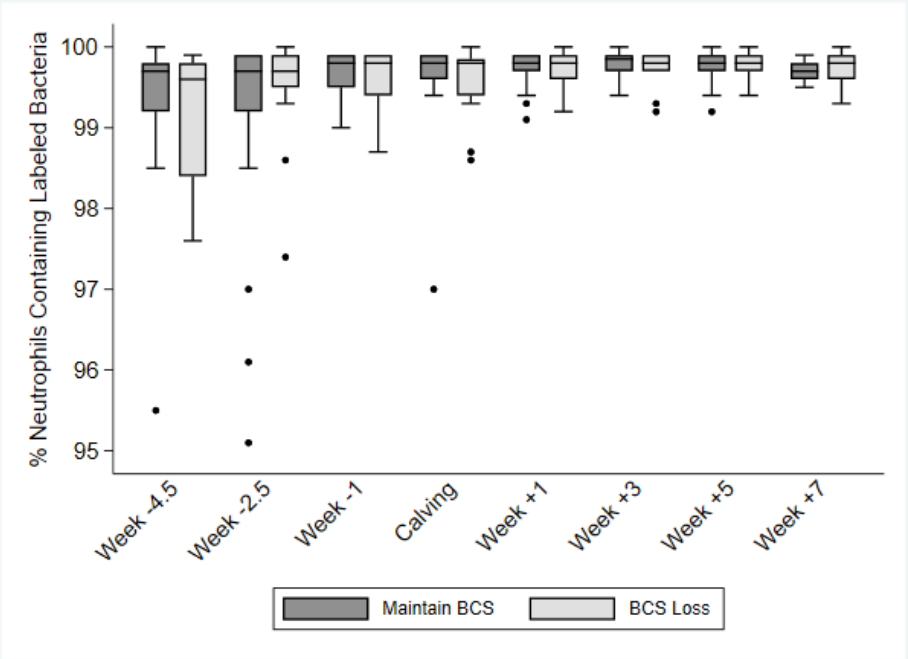


Figure 3.8 Percentage of neutrophils (median and interquartile range) containing phagocytosed bacteria at each timepoint grouped by (A) the presence or absence of subclinical endometritis at 5 weeks postpartum, and (B) the maintenance or loss of ≥ 0.5 BCS points in the 5 weeks following calving.

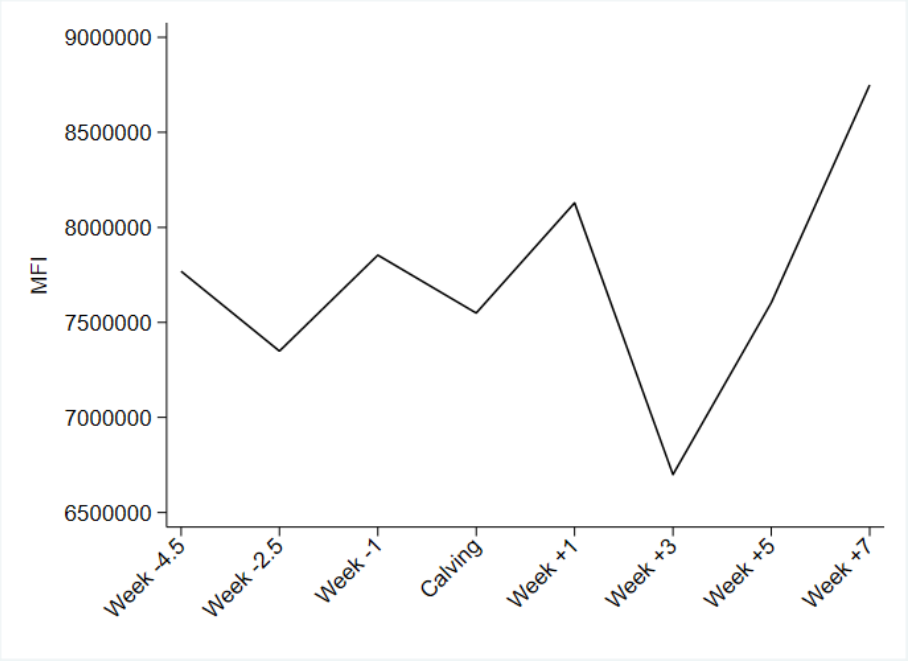
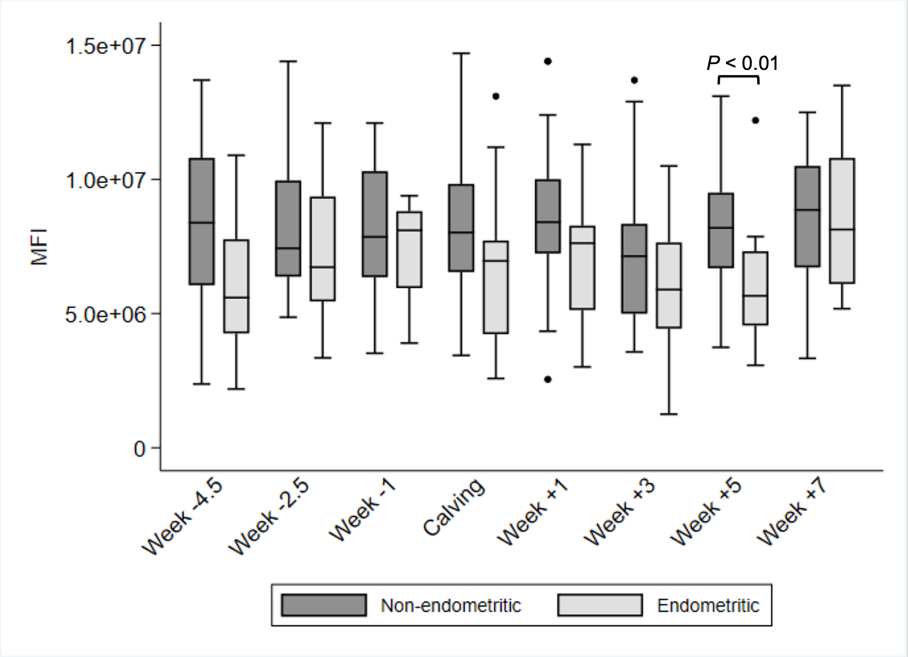


Figure 3.9 Median MFI of the phagocytosis assay for all cows (n = 40) during the study period. No statistically significant differences were found between the median MFI at calving and at other timepoints.

A



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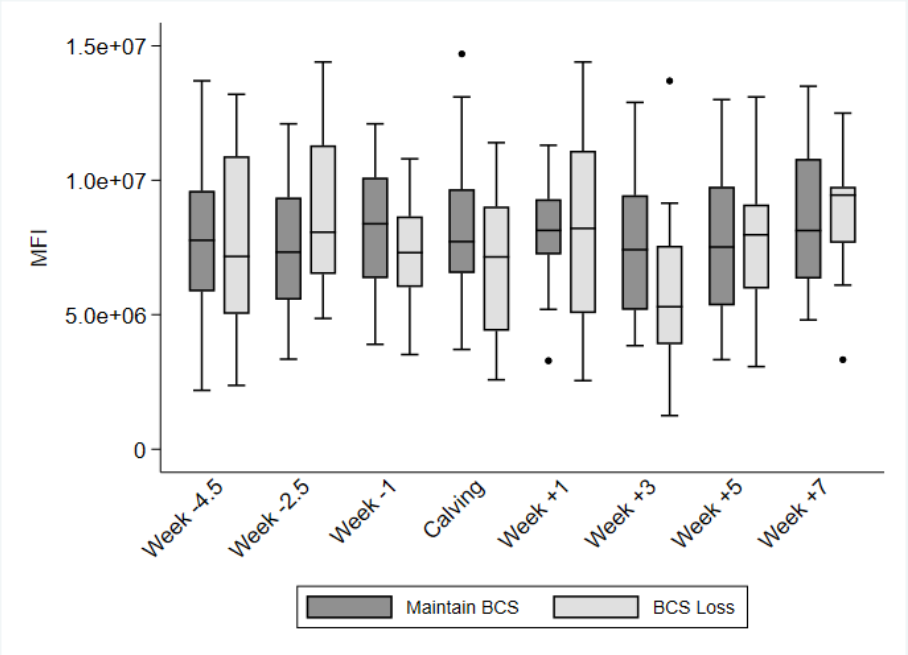


Figure 3.10 MFI (median and interquartile range) for the neutrophil phagocytosis assay at each timepoint grouped by (A) the presence or absence of subclinical endometritis at 5 weeks postpartum, and (B) the maintenance or loss of ≥ 0.5 BCS points in the 5 weeks following calving.

3.3.3 *Neutrophil Oxidative Burst Assay*

An undetermined technical error led to significant differences between data collected in 2019 vs. 2020, rendering it unsuitable for use. A summary of the data is presented in Supplemental Figures 3 and 4.

3.4 Discussion

This study described the temporal changes in blood leukocyte profile and neutrophil function during the periparturient period in a cohort of dairy cows. We identified statistically significant differences in total neutrophil and lymphocyte counts and neutrophil phagocytic ability between healthy cows and those that developed subclinical endometritis. Endometritic cows had lower neutrophil counts and reduced neutrophil phagocytic ability at one week postpartum, and lower lymphocyte counts at 2.5 weeks prior to parturition when compared to their non-endometritic counterparts.

The previously described trend of a leukocytosis prior to parturition followed by a decrease in the first week postpartum[44] was also observed in our cohort of cows. However, published studies assessing differences in the leukocyte profile of endometritic cows are lacking. In our study, endometritic cows had lower lymphocyte counts 2.5 weeks prior to parturition and lower neutrophil counts one week following parturition. It seems plausible that the lower neutrophil count one week following parturition in endometritic cows is due to a greater influx of neutrophils into peripheral tissues – namely, the uterus and/or mammary gland and, therefore, indicates a more severe inflammatory state than that experienced by non-endometritic cows. The cause of the lower lymphocyte count 2.5 weeks prior to parturition in endometritic cows is less clear. One of the most common causes for lymphopenia in domestic mammals is glucocorticoid-induced apoptosis of lymphocytes.[112] Therefore, this finding may indicate that endometritic cows experience greater physiologic stress and the associated release of cortisol in the weeks leading up to parturition than their non-endometritic counterparts, although this was not reflected in our results assessing cortisol concentrations in Chapter 2. In contrast to our findings, a study comparing the blood leukocyte profile of endometritic vs. non-endometritic cows in the four weeks following parturition found that endometritic cows had significantly higher total leukocyte, neutrophil, lymphocyte and monocyte counts than non-endometritic cows at all time points examined.[43] Like the present study, the number of cows included in their endometritic group was low (n = 11). Therefore, larger studies

are required to establish more robust relationships between the blood leukocyte profile around the time of parturition and the development of endometritis.

As discussed previously, BCS is widely used as a proxy for the overall nutritional status of the cow and their risk of metabolic disease following parturition.[88] However, few studies have assessed the effect of gain or loss of BCS during the periparturient period on the leukogram of dairy cows. This study demonstrated that cows classified as losing body condition had lower neutrophil counts at 2.5 weeks prior to parturition and lower lymphocyte counts at five weeks following parturition than cows that maintained body condition. It is possible that because these cows often experience a greater degree of metabolic stress, cortisol-induced apoptosis of lymphocytes occurs to a greater degree than in cows that maintain body condition.[112] However, the cause for the decreased neutrophil count at 2.5 weeks prior to parturition is not immediately apparent. In line with our findings, a separate study found higher BCS around the time of parturition had a weak negative correlation with total leukocyte and lymphocyte count; however, the overall effect of BCS on blood leukocytes was found to be minimal.[113]

The phagocytic ability of blood neutrophils has been studied as an indicator of neutrophil function.[44,51,114] In these studies, the phagocytic percentage remained above 80 % during the periparturient period;[44,51,114] and this trend was also reflected in our results with the majority of our samples having a phagocytic percentage above 90 %. We also found that endometritic cows had a lower percentage of neutrophils containing phagocytosed bacteria at one week postpartum than their non-endometritic counterparts and this suggests a reduced functionality of these cells. Our observations are similar to previous studies where blood neutrophil phagocytic ability was found to be reduced in endometritic cows during the immediate periparturient period.[43,52] However, although we observed statistically significant differences in neutrophil phagocytic ability, it is arguable whether these differences are of sufficient magnitude to be biologically significant. Much like in our study, Mateus *et al*.[44] found that blood neutrophil phagocytic activity remained consistently high throughout the periparturient period both in cows that later developed metritis or endometritis and in cows that remained healthy. They concluded that blood neutrophil phagocytic ability is not likely to be relevant to the establishment and persistence of endometritis.[44] Furthermore, it has yet to be determined whether indicators of blood neutrophil function can be extrapolated to neutrophil function within the uterine environment. For example, Lietaer *et al*.[115] found that the correlation between blood and uterine neutrophil function was poor when assessed

via flow cytometry; however, their study only included healthy cows from 9 days postpartum. Further studies are needed to assess the correlation between blood and uterine neutrophil function in clinically healthy and endometritic cows.

Another method of inferring the phagocytic ability of neutrophils is assessing the MFI. In contrast to the proportion of neutrophils containing labelled bacteria, the MFI value instead indicates the number of bacteria engulfed per cell. Therefore, a higher MFI value indicates more FITC labelled bacteria have been phagocytosed. In our study, endometritic cows had a lower MFI value than non-endometritic cows at five weeks postpartum. This suggests that the blood neutrophils in endometritic cows had reduced phagocytic ability at the time when normal uterine involution is nearing completion. If this finding translates into reduced phagocytic ability within the endometrium, this may provide evidence that reduced neutrophil function is involved in the persistence of bacterial infection within the uterus and the development of endometritis. However, this finding should be interpreted with caution due to the limitations discussed earlier.

In summary, our results suggest that the development of endometritis may be associated with reduced neutrophil phagocytic ability, as indicated by a lower percentage of neutrophils containing bacteria at one week postpartum and lower MFI five weeks postpartum in endometritic cows. This information may be useful in understanding the pathophysiology behind the development of endometritis in dairy cows, and aid in the development of preventative regimes.

4 General discussion and future directions

4.1 Discussion

Our studies identified differences in both key metabolites (glucose, calcium and BHB) and blood neutrophil phagocytic ability in endometritic cows when compared to their non-endometritic counterparts. Interestingly, most of the significant differences detected between these groups were observed one week on either side of parturition, further emphasizing the importance of this period for long-term uterine health.[38] Although these results were presented and discussed separately, the development of endometritis is almost certainly multifactorial with a combination of environmental, nutritional and individual variables all interacting to result in disease.[7] For example, all three of the metabolites associated with the development of endometritis in this study not only influence neutrophil phagocytic ability,[38] but also play key roles in intracellular signal transduction and muscle contractility (calcium)[68] and the provision of substrates for ATP production to peripheral tissues (glucose and BHB).[59] When translating our findings and those of previous studies into clinical applications, the evidence seems to support the use of management practices aimed at minimizing negative energy balance and the incidence of hypocalcemia in the periparturient period to maintain appropriate immune function. However, there is insufficient data to make specific recommendations that would purposely enhance neutrophil function or reduce the incidence of diseases thought to be consequences of impaired immune function.

4.2 Limitations

There were several limitations to this study. The small sample size of endometritic cows limited the statistical power of the analyses, meaning that statistically significant differences between groups may have been missed. This is particularly important when assessing indices that have low variability and/or tight homeostatic control, such as serum total calcium and glucose concentration. Furthermore, due to the small sample size and limited study population available, we were also unable to group cows based on parity or exclude cows that had other comorbidities

that may complicate the interpretation of our findings. It is thought that primiparous cows are at decreased risk of profound negative energy balance due to lower lactational demands,[48] whereas multiparous cows experience a greater negative energy balance,[81] which may lead to different metabolic profiles between the two groups. Similarly, cows with comorbidities such as mastitis may also show alterations in their metabolic and leukocyte profile that are not directly related to endometritis or body condition. Ideally, future studies would include a larger sample size, equal numbers of primiparous and multiparous cows and exclude those that develop other comorbidities so that specific recommendations that would purposely enhance neutrophil function can be made. However, many cows in a typical commercial farm setting will often experience one or more comorbidities.[96] Therefore, exclusion of such cows may not be required for the specific purpose of making general recommendations to farmers to reduce the incidence of endometritis on their farm.

Finally, the identification of subclinical endometritis using cytology may be affected by the presence of high numbers of epithelial clumps within the sample. When performing the cell differential in such samples, large clumps of epithelial cells were excluded which could, therefore, introduce bias. In these circumstances, resampling to obtain a well-prepared sample may be optimal but is often not practical. In our study, the effect of epithelial clumping is likely minimal as the majority of endometritic cows had clear cytologic evidence of inflammation.

4.3 Future Directions

This study characterized the temporal changes in key metabolites and blood neutrophil function in endometritic and non-endometritic cows and, in doing so, identified several timepoints at which statistically significant differences exist between these two groups. Further analysis of the data will include multiple logistic regression to identify variables that best predict the development of endometritis, and correlate neutrophil phagocytic ability to the concentrations of each metabolite. It is hoped that this information will lead to improved detection of at-risk cows, allowing early intervention and overall improvement of the health, productivity, and longevity of the herd.

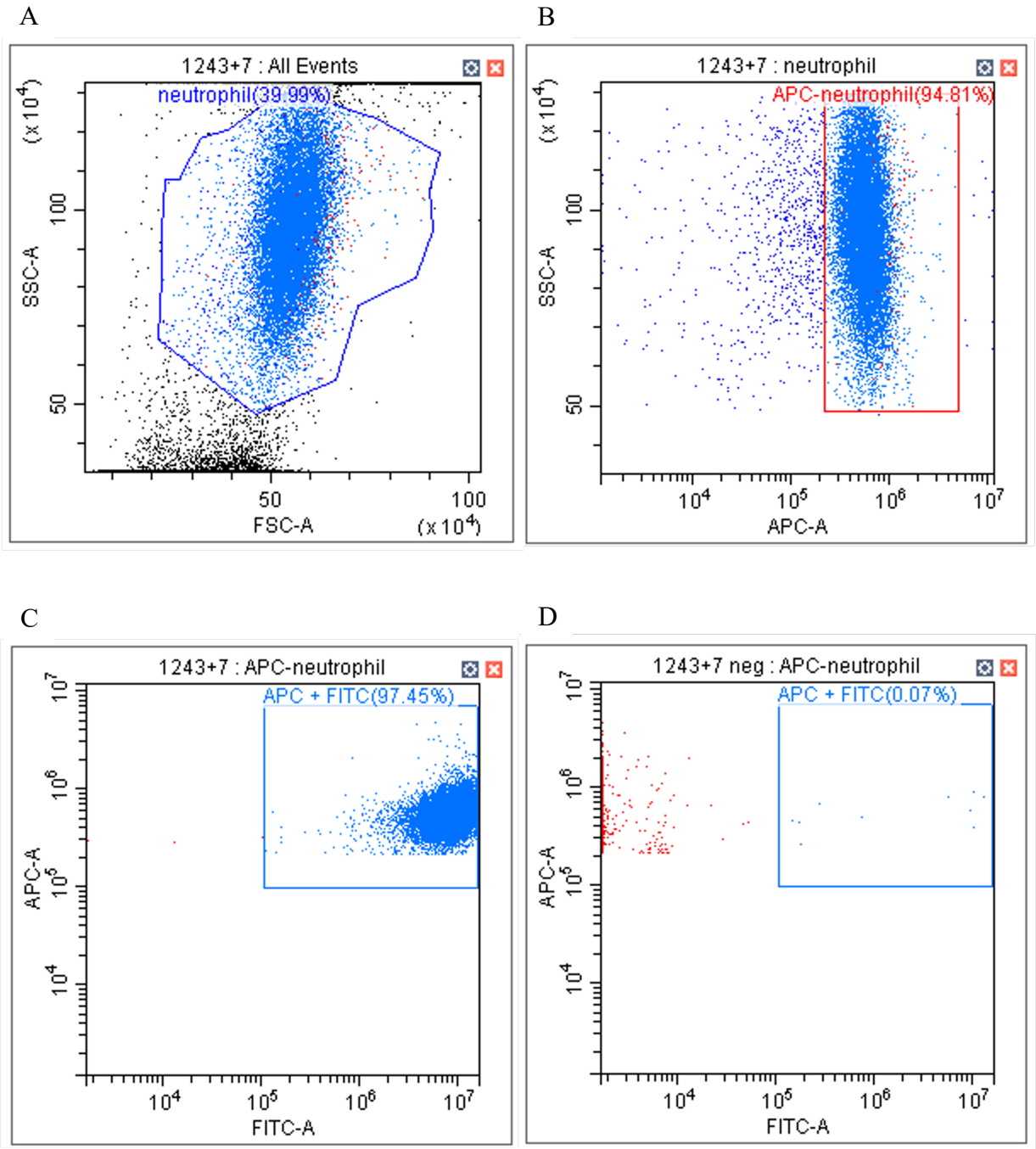
Several questions were raised from the findings of this study which may form the basis for future research. Unfortunately, meaningful conclusions could not be derived from the neutrophil oxidative burst data due to an undefined technical error. As oxidative burst is an important

indicator of neutrophil bactericidal ability, it would be worthwhile to investigate whether this is affected to a similar or different degree compared to phagocytic ability. In addition, the functionality of neutrophils obtained from the endometrium in endometritic and non-endometritic cows could also be compared to that of blood neutrophils to determine if meaningful differences exist between the two populations. The ability to correlate blood neutrophil function to what occurs in the endometrium would provide insight to the pathophysiology of endometritis and allow appropriate interpretation of prior studies investigating blood neutrophil function.

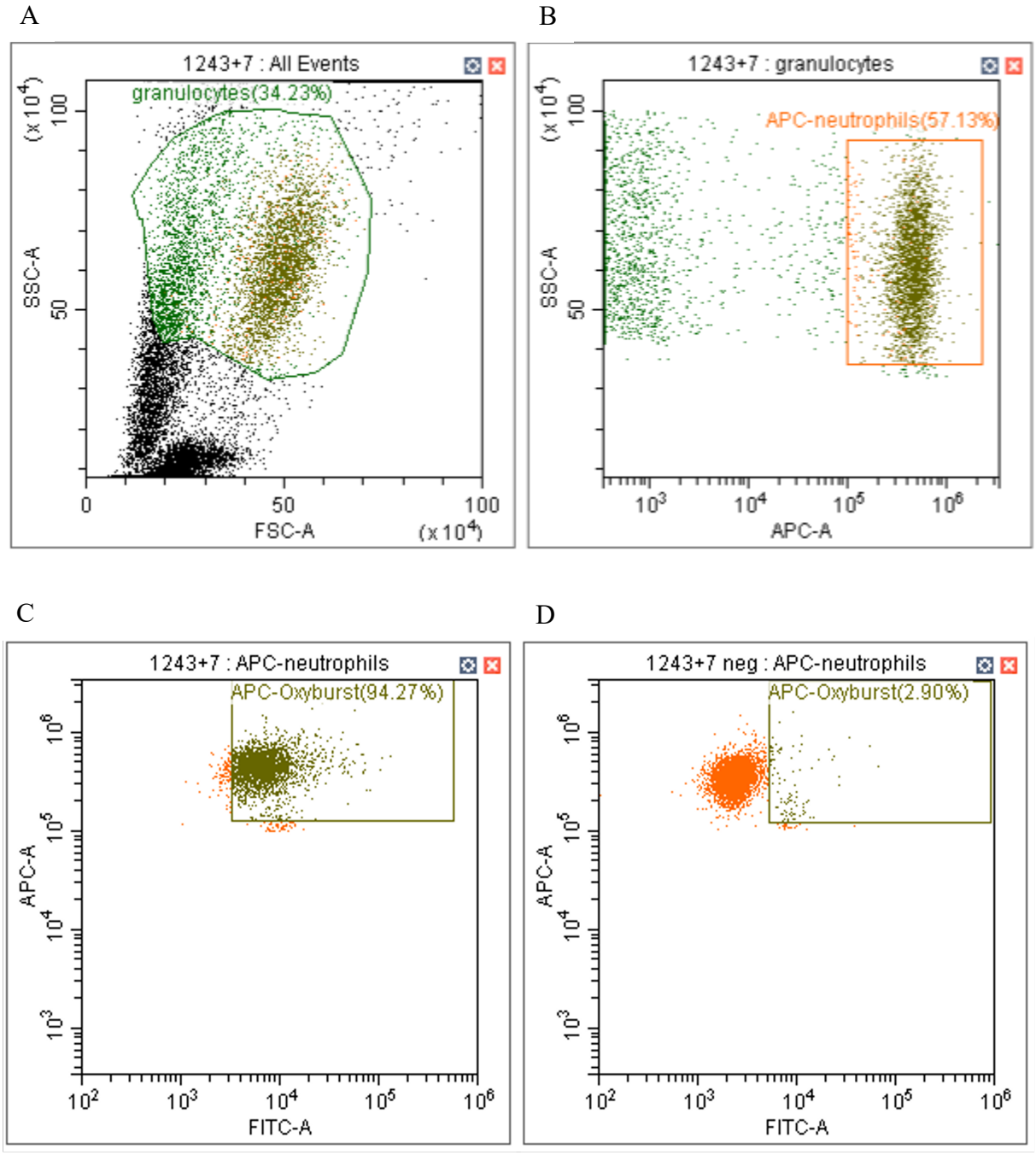
Other aspects of neutrophil function could also be assessed, including migration capacity, neutrophil extracellular trap (NET) function, apoptosis, interactions of neutrophils with the adaptive immune system and the role of neutrophils in the downregulation of inflammation following re-epithelialization of the endometrium. Furthermore, the role of other uterine immune cells, such as macrophages, in the development of endometritis could also be investigated. Macrophages serve an essential role in the regulation of the innate and adaptive immune responses through phagocytosis, antigen presentation and the initiation of tissue repair, and may also prove to be implicit in the persistence of an inflammatory state.[7]

Finally, many aspects of neutrophil function explored in mice and humans have yet to be investigated in cattle, including uterine tissue-specific recruitment mechanisms for leucocytes and tissue-resident leukocytes. Finally, it may be of practical importance to understand the effects of aberrations in glucose, calcium, BHB and/or NEFA on developing granulocytes during myelopoiesis.

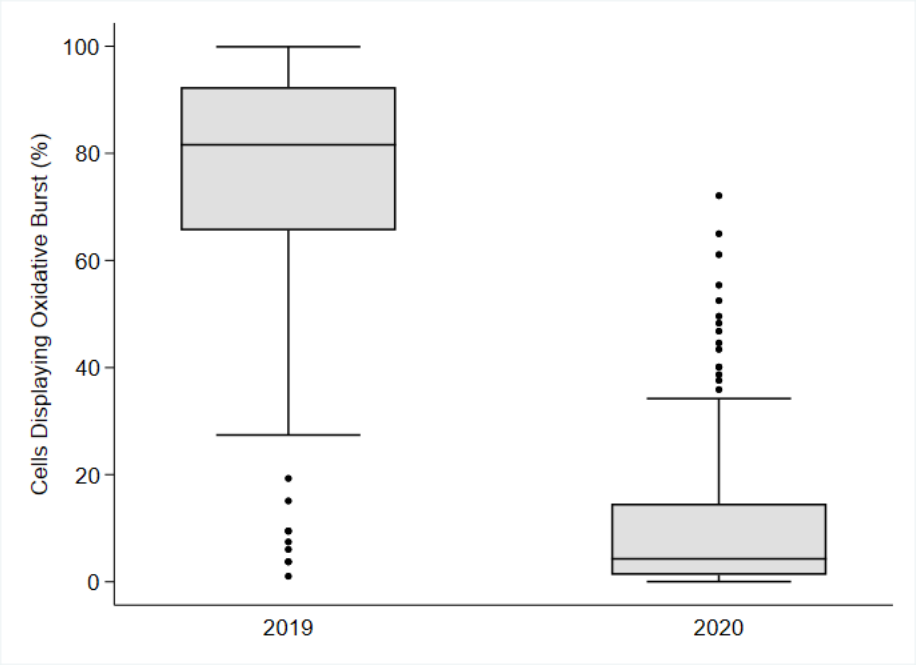
Appendix: Supplemental Figures



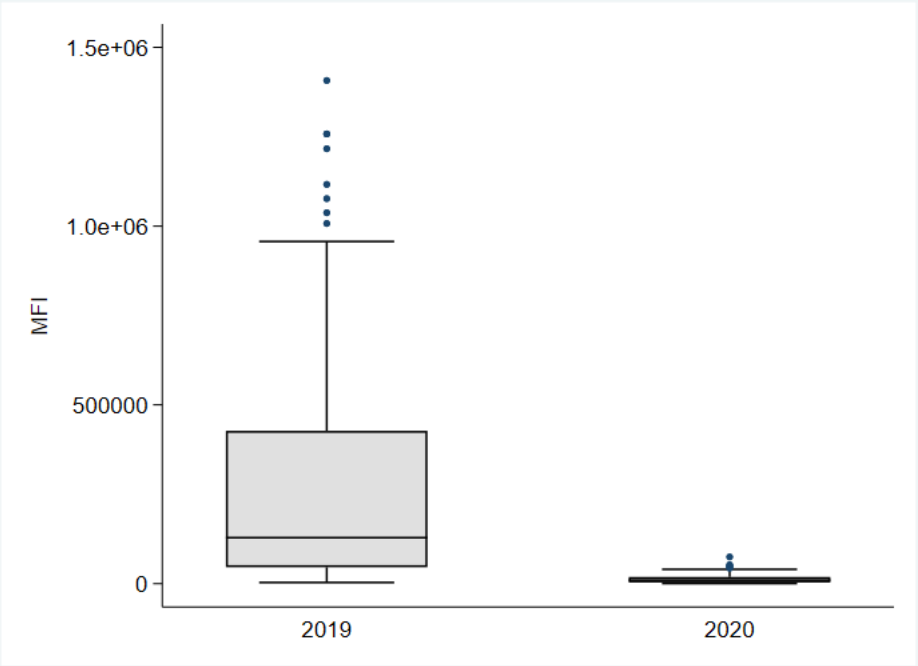
Supplemental Figure 1: Gating strategy for the flow cytometric assessment of neutrophil phagocytic ability. **A**, all events; **B**, selection for APC-neutrophils; **C**, selection for neutrophils containing FITC-bacteria; **D**, control sample without the addition of bacteria. Representative data from one cow taken at 7 weeks postpartum.



Supplemental Figure 2: Gating strategy for the flow cytometric assessment of neutrophil oxidative burst ability from a successful 2019 experiment. **A**, all events; **B**, selection for APC-neutrophils; **C**, selection for neutrophils displaying oxidative burst activity; **D**, control sample without the addition of PMA. Representative data from one cow taken at 7 weeks postpartum.



Supplemental Figure 3: Percentage of neutrophils (median and interquartile range) displaying oxidative burst activity grouped by the year the data was collected, all timepoints combined. Experiments conducted in 2019 (n = 13 cows) performed as expected with > 80% of cells displaying oxidative burst activity; however, the majority of those conducted in 2020 (n = 27 cows) failed to show oxidative burst activity. An undefined technical error is thought to have led to this discrepancy.



Supplemental Figure 4: Mean fluorescent intensity values (median and interquartile range) obtained from the neutrophil oxidative burst experiments grouped by year the data was collected, all timepoints combined. Experiments conducted in 2019 (n = 13 cows) performed as expected; however, the majority of those conducted in 2020 (n = 27 cows) failed to show oxidative burst activity as demonstrated by the low MFI values. An undefined technical error is thought to have led to this discrepancy.

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