The effect of a non-steroidal anti-inflammatory drug on subclinical endometritis in dairy cows and the identification of at-risk cows

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Abstract of a thesis submitted in partial fulfilment of the
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Subclinical endometritis (SCE) is a uterine pathology characterised by an increased proportion of polymorphonuclear cells (PMN) in the uterus after calving, and it is known that SCE has negative effects on dairy cow reproductive performance. However, the mechanism by which SCE affects reproductive performance in New Zealand dairy cows appears to be different from that reported in international literature. This provided the basis for the research reported herein, which sought to investigate the mechanism by which SCE reduces reproductive performance of New Zealand dairy cows. Furthermore, the need for a practical method to detect or diagnose SCE was identified.

The objective of the first experiment was to determine if the inflammation associated with SCE, both uterine and systemic, is a part of the mechanism by which reproductive performance is reduced in cows with this disease. The hypothesis was that reducing this inflammation with a non-steroidal anti-inflammatory drug (NSAID) would reduce the severity of uterine inflammation (average PMN %), and improve reproductive performance. Dairy cows (n = 213) were paired by calving date and day 14 uterine PMN %, and randomly assigned to either the NSAID treatment (administered 21 - 31 days postpartum) or control group. Cows with ≥ 14% PMN in the cytological sample collected at day 14 postpartum were defined as having SCE. Treatment with a NSAID increased pregnancy rate in SCE cows and reduced metabolic indicators of systemic inflammation. There was, however, no effect of NSAID treatment on day 42 PMN %, postpartum anovulatory interval, or milk production. Further research is required to determine the effect of NSAID on SCE, and evaluate the influence of timing of drug application on treatment effectiveness.

The objective of the second experiment was to determine whether a model could be developed, based on known associations between SCE and serum metabolites and cow body condition score, to

both predict cows at risk of SCE and reduce the number of cows to be submitted for cytological examination to a manageable level. Models were developed based on either a single week's data (week relative to calving; -4, -3, -1, +1, +2), or the herd's planned start of calving date (27th June, and a week later; 4th July). The optimum PMN % threshold was determined for the model with the highest predictive value (R^2; week +1). The optimum PMN % threshold for the week +1 model's fitted values was 10.7% (sensitivity = 58%, specificity = 81%). This threshold, and all other thresholds investigated, however, resulted in combinations of sensitivity and specificity where either too few SCE cows were identified, or too many cows would be submitted for cytological examination. These results indicate that although a model was generated that could predict all SCE cows, the dual aim of predicting cows at-risk and enabling only a subset of cows to be submitted for cytological examination was unable to be achieved with the serum and physical parameters evaluated. Further research into the aetiology of SCE may provide better biological markers to use for prediction of SCE.

Keywords: subclinical endometritis, anti-inflammatory, reproduction, prediction, serum metabolites, body condition score, dairy cow

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List of abbreviations

AI	Artificial insemination
AGR	Albumin:globulin ratio
-APP	Negative acute phase protein
+APP	Positive acute phase protein
AS	Acetylsalicylate
ASAT	Aspartate aminotransferase
BCS	Body condition score
CL	Corpus luteum
COX	Cyclooxygenase enzyme
CV	Coefficient of variation
D 0	Day of calving
D 4	Four days after calving
DIM	Days in milk
DMI	Dry matter intake
FSH	Follicle stimulating hormone
GDH	Glutamate dehydrogenase
GnRH	Gonadotrophin releasing hormone
LH	Luteinising hormone
LPS	Bacterial lipopolysaccharide
NEFA	Non-esterified fatty-acids
NSAID	Non-steroidal anti-inflammatory drug
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PMN	Polymorphonuclear cell(s)
PPAI	Postpartum anovulatory interval
ROC	Receiver operator characteristic curve
SCE	Subclinical endometritis
SED	Standard error of the difference
TMR	Total mixed ration

Chapter 1

Introduction

New Zealand's dairy industry is a major contributor to the New Zealand economy as it is the country's biggest export earner, accounting for more than 25% of national export earnings (Burke and Verkerk 2010). As greater than 90% of the milk produced is exported at world market prices without subsidies, to remain profitable, tight economic constraints are placed on New Zealand dairy farmers (Holmes et al. 2002). To remain profitable within these economic constraints, the cost of milk production must be kept down. In New Zealand the cost of production is kept low by the use of grazed pasture as the main constituent of dairy cow diets, because pasture grazed in situ is the lowest cost feed available (Holmes et al. 2002; Blackwell et al. 2010). Therefore, to maximise pasture utilisation, and in turn profitability, the changing herd feed demands need to be synchronised with the growth pattern of pasture as pasture growth also varies throughout the year (Figure 1) (Parsons 1988; Burke and Verkerk 2010). This synchrony between pasture availability and herd feed demand is achieved by seasonal calving, where cows calve in spring when herd feed demand is greatest and pasture growth is maximal (Holmes et al. 2002; Blackwell et al. 2010).

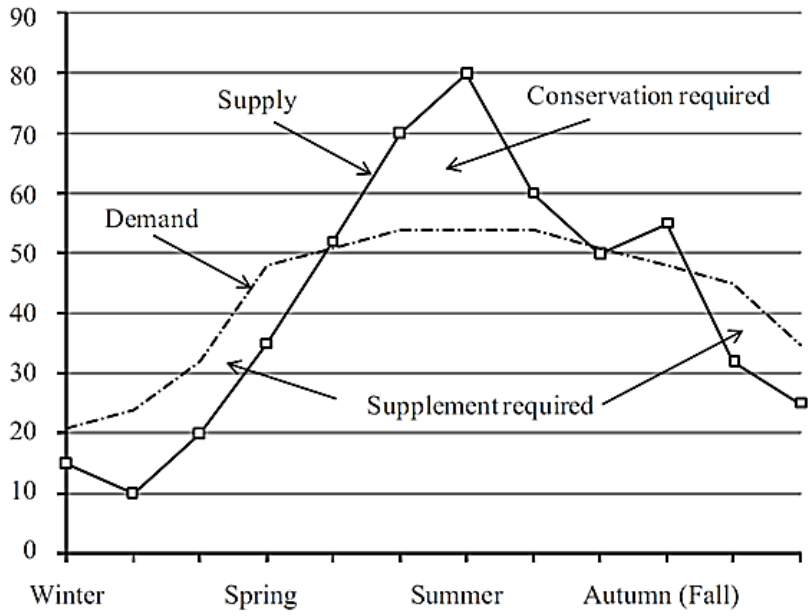

Figure 1: Representation of annual herd feed demand and pasture supply for a pasture-based seasonal calving system. Burke and Verkerk (2010), reproduced with permission from Reproduction in Domestic Ruminants 7, 2010, published by the Society of Reproduction and Fertility, Nottingham, United Kingdom.

In New Zealand, more than 95% of milk produced comes from strictly seasonal calving, grazed pasture systems (Blackwell et al. 2010). To maximise the number of days in milk and thus milk production, cows must calve every year in a tight calving pattern and have a 365 day inter-calving interval (Blackwell et al. 2010). This means that farm productivity is directly related to cow reproductive performance. To achieve this 365 day inter-calving interval, cows must conceive by 83 days after calving (Rhodes et al. 2003). This requires cows to resume cycling, display oestrous behaviour, be mated during the early part of the breeding period, and in a seasonal calving system, conceive by 83 days after the herd's planned start of calving irrespective of time postpartum (Rhodes et al. 2003; Blackwell et al. 2010). Cows that cannot maintain this 365 day inter-calving interval are at risk of being culled (Blackwell et al. 2010).

The New Zealand dairy industry has set targets to achieve optimal reproductive performance. These targets are a78% 6-week in-calf rate (78% of the cows pregnant after 6 weeks of mating), 90% 3-week submission rate (90% of cows mated within the first 3 weeks of the mating period), 60% conception rate, and 6% not-in-calf rate after 12 weeks of mating (6% of cows not pregnant by the end of the mating period; empty rate) (Burke et al. 2007). However, at an industry level, these targets are not being met; in fact herd reproductive performance has been declining (Harris et al. 2006). The reduction in reproductive performance is partially attributed to the increase in the number of cows that are anoestrous at the start of the mating period (Verkerk et al. 2000). This increase in the number of anoestrous cows has a 2-fold impact on reproductive performance, in that both the 3-week submission rate and the conception rate would be negatively affected. The 3-week submission rate would be reduced because there would be fewer cows cycling and thus submitted for mating within the first 3 weeks of the mating period. The conception rate would be reduced because fertility increases as the number of oestrous cycles postpartum increases (up to ~4 cycles) (Thatcher and Wilcox 1973), therefore, the cows that were anoestrous at the start of the mating period will not have cycled four times prior to mating, and will therefore have reduced conception rates. The reduction in submission and conception rates may result in these cows being culled for not getting in calf, or for being a late calver in the current season or the subsequent season.

The increased risk of culling as a result of being anoestrous at the start of the mating period highlights the fact that cows need to calve in the early part of the calving season and resume cycling as early postpartum as possible. One factor that can delay the resumption of oestrous activity, and negatively affect subsequent conception and pregnancy rates, is uterine disease. The uterine disease that is investigated in this thesis, as a part of a larger programme of work, is subclinical endometritis (SCE). Subclinical endometritis is a uterine pathology characterised by an increased proportion of polymorphonuclear cells (PMN) present in the uterus after calving. International literature has identified this disease as a factor that reduces reproductive performance; however, as there was a

knowledge gap in the New Zealand context around this disease, the impact of SCE on dairy cow reproductive performance in New Zealand was investigated. Previous studies by Burke et al. (2010) and McDougall et al. (2011) reported that this disease was present in New Zealand and that it was reducing reproductive performance. However, the mechanism by which SCE affected reproductive performance in New Zealand dairy cows appeared to be different from that reported in the international literature. This has provided the basis for the research reported in this thesis, which sought to investigate the mechanism by which SCE reduces reproductive performance in New Zealand dairy cows.

In addition to the need for further research into how SCE is reducing reproductive performance, the need for a practical method to detect and diagnose SCE has also been identified. In New Zealand, it is common practice for farmers to check their herd for clinical endometritis (Burke et al. 2007). They are able to do this using the Metricheck^{TM} device. However, the Metricheck^{TM} device cannot detect SCE (McDougall et al. 2011), therefore, currently there is no practical and cost-effective method for on-farm detection and diagnosis of SCE. This has led to the investigation of using biological markers in serum and cow body condition score to predict cows at risk of SCE.

The objectives of the research reported in this thesis are two-fold: (1) to attempt to determine if the inflammation associated with SCE (both uterine and systemic) is the mechanism by which reproductive performance is reduced in cows with SCE, and (2) to validate the usefulness of biological markers in serum and cow body condition score for the identification of cows at risk of SCE. The specific hypotheses for the research reported in this thesis are: (1) that treatment with non-steroidal anti-inflammatory drug (NSAID) between 21 and 31 days postpartum will reduce the severity (average polymorphonuclear cell (PMN) %) of uterine pathology at 42 days postpartum, without lengthening the postpartum anoestrous interval (PPAI), and will mitigate the negative association of SCE on reproduction and milk production by reducing inflammation and improving liver function, and (2) that metabolic and physical characteristics could be used to predict cows at risk of SCE and enable the identification of a subset of cows for cytological examination or precautionary treatment.

Chapter 2

Literature review

2.1 Introduction

As explained in the previous section, the research described in this thesis was designed to test two different hypotheses. Because of this, the literature review covers two essentially separate, but related, subjects: i.e. postpartum uterine disease and validation of biological markers. The topics that are reviewed in the postpartum uterine diseases section are: endometritis (specifically SCE), its prevalence, the effect of this disease on reproductive performance and milk production, the proposed mechanisms of how factors associated with SCE reduce reproductive performance, and the various treatment options that have been investigated for SCE. In the validation of biological markers section, investigations of markers that have been trialled to detect/diagnose SCE are discussed.

2.2 Postpartum uterine diseases

Uterine disease can occur in the early postpartum period and manifests as conditions such as metritis, pyrometra, or endometritis. An infection of the uterus within the first two weeks after calving is called metritis (Sheldon et al. 2006). Metritis involves inflammation in all the layers of the uterus: endometrial mucosa and submucosa, muscularis, and serosa (Bondurant 1999). Cows with metritis show signs of systemic illness, e.g. fever, depression, weakness, inappetence, and decreased milk yields (Bondurant 1999; Sheldon et al. 2006). Pyrometra is a chronic infection of the uterus, with accumulation of purulent or mucopurulent material in the uterine lumen in the presence of a corpus luteum (CL) (Sheldon et al. 2006; Parkinson et al. 2007).

Endometritis is defined as superficial inflammation of the endometrium, extending no deeper than the stratum spongiosum (Bondurant 1999), that occurs more than two to three weeks postpartum (Sheldon et al. 2006; LeBlanc 2008). The reason for stipulating a time frame for this disease is that during the two to three weeks immediately after calving, a cow's uterus undergoes an involution process involving tissue remodelling and inflammation. The reported time to gross uterine and cervical involution varies greatly from 25 to 47 days in milk (LeBlanc 2008). Thus it is inflammation of the uterus that extends beyond two to three weeks postpartum that is termed endometritis (Sheldon et al. 2006). Endometritis can be classified as either clinical or subclinical.

2.2.1 Clinical endometritis

Clinical endometritis in cows is defined as inflammation and infection of the uterus more than two to three weeks postpartum without systemic signs of illness. Affected cows exhibit visual signs such as

purulent material on the tail and/or vulva, purulent (21 days postpartum) or mucopurulent (26 days postpartum) material accumulated in the vagina, a cervical diameter > 7.5 cm after 20 days postpartum, and malodorous mucus (LeBlanc et al. 2002a; Barlund et al. 2008; McDougall et al. 2011). It must be noted that the criteria for diagnosis, e.g. cervix size, changes with increasing time postpartum (LeBlanc et al. 2002a).

Endometritis, both clinical and subclinical, causes significant economic losses because of decreased reproductive performance and reduced milk yield (discussed in later sections), and increased culling (Pleticha and Heuwieser 2009), therefore it is a condition that needs to be addressed. Clinical endometritis is easy to detect and it is common practice for whole herds to be routinely checked with the Metricheck^{TM} device (Burke et al. 2007); therefore cows with clinical endometritis will most likely be detected and treated. However, SCE is not easy to detect, which, therefore, means it can go untreated, resulting in a greater probability of poor reproductive performance for the affected cows. It is for this reason that the research reported in this thesis, where possible, is focused on SCE.

2.2.2 Subclinical endometritis

Subclinical endometritis is defined as cows with no systemic signs of illness or clinical infection (Barlund et al. 2008), but that have inflammation of the endometrium, which is characterised by an elevated population of PMN present in the uterus postpartum (Sheldon et al. 2009). Subclinical endometritis has also been described as 'cytological endometritis' (Gilbert et al. 2005; Dubuc et al. 2010). Dubuc et al. (2010) defined cytological endometritis as "an increased proportion of PMN in endometrial cytology samples obtained by endometrial cytobrush or low-volume uterine lavage". The authors indicated that cytological/subclinical endometritis and clinical endometritis may actually represent different manifestations of reproductive tract disease and inflammation, rather than a difference in severity of the same disease. Cows in the Dubuc et al. study were classified as having cytological endometritis only, clinical endometritis only, or both cytological and clinical endometritis. The results demonstrated that cytological endometritis only and clinical endometritis only had similar detrimental effects on reproductive performance. However, for cows that had concurrent cytological and clinical endometritis, the detrimental effects of both conditions were additive (Figure 2). This finding is important as it may have implications for the way either form of endometritis is detected and treated.

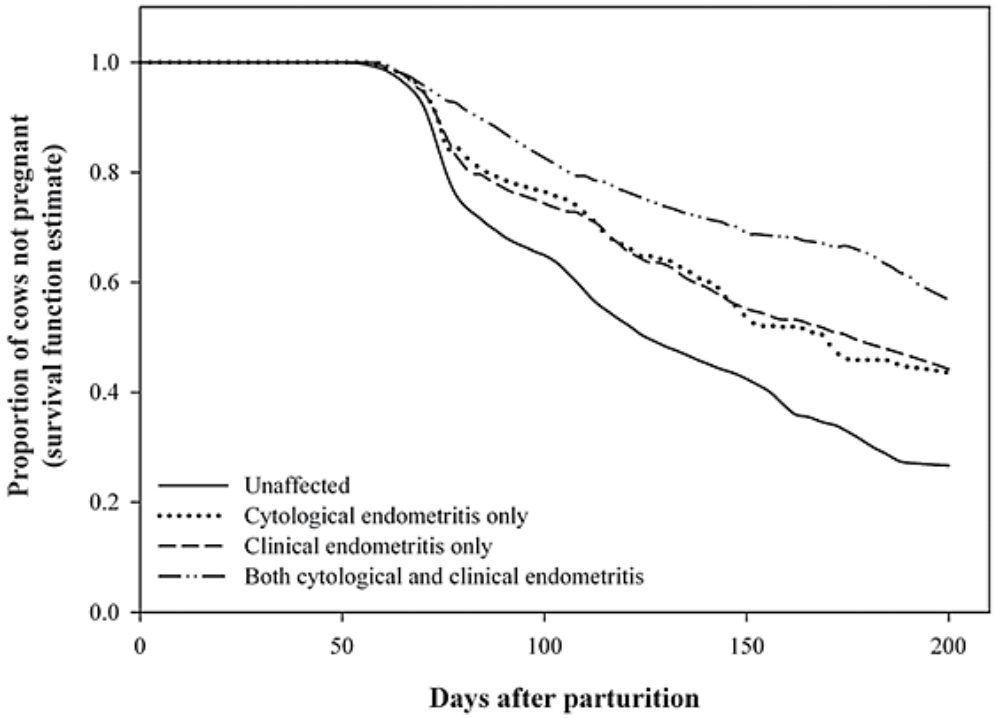

Figure 2: The effect of cytological endometritis, clinical endometritis, or both, on the proportion of cows pregnant. Dubuc et al. (2010), reproduced with permission from Elsevier.

As mentioned above, SCE is superficial inflammation of the endometrium (Sheldon et al. 2006; LeBlanc 2008). The inflammation is present because inflammation is, as part of the innate immune system, the body's response to injury or infection (Serhan et al. 2010). Some of the processes that occur during an inflammatory event include recognition of an injury or a foreign invading particle (antigen), activation of endothelial cells to release pro-inflammatory cytokines and chemokines, influx of polymorphonuclear cells (in particular neutrophils) to the site of the injury/antigen via chemotaxis in response to the cytokines and chemokines, stimulation of release of acute-phase proteins from the liver in response to proinflammatory cytokines, and phagocytosis of foreign invading particles by PMN (Hussain 1989; Sheldon et al. 2009; Serhan et al. 2010; Galvão et al. 2011). Inflammation is induced by prostaglandins that stimulate the release of cytokines (Bos et al. 2004). Prostaglandins are bioactive lipids that are derived from the conversion of arachidonic acid by the cyclooxygenase enzyme (COX) into PGH_2 (Figure 3), which is then further metabolised into various prostaglandins such as series F, E, I, and D prostaglandins (Mitchell et al. 1993; Sales and Jabbour 2003; Drillich et al. 2007). The COX-1 enzyme is constitutively expressed in cells; COX-2 is not, but its expression is induced by some cytokines, endotoxins and inflammation (Mitchell et al. 1993; Drillich et al. 2007).

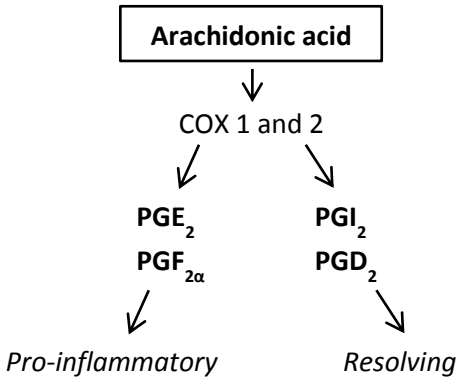

Figure 3: The production of prostaglandins (PGE_2, $PGF_{2\alpha}$, PGI_2, PGD_2) via the conversion of arachidonic acid by the cyclooxygenase enzyme (COX) -1 and -2 (Sordillo et al. 2009).

In the case of bacterial infection, an inflammatory response is elicited by bacteria infecting the uterine endometrium (Galvão et al. 2011). Once a pathogen has come into contact with the endometrium, the endometrium is stimulated to produce cytokines and chemokines that attract immune cells, in particular PMN, into the uterus and activate these cells once they are there (Galvão et al. 2011). There are three kinds of PMN: neutrophils, eosinophils and basophils (Table 1). Neutrophils are the main PMN involved in uterine inflammation (Barlund et al. 2008). Neutrophils are adept at seeking out and ingesting (phagocytising) bacteria and dead cells (Solomon et al. 2004). Most of the cytoplasmic granules in neutrophils contain enzymes that digest the ingested material (Solomon et al. 2004; Serhan et al. 2010).

Table 1: The three types of polymorphonuclear cells[1]

Cell type	Diagram	Main targets	Granules	Lifespan
Neutrophil		Bacteria and Fungi	Fine, faintly pink (H & E Stain)	6 hours – few days (days in spleen and other tissues)
Eosinophil		Larger parasites Modulate allergic inflammatory responses	Pink-orange (H & E stain)	8 – 12 days (circulate for 4 – 5 hours)
Basophil		Release histamine for inflammatory responses	Large blue	A few hours to a few days

[1](Alberts et al. 2007; Serhan et al. 2010)

The main function of PMN is phagocytosis and killing of invading bacteria. This involves four stages: chemotaxis, adherence and attachment, ingestion, and digestion (Hussain 1989). Chemotaxis is the

directed movement of a cell towards or away from a chemical source (Hussain 1989). When PMN are exposed to cytokines and chemokines, the PMN become polarised and move towards the invading bacteria (Hussain 1989; Galvão et al. 2011). It appears that the main cytokine involved in recruiting PMN into the uterus is interleukin 8 (Ghasemi et al. 2012). Ghasemi et al. (2012) reported that cows with SCE had a 50-fold higher gene expression of interleukin 8 than cows without SCE. Once at the site of infection, PMN attach to and ingest each bacterium by enclosing it in a phagosome (Hussain 1989). The bacteria are digested within phagosomes by lysosomal enzymes that are contained in the cytoplasmic granules (Hussain 1989). It is because of these cytoplasmic granules that PMN are also known as granulocytes. Polymorphonuclear cells are of particular interest with respect to SCE because PMN are used to diagnose SCE.

2.3 Diagnosis of subclinical endometritis

Subclinical endometritis is diagnosed by determining the proportion of cells that are PMN within an uterine cytology sample, i.e. PMN % (Sheldon et al. 2009). Uterine cytology is the collection and counting of cells obtained from the uterus, and has become the standard to which other techniques are compared (Barlund et al. 2008; Dubuc et al. 2010). There are two cytological techniques commonly used to diagnose SCE (Kasimanickam et al. 2005a), the cytobrush method and the uterine flushing/lavage method. Briefly, the cytobrush method involves a cytobrush being passed through the cervix and gently rotated against the endometrium; the cellular material is then rolled on a slide for microscopic evaluation to determine the proportion of cells that are PMN within the sample (Kasimanickam et al. 2004; Barlund et al. 2008; McDougall et al. 2009). Uterine flushing involves the uterine lumen being flushed with a small volume of saline solution, which is recovered and examined by microscopy for the proportion of cells that are PMN within the sample (Gilbert et al. 2005). Kasimanickam et al. (2005a) concluded that the cytobrush technique is a more consistent and reliable technique than uterine lavage, the latter having a 17% failure rate in retrieving samples, a reduced number of PMN recovered, and greater distortion of the recovered cells. For this reason, the cytobrush technique will be used for the research conducted in this thesis.

To determine whether a cow has SCE or not, a diagnostic threshold for PMN % must be set. The PMN % threshold used to define cows as having SCE varies depending on the time postpartum that the cytology sample was collected and the outcomes being investigated. In general, the PMN % threshold used decreases as time postpartum increases (Kasimanickam et al. 2005a; Gabler et al. 2010). This is because uterine inflammation decreases as time postpartum increases as a result of the progression of the involution process towards completion (Gilbert et al. 2005; Senosy et al. 2009). Studies have used different approaches to determine what PMN threshold to use, such as using a PMN % that was associated with a negative impact on reproductive performance or grouping cows into quartiles based on PMN %. Kasimanickam et al. (2004) used the first approach. The authors used survival

analysis to determine the lowest PMN % that was associated with a negative effect on reproductive performance; it was determined that > 18% PMN at 20 to 33 days postpartum and > 10% PMN at 34 to 47 days postpartum defined a cow as having SCE. Burke et al. (2010) used the second approach, where the PMN % threshold was determined by categorising the cows into quartiles; the highest PMN % quartile (> 6% PMN at day 42 postpartum) was used to identify cows with SCE. Both approaches have positives and negatives. The first approach is good in that it determines its PMN % threshold based on a tangible outcome, e.g. pregnant or not by a specified time after the start of the mating period, rather than using an arbitrary cut-off. However, as many factors other than PMN %, such as time from calving to mating and breeding management (Barlund et al. 2008; LeBlanc 2008), will influence whether a cow gets pregnant by the specified time, a threshold based on reproductive parameters may only be relevant for that specific study population. The benefit of the second approach is that it avoids the circular argument of determining a threshold based on the reproductive performance of the study population and then using that threshold to assess the reproductive performance of that population (McDougall et al. 2011), but there is the potential that the upper quartile PMN % may not be associated with reproductive performance at all. Therefore, both approaches have merit, but which approach is best will be determined by the research question that is being investigated.

Polymorphonuclear cells are used to diagnose SCE because they are a good indicator of the stage and the degree of uterine inflammation as they are the predominant inflammatory cells found in intrauterine fluid accumulations (Barlund et al. 2008). Anderson et al. (1985) and Kluciński et al. (1990) indicated that there is almost a doubling (~90%) in the number of PMN present in the uterus during clinical and subclinical uterine infection. These cells are a part of the body's innate immune system and provide the first line of defence against invading pathogenic organisms (Paape et al. 2002). Thus, when uterine infection or damage occurs, there is an influx of PMN into the uterine lumen (Kasimanickam et al. 2004).

2.4 Risk factors for endometritis

The main risk factors for the establishment of uterine infection and inflammation can be grouped into four broad categories: reproductive tract damage, metabolic conditions, bacteria, and other factors (Table 2). The most common risk factors reported are those associated with calving. Cows that experience a difficult calving are at risk of developing endometritis postpartum. This is because physical barriers such as the cervix, vagina, and vulva, which are the first line of defence against bacteria, are breached and may be damaged during parturition (Sheldon and Dobson 2004). This provides the opportunity for bacteria to invade and establish infection (Sheldon and Dobson 2004). Uterine tissue damage and devitalisation of the constitution of the birth canal can be caused by

dystocia and may result in tissue that is more susceptible to bacterial infection (Parkinson et al. 2007).

Table 2: Risk factors for the establishment of endometritis[1]

Reproductive tract damage	Assisted calving Twins Dystocia Retained fetal membranes Still birth Delayed uterine involution Caesarean section Premature calving Calving stress
Metabolic conditions	Milk fever Ketosis Left displaced abomasum
Bacteria	Type of bacterial flora in the uterine lumen Level of bacterial load
Other factors	Increasing cow age Trauma Endotoxin from the gut Injuries Mammary gland oedema Late calving Immune response of the cow Uterine endocrine environment Level of hygiene of the environment Delayed ovarian activity

[1] Results collated from: Knutti et al. (2000), McDougall (2001), Sheldon and Dobson (2004), Drackley et al. (2005), Drillich et al. (2005), Sheldon et al. (2006), LeBlanc (2008), Trevisi and Bertoni (2008), McDougall et al. (2009), Sheldon et al. (2009), Burke et al. (2010), Cheong et al. (2011a), McDougall et al. (2011).

The distinction must be made, however, between contamination and infection, as not all cows that have bacteria present in their uterus have an infection (Sheldon et al. 2006). An infection requires adherence of pathogenic organisms to the mucosa, colonisation or penetration of the epithelium by the bacteria, and/or the release of bacterial toxins that lead to establishment of uterine disease (Janeway et al. 2001). The establishment of infection depends on the bacterial load and species, as well as the immune response of the cow (Sheldon et al. 2006; Parkinson et al. 2007).

The bacterial load within the uterus depends on two main factors; level of contamination and species of bacteria (Parkinson et al. 2007). Contamination of the uterus with bacteria occurs during calving for most cows. However, cows that suffer from peripartum disorders, such as dystocia and retained fetal membranes, will have greater levels of contamination (Parkinson et al. 2007) and are at greater

risk of SCE. It must be noted though that although many species of bacteria may have entered the uterus after calving, only certain species of bacteria are associated with SCE. Five main bacterial species have been identified as the most common pathogenic bacteria isolated from the uterus in total mixed ration (TMR) -based farm systems: *Arcanobacterium pyogenes, Prevotella melaninogenica, Escherichia coli, Fusobacterium necrophorum* and *Proteus spp.* (Sheldon and Dobson 2004; Williams et al. 2007). The most common bacterial species for pasture-based farm systems have not been reported. Of these five species, LeBlanc (2008) reported that commonly cows with endometritis will have an infection predominated by *Escherichia coli* in the first week postpartum and *Arcanobacterium pyogenes* in the second week. However, whether the invasion of the uterus by these bacterial species results in infection depends on the immune response of the cow.

The immune response of a cow depends on many factors including her overall health status and endocrine environment (Sheldon et al. 2006; Parkinson et al. 2007). A cow with compromised health may be at greater risk of infection as she may not be able to mount a sufficient immune response (Kasimanickam et al. 2004). Thus, the metabolic conditions and other factors mentioned in Table 2 could negatively affect a cow's immune status and immune function, allowing bacteria to create an infection. However, it is not only cows showing systemic signs of illness that may be at risk of SCE. It has been reported by Green et al. (2009) and Burke et al. (2010) that cows that are retrospectively classified as having SCE (based on PMN %) have indications of systemic inflammation and impaired liver function. The indicators reported were a lower albumin concentration, a lower albumin:globulin ratio, and elevated plasma concentrations of aspartate aminotransferase (ASAT) and glutamate dehydrogenase (GDH). This systemic inflammation and impaired liver function may be negatively affecting immune function, but this assertion has yet to be tested or reported. The endocrine environment exerts its influence on immune function through progesterone and oestrogens. Progesterone is immunosuppressive, which impairs the cows' ability to mount an appropriate immune response to bacteria, whereas oestrogens support immune function (Hussain 1989; Lewis 2003; LeBlanc 2008). This is in part because high oestrogen levels intensify PMN function, whereas high progesterone levels inhibit PMN function (Hussain 1989; LeBlanc 2008). The interaction between bacterial factors and cow immune function will affect the prevalence of SCE in the herd.

2.5 Prevalence of subclinical endometritis

Studies over the past 10 years in both pasture-based and TMR-based dairy systems have reported the prevalence of SCE to range from 6.5 to 53% (Table 3).

Table 3: The prevalence of subclinical endometritis diagnosed by cytobrush or lavage cytology reported in the literature

DIM[1]	Threshold[2]	SCE[3] prevalence	Study	# of cows
Cytobrush cytology				
18-38	> 5% PMN	38%	Plöntzke et al. (2010)	194 (3 herds)
18-24	> 18% PMN	35%	Lopdell et al. (2011)	46
20-33	> 18% PMN	35.1%	Kasimanickam et al. (2004)	228
21-31	> 18% PMN	13.5%	Heidarpour et al. (2012)	90
21-33	> 8% PMN	21.5%	Madoz et al. (2013)	418 (4 herds)
28-41	> 8% PMN	11.8%	Barlund et al. (2008)	221 (8 herds)
31-38	> 6% PMN	19.3%	Dubuc et al. (2010)	1044 (6 herds)
32-52	> 5% PMN	19%	Plöntzke et al. (2010)	194 (3 herds)
34-47	> 10% PMN	34%	Kasimanickam et al. (2004)	228
34-47	> 6% PMN	16%	Madoz et al. (2013)	418 (4 herds)
35	> 18% PMN	12.4%	Kaufmann et al. (2010a)	209
39-45	> 18% PMN	7%	Lopdell et al. (2011)	46
48-62	> 4% PMN	16%	Madoz et al. (2013)	418 (4 herds)
53-59	> 4% PMN	11.1%	Dubuc et al. (2010)	1018 (6 herds)
Lavage cytology				
25-31	> 25% PMN	51.8%	Hammon et al. (2006)	83
28-41	> 8% PMN	15.8%	Barlund et al. (2008)	221 (8 herds)
31-38	> 8.5% PMN	38.2%	Galvão et al. (2009a)	445 (5 herds)
40-60	> 5% PMN	53%	Gilbert et al. (2005)	141 (5 herds)
46	> 10% PMN	25.9%	Cheong et al. (2011a)	812 (39 herds)
46-52	> 6.5% PMN	37.2%	Galvão et al. (2009a)	445 (5 herds)

[1]DIM = days in milk

[2]Threshold = polymorphonuclear cell (PMN) % cut-off value used for diagnosing subclinical endometritis

[3]SCE = subclinical endometritis

From Table 3 it can be seen that the prevalence of SCE, in general, decreases with increasing time postpartum. This is because many affected cows recover spontaneously (Gautam et al. 2009; Burke et al. 2010). Green et al. (2009) reported a self-cure rate of 73% for cows with SCE. This can occur up to four (LeBlanc et al. 2002b) to eight (Bretzlaff 1987) weeks postpartum. The spontaneous resolution of endometritis is usually due to oestrus. When a cow ovulates, there is a spike of oestrogens which stimulates uterine motility and PMN function, helping to clear uterine infection (Hussain 1989; LeBlanc 2008). The high rate of self-cure in cows with endometritis is a factor that needs to be taken into account when interpreting any results reported for the treatment of endometritis.

It is important to note that although the inflammation/infection may have been resolved, its negative impact on cow performance may still remain.

2.6 The effect of subclinical endometritis on milk production

It was first hypothesised that endometritis would have no effect on milk production. This hypothesis was initially supported by the results of Fourichon et al. (1999) who reported no loss of milk production in cows with endometritis, and Dubuc et al. (2011) reported no reduction in milk production for cows with SCE. However in studies conducted by Burke et al. (2010) and McDougall et al. (2011), daily milk yield in cows with SCE was reduced by 0.6 – 1.03 kg/cow/day, and differences in milk composition and somatic cell counts were also reported (Table 4).

Table 4: The impact of endometritis on milk composition and somatic cell count

Result reported	Impact	Reference
Protein percentage	↓	Green et al. (2009), Burke et al. (2010)
Fat percentage	↓	Green et al. (2009)
Somatic cell count	↑	Green et al. (2009)

The exact mechanism and nature of the link between endometritis and reduced milk production is yet to be established. Preliminary theories revolve around reduced dry matter intake (DMI) for cows with endometritis, which results in reduced milk production (Bertoni et al. 2008). The proposed mechanism is that proinflammatory cytokines, which induce the inflammation associated with endometritis, induce anorexia which reduces DMI and, therefore, milk production (Johnson and Finck 2001). This theory was supported by the reduced DMI and milk production observed in cows with inflammation or uterine infection (Bell and Roberts 2007; Huzzey et al. 2007; Bertoni et al. 2008). It therefore seems plausible that reducing inflammation would increase milk production. Support for this hypothesis is provided by a study reported by Trevisi and Bertoni (2008). Half of the cows in the study were untreated controls, the other half were treated with acetylsalicylate, an anti-inflammatory drug, for the first five days postpartum. The treated cows had higher milk production, higher peak milk yield (46.3 vs 40.9 kg/day), and reached peak daily milk production earlier (50.5 vs 57 days in milk) than control cows. These results indicate that the reduction of inflammation in the early postpartum period is beneficial to milk production.

Compared with the inconsistent effect of SCE on milk production, there is substantial evidence that SCE has a negative effect on reproductive performance.

2.7 The effect of subclinical endometritis on reproductive performance

The aim of reproductive management is to achieve good reproductive performance, specifically that cows become pregnant at a biologically optimal time, in an efficient manner, and at an economically beneficial interval after calving (LeBlanc et al. 2002a; Sheldon et al. 2006). In the New Zealand seasonal calving system, this means cows must conceive by 83 days postpartum to maintain the required a 12 month inter-calving interval, which requires the resumption of normal cyclicity within a

few weeks after calving (Opsomer et al. 2000; Rhodes et al. 2003). The ability to resume cycling, and other reproductive parameters, has been reported to be negatively affected by SCE (Table 5).

Table 5: The impact of subclinical endometritis on reproductive performance

Reproductive measure	Impact of SCE [a]	Reference [b]
Postpartum anovulatory interval	Increased	2, 4, 6
Submission rate	Decreased	6
First service conception rate	Decreased	2, 3, 6
Services per conception	Increased	2, 3
Days open	Increased	1, 2, 3
Overall pregnancy rate	Decreased	1, 2, 3, 5, 6

[a] SCE = subclinical endometritis

[b] Results collated from: 1) Kasimanickam et al. (2004), 2) Gilbert et al. (2005), 3) Barlund et al. (2008), 4) Burke et al. (2010), 5) Dubuc et al. (2010), 6) McDougall et al. (2011).

Despite being a subclinical disease, there is comprehensive evidence that SCE has a negative effect on reproductive performance (Table 5). These negative effects have been reported in both pasture-based and TMR-based dairy systems. In a study reported by Gilbert et al. (2005), conducted in a TMR-based dairy system, cows with SCE had a lower overall pregnancy rate at 300 days postpartum (63% vs 89%), reduced probability of first-service conception (11% vs 36%), delayed days to first service (median 101 vs 80 days), more services per conception required (median 3 vs 2 services; Figure 4)and more median days open (206 vs 118 days), compared with cows without SCE.

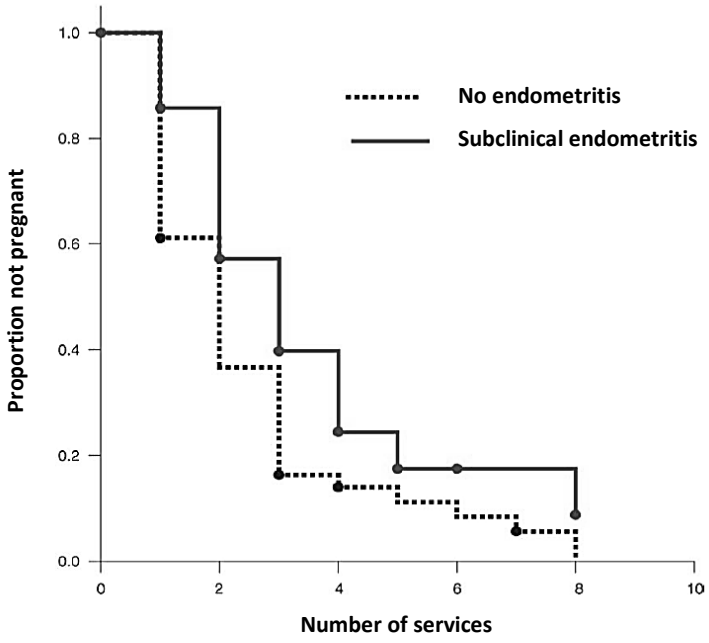

Figure 4: The number of services required per conception for cows with and without subclinical endometritis. Gilbert et al. (2005), reproduced with permission from Elsevier.

In a study reported by McDougall et al. (2011), conducted in a pasture-based dairy system, cows with SCE had a lower proportion (0.57 vs 0.97) pregnant overall, decreased pregnancy to first service, and took longer to conceive (56.1 vs 32.6 days from the planned start of mating), compared with cows without the condition.

From the studies reported in this section it is clear that SCE has a negative effect on reproductive performance; the question is, how?

2.8 Factors associated with endometritis that impair reproductive performance

The mechanisms by which SCE/endometritis reduces reproductive performance are still being elucidated. This is because the interactions among infectious uterine bacteria, the immune system and inflammatory response, and ovarian and uterine function are complex and not entirely understood. Parts of these complex interactions are summarised in Figure 5, and explored in greater detail in subsequent sections. The proposed mechanisms can be grouped into two broad categories: those associated with bacteria, and those associated with the inflammation that accompanies SCE.

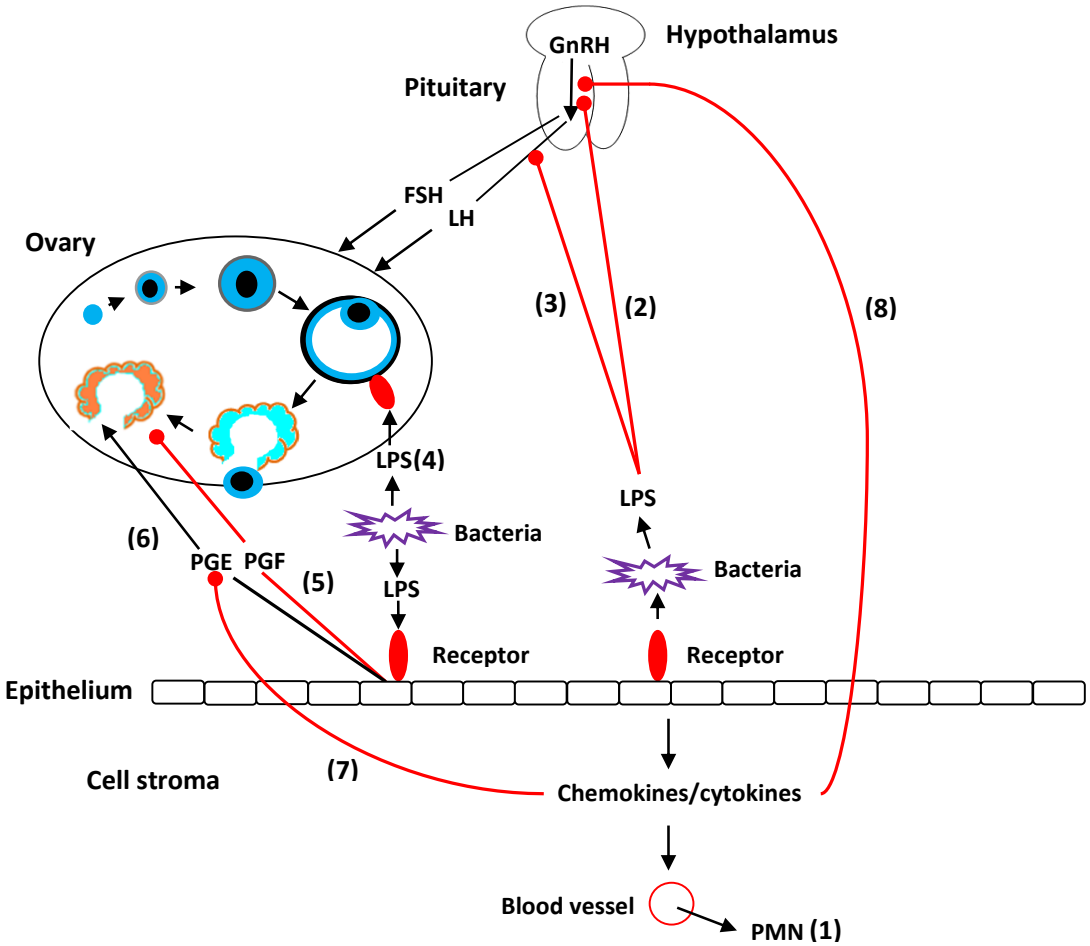

Figure 5: Mechanisms by which bacteria and inflammation affect reproductive performance in cows (modified, with permission, from Sheldon et al. (2009)). (1) Chemokines and cytokines induce polymorphonuclear cells (PMN) to leave the blood and enter the uterine

endometrium. (2&3) Bacterial lipopolysaccharide (LPS) inhibits the secretion of gonadotrophin releasing hormone (GnRH) and luteinizing hormone (LH), respectively, but has no effect on follicle stimulating hormone (FSH). (4) Bacterial LPS binds to a receptor on the ovary and suppresses the production of oestradiol. (5&6) Binding of LPS to endometrial cells causes these cells to secrete E series prostaglandins (PGE_2) instead of the F series prostaglandins ($PGF_{2\alpha}$). (7) Cytokines reduce the mRNA expression for PGE_2. (8) Cytokines inhibit the secretion of GnRH.

2.8.1 Bacteria

Parkinson et al. (2007) reported that > 90% of cows have bacterial contamination of the uterus for the first two weeks after calving, with the prevalence decreasing with increasing time postpartum (Table 6).

Table 6: The proportion of cows' uteri that are contaminated with bacteria during the postpartum period[1]

Days after calving	Contaminated uteri
1-7	92%
8-14	96%
15-21	76%
22-28	64%
29-35	30%
36-42	30%
43-49	25%

[1]Parkinson et al. (2007), reproduced, with permission, from *2007 Proceedings of the Society of Dairy Cattle Veterinarians of the New Zealand Veterinary Association*, published by VetLearn, Wellington, New Zealand.

It has been widely reported that the invasion of the uterus by infection-causing bacteria has a negative impact on reproductive performance. Bacteria exert their impact on reproductive performance in a number of ways: by directly damaging the uterine endometrium (Herath et al. 2007; Williams et al. 2007) which delays uterine involution (McDougall 2001), through bacterial lipopolysaccharide (LPS) disruption of follicle development and steroidogenesis which affects subsequent ovulation (Herath et al. 2007), through disruption of the hypothalamus or pituitary gland secretions (Battaglia et al. 1999), and through stimulation of the endometrium to secrete prostaglandin E_2 (PGE_2) which leads to persistence of the CL (Williams et al. 2007; Sheldon et al. 2009).

Infection of the uterus with bacteria does not disrupt secretion of follicle-stimulating hormone (FSH), thus, the first wave of ovarian follicles emerge approximately 10 days after parturition (Macmillan 1998; Sheldon et al. 2002). However, cows with endometritis have slower growth of dominant follicles and lower peripheral plasma oestradiol concentrations, which reduces the likelihood of ovulation (Sheldon et al. 2002). Oestrogens are essential for ovarian follicular growth, development and function, and have a central role in nurturing the oocyte and ovulation (Schams and Berisha 2002). The granulosa cells that surround the oocyte produce oestradiol by aromatisation of androstenedione derived from the theca cells, under the regulation of FSH (Fortune 1994). Bacteria suppress production of oestradiol from the granulosa cells via the LPS molecules they express on their surface (Herath et al. 2007). The LPS migrates to the ovary where it binds to the Toll-like receptor-4 /CD14/MD-2 receptor complex, that is expressed by granulosa cells, and down-regulates the expression of aromatase genes which results in suppression of oestradiol production and failure of ovulation (Herath et al. 2007).

Another way that bacteria potentially disrupt follicle ovulation is by inhibiting the release of gonadotrophin-releasing hormone (GnRH) and luteinising hormone (LH) from the hypothalamus and the pituitary gland, respectively (Battaglia et al. 1999; Williams et al. 2001; Herath et al. 2009). Prior to ovulation, plasma oestradiol concentrations rise; this signals the hypothalamus and the pituitary gland to secrete the pre-ovulatory GnRH and LH surges which precede ovulation of the dominant follicle (Moenter et al. 1990). However, the signal from oestradiol required for the surges of GnRH and LH can be blocked by LPS (Battaglia et al. 1999), thus preventing ovulation. The mechanism by which LPS block the oestrogen signal has not been reported. However, as LPS was administered intravenously in this study, perhaps the LPS gained accessed the hypothalamus and pituitary to supress the surges through the hypophysial portal blood system. It must be noted though, that LPS only suppresses the secretion of gonadotrophins if the signal from the oestradiol is blocked before the onset of GnRH/LH surge release. Once the surges have begun, LPS has no effect (Battaglia et al. 1999).

Once cows have successfully completed their first ovulation after calving, it is common for those with uterine infections to have prolonged luteal phases, thus preventing the next ovulation from occurring and delaying conception (Opsomer et al. 2000; Williams et al. 2007). If the infection is not cleared during the follicular phase and remains during the following dioestrus, the CL becomes persistent and has prolonged progesterone secretion (Parkinson et al. 2007). Progesterone suppresses immune function and propagates continued infection (Parkinson et al. 2007), further reducing reproductive function. Persistence of the CL is due to insufficient secretion of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), which is required to regress the CL and terminate the luteal phase (Opsomer et al. 2000; Parkinson et al. 2007; Williams et al. 2007; Herath et al. 2009). Prostaglandin $F_{2\alpha}$ is secreted from oxytocin-stimulated

endometrial epithelial cells and is luteolytic, whereas PGE_2, which is also secreted by endometrial epithelial cells, is luteotrophic (Arosh et al. 2004). Insufficient secretion of $PGF_{2\alpha}$ can be due to LPS binding to endometrial cells and stimulating them to secrete PGE_2 instead of $PGF_{2\alpha}$ (Herath et al. 2009). This results in low concentrations of $PGF_{2\alpha}$ and persistence of the CL.

In contrast to potentiating CL function, Williams et al. (2007) reported reduced luteal function in cows with uterine infections. In this study, cows with uterine infections, that had ovulated, had smaller corpora lutea and lower peripheral plasma concentrations of progesterone than normal fertile animals. Lower levels of progesterone would inhibit conception and the survival of embryos, as sufficient concentrations of progesterone are required to maintain pregnancy (McLeod and Phillips 1998). The CL of cows with uterine infections were smaller because cows with uterine infections ovulate smaller first dominant follicles, which produce smaller CL (Williams et al. 2007; LeBlanc 2008).

While several studies reporting how bacteria negatively affect reproduction have been discussed in this section, not all studies investigating the mechanisms by which endometritis affects reproduction have found a link with bacteria. In contrast to the results reported in the TMR studies, the results reported by McDougall et al. (2011) indicated that the five most common pathogenic bacteria isolated from the uterus in TMR-based farm systems (*A. pyogenes, P. melaninogenica, E. coli, F. necrophorum* and *Proteus spp.*) were not the major contributing factor to the poor reproductive performance observed in cows with SCE in New Zealand dairy systems. This contrasting result may be due to differences in management practices and environmental conditions. The authors concluded that in cows with SCE, uterine inflammation (as measured by PMN %) is more closely related to the negative reproductive outcomes associated with SCE than with isolation of bacteria from the uterus. This conclusion is also supported by Barański et al. (2012). Therefore, the results reported by McDougall et al. (2011) and Barański et al. (2012) suggest that there are inflammatory mechanisms which reduce reproductive performance that are not necessarily related directly to bacteria.

2.8.2 Inflammation

Inflammation can be considered as a healing process if it is restrained within certain limits (e.g. length of time the inflammation remains and the severity of the inflammatory response) but might be harmful if those limits are exceeded (Grimble 2001). These limits appear to be exceeded in some cows, as uterine inflammation has been reported to disrupt ovarian function, hypothalamic and pituitary gland function, and reduce embryo survival.

The potential disruption of ovarian function by uterine inflammation was reported by Sheldon et al. (2002). The authors reported that cows with uterine inflammation had reduced numbers of first and

second dominant follicles in the ovary ipsilateral to the previously gravid horn, reduced dominant follicle growth rates and decreased oestradiol secretion. The reduced dominant follicle growth rate and oestradiol secretion would most likely result in failure of the dominant follicle to ovulate. A possible reason for the reduced oestradiol secretion recorded in the Sheldon et al. (2002) study may be that the release of proinflammatory cytokines by endometrial cells impairs granulosa cell steroidogenesis (Spicer and Alpizar 1994; Sheldon et al. 2009). It has also been postulated that the cytokines secreted by the inflamed endometrium are the reason for the reduced peripheral plasma progesterone levels of cows with uterine inflammation (Sheldon et al. 2009). This is because bovine luteal cells are highly responsive to a range of cytokines (Petroff et al. 2001; Okuda and Sakumoto 2003; Sheldon et al. 2009).

Rivest et al. (1993) reported that cytokines disrupted the hypothalamic-pituitary axis. In a study using rats, the cytokine interleukin-1ß was reported to exert an effect on the hypothalamic-pituitary axis by altering GnRH neuronal activity and inhibiting the release of hypothalamic GnRH. This would prevent occurrence of the pre-ovulatory surge of GnRH that is essential for ovulation.

Uterine inflammation has been shown to have the potential to reduce embryo survival. Cows with uterine inflammation have a suboptimal uterine environment for embryo survival, which contributes to poor reproductive performance through embryonic loss. In a study conducted by Hill and Gilbert (2008) embryos were cultured in media conditioned with the products recovered from either an inflamed or a normal cow endometrium. The embryos cultured in inflamed conditioned media had reduced blastocyst quality as determined by lower total trophectoderm cell numbers (83.1 vs 99.8 cells for inflamed vs non-inflamed conditioned media). This reduction in trophectoderm cell numbers would be expected to result in reduced embryo viability and conception rate. The authors proposed that the reduction in trophectoderm cell numbers could leave the inner-cell mass of the blastocyst susceptible to damage from components such as inflammatory cytokines, and affect placental size and function. This hypothesis is supported by Soto et al. (2003) who found that tumour necrosis factor-α increased blastomere apoptosis and can thus compromised the development of the resultant embryo.

A potential mechanism for the reduction in embryo quality in cows with uterine inflammation/SCE was reported by Back et al. (2011). The cows with SCE in the study had increased concentrations of histidine, alanine, aspartate, and serine in follicular fluid. Histidine is important in initiation of the inflammatory response and can reduce the production of anti-inflammatory molecules; the other three amino acids affect the function of cells of the innate immune system (Li et al. 2007). Back et al. concluded that these changes in the amino acid profile could potentially result in altered oocyte viability - which in turn could reduce embryo quality and survival.

Inflammatory responses are mediated by molecules such as PGE_2, which regulate the production of various cytokines such as tumour necrosis factor-α and interleukin 6 (Bos et al. 2004; Gabler et al. 2009). In addition to its immunomodulatory role, PGE_2 is considered an important factor to prevent rejection of the embryo, as it suppresses the immune system to allow the establishment of pregnancy by inhibiting lymphocyte production (Low and Hansen 1988; Bos et al. 2004; Herath et al. 2009). In a study reported by Gabler et al. (2009), uterine mRNA expression of three PGE_2 synthases and the synthase of another inflammation mediator, prostaglandin D_2 (PGD_2; an anti-inflammatory molecule), were investigated in cows with or without endometritis. The mRNA expression of all components investigated differed in cows with endometritis from those without. Specifically, cows with endometritis had lower mRNA expression of the cytosolic PGE_2 synthase. This reduction in PGE_2 synthase may mean that insufficient concentrations of PGE_2 would be secreted, resulting in failure to suppress the maternal immune system and thus embryo loss.

The cause/source of uterine inflammation in the postpartum period has been widely discussed. Reported causes of inflammation and pro-inflammatory cytokine release within the cow include: metabolic diseases, parasites, trauma, endotoxin from the gut, injuries, mammary gland oedema, calving stress, and bacteria (Drackley et al. 2005; Sheldon et al. 2006; Bertoni et al. 2008). It is well known that bacterial infection of the uterus induces an inflammatory response in the uterus (Sheldon et al. 2006). However, not all cows that have uterine inflammation have bacteria. Uterine inflammation detected in the absence of bacteria has been reported by McDougall et al. (2011) and Barański et al. (2012), where not all cows with detectable uterine inflammation (PMN %) were culture-positive for bacteria. In addition, Sagartz and Hardenbrook (1971) reported that a higher proportion of cows that experienced poor reproductive performance had uterine inflammation rather than uterine infection. However, it must be noted, that just because bacteria were not able to be cultured, it does not mean that there were no bacteria present.

Although the cause of this uterine inflammation in the absence of bacteria is yet to be determined, one hypothesis has been proposed. Sheldon et al. (2009) suggested that the uterine inflammation observed without concurrent bacterial presence could be due to the inflammation, as defined by a high PMN count, persisting after the bacteria have been cleared. The inflammation may be persisting because it has become dis-regulated. This hypothesis is supported by the results reported by Gabler et al. (2009). In this study, cows with SCE had reduced mRNA expression of PGE_2 and increased expression of PGD_2. The authors suggested that these changes in the prostaglandin pathway indicated a dis-regulation of the PGE_2 and/or PGD_2 profile. As PGE_2 modulates inflammation, a lower level of PGE_2 synthesis could lead to inflammation being improperly controlled. Therefore perhaps this dis-regulation of the prostaglandin pathway could be the cause of the persistent uterine inflammation seen in cows with SCE.

Whatever the cause/source of the uterine inflammation, the fact that it has a negative impact on reproductive performance is what is important. As stated previously, it has been reported that the reduced reproductive performance associated with SCE are more closely related to uterine inflammation than the presence of bacteria in the uterus. This indication that inflammation rather than presence of bacteria is the cause of poor reproductive performance in cows with SCE is also supported by the results reported by Bertoni et al. (2008). When the cows in this study were grouped according to inflammation indices, not clinical problems, cows with the highest inflammation indices had reduced reproductive performance and milk production. The authors suggested that the systemic effects of diseases mediated by proinflammatory cytokines (e.g. endometritis) could be a common mechanism for reproductive impairment. The conclusion that inflammation may be the more important factor in the poor reproductive performance of cows with SCE than bacterial presence in the uterus has important implications for how SCE is treated, and indicates that the treatment of SCE should focus on eliminating inflammation rather than bacteria.

2.9 Treatment of endometritis

Treatment of endometritis has been the subject of considerable discussion, particularly with respect to which therapy to use, which cows to treat (LeBlanc et al. 2002b), and when to treat them. The treatments used have focused on; eliminating bacteria (antibiotics, proteolytic enzymes and disinfectants), eliminating anoestrus by stimulating oestrus (prostaglandin, $PGF_{2\alpha}$), resumption of normal ovarian activity by removing persistent corpora lutea ($PGF_{2\alpha}$), and reducing inflammation and its negative effects on reproductive performance (e.g. use of a NSAID). Treatments for SCE that have been investigated include antibiotics (ceftiofur and cephapirin) and prostaglandins ($PGF_{2\alpha}$ and cloprostenol). These treatment options, plus proteolytic enzymes and disinfectants, have also been investigated for clinical endometritis.

2.9.1 Antibiotics

As bacterial infection appears to be one of the causes of endometritis, it seems logical to use antibiotics for therapy. However, there are several things to consider. The formulations should: be effective against the pathogens present, not inhibit uterine defence mechanisms, be effective in a pyogenic environment, leave no milk or meat residue, have an adequate concentration, incorporate an adequate number of treatments, and be cost effective (Drillich et al. 2005; Kasimanickam et al. 2005b). The proposed theory for antimicrobials working is not that they sterilise the uterus, but that they reduce the bacterial load to a level where the cows' natural defences can gain dominance and the inflammatory stimulus is reduced (LeBlanc 2008).

It has been reported by Kasimanickam et al. (2005b) that treatment with a single intrauterine administration of cephapirin increased pregnancy rate by 62% for cows with SCE compared with

control cows. Cephapirin is a first generation cephalosporin antibiotic that is predominantly active against gram-positive organisms and anaerobic bacteria (Kasimanickam et al. 2005b). However, several other studies have reported no beneficial effect on reproductive performance or clinical cure rate from the use of antibiotics for either clinical or subclinical endometritis (Knutti et al. 2000; LeBlanc et al. 2002b; Galvão et al. 2009b; Kaufmann et al. 2010b). These mixed results indicate that antibiotics are not entirely effective as a treatment option for endometritis. This may be the due to the fact that not all cows with endometritis have bacteria present in the uterus, and/or that the presence of bacteria does not necessarily mean that there is an infection.

2.9.2 Prostaglandins

The proposed mechanism by which $PGF_{2\alpha}$ (or one of it analogues such as cloprostenol) treatment is effective is that $PGF_{2\alpha}$ induces luteolysis in cows with a responsive CL (Knutti et al. 2000; LeBlanc et al. 2002b; Kasimanickam et al. 2005b). This is beneficial especially if the luteal phase has been prolonged by endometritis as explained earlier. The decrease in progesterone levels would help the uterus to clear any infection as progesterone decreases uterine phagocyte function (Hussain 1989; Lewis 2003; LeBlanc 2008). Prostaglandins also stimulate uterine motility and induce oestrus, which will help to clear out purulent material from the uterus (Lewis 1997; Knutti et al. 2000; Kasimanickam et al. 2005b).

Galvão et al. (2009a) investigated the effect of $PGF_{2\alpha}$ treatment administered at 21, 35 and 49 days postpartum on the cure rate of SCE and subsequent reproductive performance. The results demonstrated that $PGF_{2\alpha}$ did not affect SCE prevalence or increase pregnancy rate at days 35 or 49 in cows with SCE. Interestingly, first service conception rate was increased by the treatment when comparing treated versus control cows (irrespective of endometritis status). However, this was attributed to an improvement in the cows without SCE. In contrast to the null result reported by Galvão et al. (2009a), Kasimanickam et al. (2005b) reported a 63% higher pregnancy rate for cows that were treated with cloprostenol at 20 to 33 days in milk (DIM). Mixed results have also been reported for the effectiveness of $PGF_{2\alpha}$ as a treatment for clinical endometritis (Knutti et al. 2000; LeBlanc et al. 2002b; Kaufmann et al. 2010b). The inconsistent effect of $PGF_{2\alpha}$ treatment may be due to therapeutic trials suffering from a lack of negative controls and small numbers resulting in low statistical power (LeBlanc et al. 2002b). On the other hand, for this treatment to work, a responsive CL is required. Thus, as the first postpartum ovulation may not have occurred for many cows, due to endometritis or other factors, not all cows would have a CL present, resulting in fewer potentially responsive cows. Therefore, the mixed results obtained from studies investigating both SCE and clinical endometritis, and the need for a responsive CL for the treatment to be effective, indicates that $PGF_{2\alpha}$ is not an entirely effective treatment option for endometritis.

2.9.3 Proteolytic enzymes and disinfectants

Increasing criticism by the public and political concern about the use of antibiotics and hormones in food-producing animals (Drillich et al. 2005) has led to research into other treatment options. Disinfectants and proteolytic enzymes are the two options discussed here. The disinfectant investigated by Nakao et al. (1988) was 2% polyvinylpyrrolidone–iodine solution. This disinfectant was used because it is less irritating than the commonly used disinfectant, Lugol's solution. However, the results demonstrated that the disinfectant had a negative effect on reproductive performance. Proteolytic enzymes were investigated as they have previously been used for the intra-mammary treatment of mastitis (Drillich et al. 2005). The enzymes used were chymotrypsin, trypsin, and papain. These enzymes have fibrinolytic and proteolytic activity in inflamed tissue which is proposed to support the cellular defence mechanism by inhibiting growth and survival of micro-organisms (Drillich et al. 2005). Drillich et al. (2005) investigated the treatment of cows with endometritis 21 to 27 DIM with an enzyme salve (containing chymotrypsin, trypsin, and papain). The results did not demonstrate any beneficial effect on clinical cure rate or reproductive performance. The results from these two studies demonstrate that neither of these two options is an effective treatment option for endometritis.

2.9.4 Non-steroidal anti-inflammatory drugs

As previously discussed, SCE is inflammation of the uterine endometrium, and this inflammation is associated with a negative impact on reproductive performance. In addition, cows with SCE have been reported to have indicators of systemic inflammation. Furthermore, Bertoni et al. (2008) reported that the results from their study suggest the systemic effects of diseases mediated by pro-inflammatory cytokines (such as endometritis) could be a mechanism for reproductive impairment and reduced milk yield occurring during and after disease. The authors also suggested that emphasis should be placed on reducing inflammation during the postpartum period. This has led to the hypothesis that reducing the inflammation associated with SCE (both uterine and systemic) will improve reproductive performance.

Inflammation is induced by prostaglandins that stimulate the release of pro-inflammatory cytokines (Elliott and Elliott 1997). Therefore, to reduce inflammation the synthesis of prostaglandins needs to be inhibited. Non-steroidal anti-inflammatory drugs block the synthesis of prostaglandins (Sales and Jabbour 2003), making NSAID a candidate to treat SCE. Prostaglandin synthesis is inhibited by NSAID via inhibition of one, or both, of the two isoforms of the cyclooxygenase enzyme, COX-1 and COX-2 (Anderson 1990). The COX-1 isoform is constitutively expressed and is considered a to be 'house-keeping' enzyme, whereas COX-2 is generally only expressed during inflammatory events (Mitchell et al. 1993; Sales and Jabbour 2003). A NSAID may inhibit either COX-1 or COX-2, or both, depending on

the selectivity of the NSAID (von Krueger and Heuwieser 2010). The selectivity of NSAID in cattle has not been reported yet.

Unfortunately there have been no studies reported specifically on treating endometritis (either clinical or subclinical) with a NSAID; however there are several studies on the use of a NSAID to improve reproductive performance. Which NSAID to use, and when to administer it, are the two main questions that researchers have investigated. The use of a NSAID post-insemination to improve embryo survival has been investigated in three studies (Erdem and Guzeloglu 2010; von Krueger and Heuwieser 2010; Heuwieser et al. 2011), and others have investigated the use of a NSAID in the period immediately after calving (Trevisi and Bertoni 2008) to reduce the negative effects of inflammation on reproductive performance.

To maintain pregnancy, $PGF_{2\alpha}$ must be prevented from being secreted by the uterus, as it induces luteolysis (Erdem and Guzeloglu 2010; von Krueger and Heuwieser 2010). This secretion is stopped by the embryo secreting interferon-τ, the maternal pregnancy recognition protein, which reduces the oxytocin-dependent pulsatile release of $PGF_{2\alpha}$ (Thatcher et al. 1995; Bazer et al. 1997). This process happens between days 14 to 17 after conception (Bazer et al. 1997). A proposed reason for embryonic loss is that there may be retardation of the embryo's development, which could result in insufficient interferon-τ being secreted for the pregnancy to be recognised and prevent luteolysis (Erdem and Guzeloglu 2010; von Krueger and Heuwieser 2010). Another proposed reason for embryonic loss is that manipulation of the reproductive tract (e.g. insemination) causes inflammatory processes, such as the release of cytokines and $PGF_{2\alpha}$, which can induce luteolysis and embryo loss (Heuwieser et al. 2011).

Erdem and Guzeloglu (2010) investigated whether embryo survival/pregnancy rate would be improved by inhibiting luteolysis as a result of suppressing the production of $PGF_{2\alpha}$ through use of a NSAID. To do this, a single dose of the NSAID meloxicam was administered at day 15 after artificial insemination (AI) and the subsequent pregnancy rate recorded. Meloxicam was chosen as it has a longer half-life, and therefore longer anti-inflammatory effect, than flunixin meglumine (Erdem and Guzeloglu 2010). The authors reported that the treatment had a negative effect on pregnancy rate when compared with the pregnancy rate of the control group. A negative effect on pregnancy rate was also reported by Heuwieser et al. (2011) in cows treated with either intrauterine carprofen, or carprofen administered subcutaneously, at the time of AI. von Krueger and Heuwieser (2010) investigated the use of flunixin meglumine or carprofen on first service conception rate. Flunixin meglumine was administered in 2 doses on days 14 to 16 after insemination, and carprofen was administered in one dose on day 15 after insemination. The authors reported no increase, relative to the control group, in conception to first service for either of the treatments.

The results from the studies in the previous paragraph indicate that treatment with a NSAID at, or near, the time of insemination is not effective for improving reproductive performance. However, treatment of inflammation with a NSAID during the early postpartum period has had more success. Trevisi and Bertoni (2008) investigated the effect of acetylsalicylate (AS) given in the first five days postpartum. The results demonstrated that cows that received AS had a higher first service conception rate, required less services per conception, and fewer cows became repeat breeders. The study also reported other interesting results: cows with the lowest inflammatory status had the highest milk yield and highest fertility indices, the AS treated cows' milk yield rose faster and remained significantly higher until 60 DIM, the AS cows recovered normal liver protein synthesis levels quicker, positive acute phase proteins were reduced quicker, and negative acute phase proteins increased and returned to normal faster than for control cows. These results indicate that treating cows with inflammation with a NSAID in the early postpartum period is an effective option for improving reproductive performance. Therefore, it is possible that treating cows with SCE with a NSAID in the early postpartum period will be an effective treatment option for the condition.

2.10 Validating biological markers

At this point in time, the most common methods for detecting SCE are uterine cytobrush cytology or uterine flushing. These methods are effective for diagnosing SCE, but collecting a sample of cells from within the uterus for cytological examination is an invasive and time consuming process. This means that although these methods are well suited for the use in scientific research, they are simply not practical for on-farm diagnosis of SCE. Therefore, more practical methods for detecting and diagnosing SCE are required.

One potential method is the use of farm cow health records. Peripartum disorders such as dystocia, twinning, stillbirth and retained placenta predispose cows to uterine infections (McDougall 2001). Therefore, the use of farm cow health records from the period around calving could potentially be used to identify cows that are at risk of uterine infection. This would reduce the cost of diagnosis by reducing the number of cows to be submitted for veterinary assessment (Sheldon et al. 2006). However, this method does not give a specific diagnosis, nor capture all affected cows. In a study reported by LeBlanc et al. (2002a), over half the cows with endometritis did not have any of the peripartum disorders listed above. This means that more specific markers are required to detect SCE.

The main focus of research into alternative detection and diagnostic methods has been on biological markers. There are two different types of biological markers, direct and indirect. A direct marker is a marker that is directly associated with the condition that is being investigated, i.e. a factor that is either a direct cause or effect of the condition being investigated. For example, a direct marker for milk fever is blood calcium concentration (Parkinson et al. 2010). No direct marker for SCE, other

than uterine PMN %, is known. An indirect marker is a marker that is not directly associated with condition, but is associated with a part of the physiological processes involved with the condition being investigated. For example, an indirect marker for inflammation is leukocyte esterase (Couto et al. 2012). A range of indirect markers have been investigated for detection and diagnosis of SCE.

2.10.1 Dry matter intake

Studies reported by Zamet et al. (1979), Bell and Roberts (2007), and Huzzey et al. (2007) have found an association between cows with uterine infection and depressed DMI, possibly due to depressed appetite. Thus, biological markers of DMI could be useful for detecting cows at risk of SCE. Markers for DMI include non-esterified fatty-acids (NEFA), urea, glucose, and ß-hydroxybutyrate. Differences have been reported in these markers, especially increased plasma NEFA concentrations, in cows with endometritis in TMR-based dairy systems (Hammon et al. 2006; Kaufmann et al. 2010a), but not in pasture-based dairy systems (Burke et al. 2010). Kaufmann et al. (2010a) investigated whether cut points associated with SCE could be established for NEFA, ß-hydroxybutyrate, bilirubin, and urea, and whether these cut points could be used as predictors of cows with SCE. Cut points associated with SCE were able to be established, but the predictive accuracy of these cut points was not satisfactory for practical use. The results from the Kaufmann et al. (2010a) study and the difficulties in measuring DMI in pasture-grazed dairy cows, means that DMI and markers of DMI are currently not suitable to use for the detection of cows with SCE.

2.10.2 Milk parameters

Milk composition and yield were investigated by Green et al. (2009) as biological markers of SCE. The significant results reported were deceased milk protein and fat % and an increased somatic cell count in cows with SCE compared with control cows. The authors suggested the results indicated that lower milk fat %, milk protein %, and milk yield in conjunction with an early Metricheck^{TM} examination could aid in the detection of SCE. Practically, measuring and tracking milk production to detect SCE is feasible. However, the problem with the use of milk production and composition for detection of SCE is that many other factors, such as other illnesses, can also impact milk parameters. In addition, the effect of SCE on milk production and composition has been variable. Dubuc et al. (2011) found no statistically significant differences in milk yield between cows with and without SCE, however other studies have reported a negative association between SCE and milk yield (Burke et al. 2010; McDougall et al. 2011). Due to the inconsistency of the effect of SCE on milk production and the multi-factorial causes for alterations in milk production, is not likely that milk production will be a good biological marker for SCE.

2.10.3 Markers in serum/blood

It has been reported by Huzzey et al. (2007), Bertoni et al. (2008), Green et al. (2009), and Burke et al. (2010) that there are links between inflammation, liver function, SCE, and reproductive performance. Although these links are more likely to be associations than cause and effect, they provide potential markers for SCE in blood/serum.

Bertoni et al. (2008) investigated the link between inflammation, liver function, and fertility by evaluating serum indices around parturition. Inflammation was identified by measuring the concentration of positive acute phase proteins (+APP) such as haptoglobin and globulin. Liver function was assessed by retrospectively classifying the cows into quartiles using a liver activity index, which was based on the plasma concentration of negative acute phase proteins (-APP). The authors reported that cows with inflammation (higher plasma concentrations of haptoglobin and globulin) had a lower liver activity index (reduced production of –APP liver proteins). It was proposed by the authors that the drop in –APP was not due to liver damage, rather that it was due to diversion of liver protein synthesis to favour +APP. The liver is still producing as many proteins as normal, but it is producing more +APP and less –APP's (Bertoni et al. 2008). This process was termed 'liver function impairment' by Bertoni et al. (2008). Impairment of liver function was indicated by lower bilirubin clearance, a normal function of the liver (Bertoni et al. 2008). This liver function impairment meant that the synthesis of the +APP in the liver was up-regulated at the expense of the –APP, resulting in lower plasma concentrations of albumin. Albumin is a -APP produced by the liver under normal conditions and helps to maintain blood volume and clotting factors (Fleck 1989; Parkinson et al. 2010). In addition, Bertoni et al. (2008) reported that cows with the lowest liver activity index (low concentrations of –APP) had the lowest milk yield and poorest reproductive performance. These results demonstrate that indicators of inflammation, the +APP liver proteins, and indicators of liver function impairment, the –APP liver proteins, are associated with reproductive performance. This indicates that the positive and negative acute phase liver proteins could be used as biological markers for identifying cows at risk of poor reproductive performance.

In addition to being markers for poor reproductive performance, the acute phase proteins have also been suggested as markers for uterine infection. Sheldon and Dobson (2004) reported that increased concentrations of non-specific markers of inflammation, such as the acute phase proteins, were associated with uterine disease and poorer fertility. In a study reported by Huzzey et al. (2009), cows with uterine infections had increased plasma haptoglobin. Haptoglobin is a +APP synthesised by the liver whose plasma concentrations is elevated during the acute phase response (Fleck 1989; McClatchey 2002; Bertoni et al. 2008). Huzzey et al. (2009) proposed that haptoglobin could be a biological marker for predicting uterine infection.

The link between inflammation, liver function, and endometritis was investigated by Green et al. (2009) and Burke et al. (2010). Green et al. (2009) investigated the association between liver proteins and SCE diagnosed at day 21 and 42 postpartum. The cows with SCE had lower plasma albumin, and lower total protein concentrations. The authors suggested the decreased plasma albumin and total protein could be possible biological markers for SCE. In a study conducted by Burke et al. (2010), blood samples were analysed for indicators of liver function (albumin and globulin), inflammation (haptoglobin, globulin, GDH, ASAT), and mineral status (Ca and Mg). Cows with SCE at day 42 postpartum (as classified by >6% PMN) had lower plasma albumin concentration, lower albumin to globulin ratio, and lower plasma Mg concentration. These cows also had higher plasma GDH and ASAT concentrations. Glutamate dehydrogenase is a liver-specific enzyme with a short plasma half-life, whose presence in serum indicates liver damage, which can occur during an inflammatory event (Taylor et al. 2010). Aspartate aminotransferase is an enzyme from the liver whose concentration in blood increases 6 – 10 hours after liver damage occurs (Keogh 2011). In contrast to the results reported by Huzzey et al. (2007), plasma concentrations of haptoglobin were not elevated in cows with SCE. The results of the Green et al. (2009) and Burke et al. (2010) studies support the existence of an association between endometritis, inflammation, impaired liver function, and reduced reproductive performance. Therefore, measures of inflammation or liver function could be potential markers for SCE.

Other associations between blood indices and SCE include the haematological measures investigated by Green et al. (2009). The haematological measures investigated included red blood cell count, haematocrit, haemoglobin, white blood cell count, fibrinogen, lymphocytes and neutrophil %. The cows with SCE had higher plasma neutrophil concentrations. Green et al. (2009) suggested the increased plasma neutrophil concentration could be a possible biological marker for SCE. Other potential markers were reported by Lopdell et al. (2011). In this study, cows with SCE had increased plasma concentrations of the amino acids serine and aspartate. Both of these amino acids have immune and energetic functions, with serine being involved in the regulation of interleukin-2 production, T-lymphocyte activation and gluconeogenesis (Li et al. 2007; Wu 2009). Aspartate also has a role in leukocyte and lymphocyte function (Wu 2009). The results from these two studies indicate that plasma neutrophils and amino acids could be potential markers for SCE.

The results from all the studies discussed above indicate that markers of inflammation such as globulin, ASAT, and GDH, could be viable candidates as biological markers for SCE. Subclinical endometritis is inflammation of the uterus; therefore it seems logical that biological markers associated with inflammation would be viable candidates as biological markers for the detection of cows at risk of SCE. Other potential markers could be indicators of liver function impairment (total protein and albumin), plasma neutrophils, and amino acids (serine and aspartate).

Although collecting a blood sample from a herd of cows for the diagnosis of SCE is not as quick and easy as Metrichecking for clinical endometritis, it is a viable option. Factors that need to be taken into consideration are the timing of collection of the blood sample, e.g. pre or postpartum, and the cost of sample analysis and thus how many metabolites should be analysed.

Chapter 3

The effect of an anti-inflammatory drug on subclinical endometritis

3.1 Declaration

The content in this experimental chapter has been published in the Journal of Dairy Science (vol 96, issue 7, pages 4323 to 4332) as an paper entitled 'The responsiveness of subclinical endometritis to a non-steroidal anti-inflammatory drug in pasture-grazed dairy cows' authored by: N. V. Priest, S. McDougall, C. R. Burke, J. R. Roche, M. Mitchell, K. L. McLeod, S. L. Greenwood, and S. Meier. Excerpts of data presented in this chapter have also been presented in two conference papers: at the New Zealand Society of Animal Production (Priest et al. 2012a), and at the Australasian Dairy Science Symposium (Priest et al. 2012b). I was involved in all aspects of the experimental data collection and collation, interpretation of results, writing of the manuscripts, and some of the statistical analyses. Furthermore, all progesterone assays were performed by me. Other analytes were assayed by Gribbles Veterinary Pathology Laboratories. The remainder of the statistical analyses were completed by Barbara Dow. The writing of this manuscript was completed with contributions from my co-authors, and has benefited from the comments and suggestions of anonymous reviewers.

3.2 Introduction

Seasonal, pasture-based dairy cows are constrained to a 365-day inter-calving interval, leaving 83 days from calving to re-establish pregnancy (Rhodes et al. 2003). A rapid restoration of the uterus to a reproductively capable state after calving is, therefore, critical. Uterine disease, such as SCE, impedes uterine recovery. Subclinical endometritis is a uterine disorder characterized by an increased proportion of PMN in the uterus after calving (Barlund et al. 2008; Sheldon et al. 2009; Dubuc et al. 2010). Subclinical endometritis has been reported to be a significant problem in both pasture-based and TMR-based dairy systems, with incidence ranging from 6 to 53% (Gilbert et al. 2005; Green et al. 2009). Negative effects of SCE on reproductive performance include a longer PPAI, lower first-service conception rate and overall pregnancy rate, and more services per conception (Gilbert et al. 2005; Barlund et al. 2008; Burke et al. 2010); lower milk production and altered milk composition have also been reported (Green et al. 2009; Burke et al. 2010; McDougall et al. 2011).

The cause of SCE and mechanistic links to reduced fertility are not well understood. Subclinical endometritis may result from bacterial infection, with lipopolysaccharide disruption of the hypothalamic-ovarian axis and uterine secretions reducing reproductive performance (Battaglia et al. 1999; Herath et al. 2007). Recent indications, however, are that PMN %-defined SCE in pasture-grazed dairy cows is associated with liver dysfunction and systemic inflammation, and that it is the

inflammation (as assessed by PMN %) associated with endometritis and not, necessarily, the presence of bacteria in the uterus, which is causing the negative impact on reproductive performance (Green et al., 2009; Burke et al., 2010; McDougall et al., 2011). A stronger association between poor reproductive performance and inflammation in SCE cows, rather than bacteria, was also reported by Barański et al. (2012) in a TMR-based dairy system. Indicators of liver dysfunction and systemic inflammation reported for SCE cows include a lower albumin: globulin ratio and elevated plasma concentrations of ASAT and GDH (Bertoni et al. 2008; Burke et al. 2010). This association of SCE with inflammation (both systemic and uterine), rather than uterine bacteria, indicates that treatment of SCE may need to focus on reducing inflammation rather than eliminating uterine bacteria; a case for the use of a NSAID. This is supported by the lack of a consistent beneficial treatment effect of antibiotics (Kasimanickam et al. 2005b; Galvão et al. 2009b) and prostaglandins (Kasimanickam et al. 2005b; Galvão et al. 2009a).

To test the role of local (and systemic) inflammation associated with SCE in reducing reproductive performance, a NSAID was used in this study. One pathway through which NSAID reduces inflammation is the COX – prostaglandin pathway. The NSAID inhibits the action of COX and prevents the secretion of prostaglandin, a pro-inflammatory molecule (Sordillo et al. 2009; Erdem and Guzeloglu 2010; Heuwieser et al. 2011). The timing of NSAID treatment is an important consideration, however, because prostaglandin-mediated inflammation is a normal part of the uterine involution process after calving (Barlund et al. 2008). For this reason, NSAID treatment was delayed until 21 days postpartum in the current study.

It is hypothesised that treatment with NSAID between 21 and 31 DIM will reduce the severity (average PMN %) of uterine pathology at 42 days postpartum, without lengthening the PPAI, and will mitigate the negative association of SCE on reproduction and milk production, by reducing inflammation and improving liver function. Additionally, the effect of the NSAID treatment on circulating metabolites and minerals previously reported to be altered in cows with SCE was investigated. The objectives of this study were to determine the effect of a NSAID on PMN % and associated effects on reproduction, metabolic indicators, and milk production.

3.3 Materials and methods

3.3.1 Experimental design

Multiparous cows (n = 213; 136 Holstein-Friesian and 77 Holstein-Friesian x Jersey) aged 5.4 ± 2.2 years (± SD) and with a mean liveweight of 445 ± 56.3 kg were enrolled at Scott Farm (DairyNZ; 37˚47’S, 175˚19’E), Hamilton, between May and October 2011. Primiparous cows were not included due to a lack of availability at the research farm. The number of cows required for the NSAID and control groups (n =100) to detect (80% power, 5% significance) a 1/3 reduction in PMN % (e.g. a

reduction from 15% to 10% PMN) from day 14 to day 42 cytology was calculated using a standard deviation of 0.45 (log_{10} of PMN %; Meier unpublished data) and a detectable difference of 0.18 (difference between the day 14 and day 42 log_{10} PMN % counts). Prior approval for animal use was obtained from the Ruakura Animal Ethics Committee, Hamilton, New Zealand (No. 12294).

To ensure that the control and NSAID treatment groups were balanced for PMN %, a uterine cytology sample was taken on day 14 to 17 postpartum (day 14 PMN %). Cows were blocked on calving date and day 14 PMN % before being randomly allocated to either the control (n = 109) or the NSAID (n = 104) treatment group. To allow time for involution to proceed unimpeded, the NSAID treatment was initiated at 21 to 25 days postpartum. The cows in the NSAID treatment group (Carprieve LA, Norbrook NZ Ltd, Auckland, New Zealand: carprofen 50 mg/mL) were given 3 injections (each injection 1.4 mg carprofen/kg liveweight; 1 mL carprofen/35 kg liveweight), at intervals of 3 days, between 21 and 31 days postpartum. The rationale for this treatment regime was to ensure an extended period of coverage since carprofen has a plasma elimination half-life of 45 to 70 hours (Ludwig et al. 1989; Norbrook 2011).

All cows were examined using the Metricheck™ procedure (McDougall et al. 2007) at day 14 and at day 42. A Metricheck™ score of ≥ 3 was determined in 22 cows at day 14 and 4 cows at day 42. None of these cows received antibiotic treatment and all were included in the analyses. Cows with calving difficulty (n = 1), retained fetal membranes (n = 4), or milk fever (n = 12) were included. Cows (n = 2) were excluded if they received a systemic antibiotic during the experiment.

3.3.2 Uterine cytology

Uterine endometrial cytology samples were collected on day 14 to 17 (day 14 PMN %) and day 42 to 45 (day 42 PMN %) postpartum as described by Burke et al. (2010). The vulva of the cow was cleaned with paper towel, then a double guarded, modified, AI pipette was passed through the cervix and into the uterus. A clean stylette with a clean cytology brush attached (Pap endocervical sample brush, EBOS Group Ltd., Christchurch, New Zealand) was used to collect a sample from the uterine wall. The stylette was then retracted into the AI sheath and all sampling equipment removed from the cow. Material recovered was rolled onto a microscope slide and air-dried. The slides were stained once completely dry (Diff-Quick, Dade Behring, Newark, Delaware, USA). The slides were examined by a veterinary pathologist (IVABS, Massey University, Palmerston North, New Zealand) for determination of the proportion of PMN present. Areas of each slide that contained small clusters of epithelial cells (5 to 20 per cluster) were preferentially selected and all identifiable nucleated cells counted. Based on day 14 PMN % results, cows were retrospectively grouped into quartiles and cows in the upper quartile (≥ 14% PMN, High PMN group) were classified as having SCE. Subclinical endometritis at day 42 was defined as a PMN of ≥ 7%, based on the results reported by Burke et al.

(2010). Cure of SCE from day 14 to day 42 was defined as a cow that had ≥ 14% PMN at day 14 but that had < 7% PMN at day 42. The < 7% PMN cut-off was based on Dubuc et al. (2010) reporting that a PMN % of < 7 at 32 to 38 postpartum did not have a negative impact on reproductive outcomes.

3.3.3 Postpartum anovulatory interval

Progesterone was measured in plasma collected weekly from 3 days postpartum to 54 days postpartum using a commercial kit (Progesterone Coat-a-Count, Siemens, Los Angeles, CA). The average intra-assay coefficient of variation (CV) for the high control was 8.4% and 14.6% for the low control, and the minimum detectable level was 0.08 ng/mL. The average inter-assay CV was 0.5% for the high control, and 9.3% for the low control. Progesterone data were used to determine the PPAI, which was defined as the interval from calving to the first sample day that plasma progesterone concentration was ≥ 1 ng/mL. The PPAI for 43 of 108 control cows and 32 of 103 NSAID cows was > 54 days as their progesterone concentrations remained < 1 ng/mL throughout the sampling period.

3.3.4 Breeding management

The seasonal breeding period was 10 weeks from 20th September to 29th November. The number of days since conception was estimated by a veterinarian at the final pregnancy test (12th January 2012). This was matched with breeding records. For 4 cows, days pregnant were not consistent with the breeding records, therefore the estimated days pregnant from the veterinarian was used instead of the days pregnant from the breeding records.

3.3.5 Blood sample collection

Duplicate venous blood samples were collected weekly from the tail-head into two evacuated blood tubes containing sodium heparin or K_2EDTA as the anticoagulant (Vaccutainer, Becton & Dickinson, New Jersey, USA) for every cow from 28 days before the estimated calving date to 46 to 54 days after calving. Additional samples were collected on the day of calving (D 0) and four days postpartum (D 4). Blood samples were collected at the same time each day relative to milking (pre-a. m. milking for weekly, pre-p. m. for D 0 and D 4 samples). After centrifugation at 1835 x g for 15 min at 4˚C, duplicate plasma samples were harvested and stored at -20˚C.

Plasma was submitted to Gribbles Veterinary Pathology Ltd (Hamilton, New Zealand) within 40 days of sampling. All assays were colorimetric and performed at 37˚C with a Roche Modular P800 analyser (Roche Diagnostics, Indianapolis, IN), using Roche Modular commercial kits. All samples were analysed for: albumin (g/L; bromocresol green reaction at pH 4.1), total protein (g/L; Biuret reaction method), ASAT (IU/L; catalysing activity of transamination of L-aspartate to oxaloacetate), GDH (IU/L; catalysing activity of NADH-dependent conversion of α-ketoglutarate to glutamate), NEFA (mmol/L; commercial kit using the acyl Co-A synthase, acyl-Co-A oxidase method, Wako, Osaka, Japan), and

Mg (mmol/L; xlidyl blue reaction). Day 0 and D 4 samples were also analysed for Ca (mmol/L; o-cresolphthalein complexone method) concentrations. The inter-assay and intra-assay CV for all assays were < 5% and < 2%, respectively.

Levels of Se (nmol/L), Zn (µmol/L), urea (mmol/L) and gamma-glutamyl transferase (IU/L) were determined by Gribbles in plasma from additional blood samples collected from 20 randomly selected cows at 2 to 3 week pre- and postpartum as a measure of herd-level status for these minerals and metabolites.

3.3.6 Milk production

Individual milk yields (kg/cow per d) were recorded twice daily (ALPRO, DeLaval, Tumba, Sweden). Milk protein, fat, and lactose yield were determined weekly (FT120, Foss Electric, Hillerød, Denmark), and SCC every second week (Fossomatic, Foss Electric, Hillerød, Denmark), by Fourier-transfer infrared spectroscopy.

3.3.7 Grazing management and body condition scoring

Cows were maintained as two separate herds, a dry (pre-calving) and a milking herd, until all cows were calved. Both herds received a generous allowance of fresh pasture (ryegrass-white clover mix) supplemented with maize silage and grass silage. Cow body condition score (BCS; 10 point scale: Roche et al. (2004)) was assessed every second week until 1st June, then weekly until the end of the experiment.

3.3.8 Statistical analyses

Polymorphonuclear cell percentage

The day 14 and day 42 PMN % were analysed using linear models. Day 14 PMN % was initially included as a covariate for day 42 PMN %, but was excluded when not significant. Angular transformation of PMN % was performed before analysis; the raw means are presented to aid interpretation.

Associations between PMN % and the variables measured in this study were analysed using three PMN % groups calculated from day 14 PMN % results: High (upper 25%, n = 53, PMN ≥ 14%), Medium (middle 50%, n = 105, PMN 2 to 13%), and Low (lower 25%, n = 53, PMN ≤ 1%).

Reproductive measures

The proportion of cows that ovulated by a specified date postpartum was analysed using generalized linear models with a binomial error distribution in GenStat 14 (VSN-International 2011). Breed (Holstein-Friesian vs Holstein-Friesian cross), age (3 yr. old vs ≥ 4 yr. old), their interaction, and PMN

group (described above) were included in the model as fixed effects. When estimating the effect of NSAID treatment, treatment and the interaction of the PMN groups with treatment were also included as fixed effects. Cow was included as a random effect. Metricheck^{TM} group (score ≤ 2 vs scores ≥ 3) was initially included in the model, but excluded when not significant. The PPAI was analysed using a proportional hazards regression to allow for censored data resulting from having only a lower bound for PPAI for cows still anovular at the end of the measurement period. For the effect of NSAID treatment, cows that had ovulated before treatment were excluded from the analyses (n = 20).

Pregnancy status was determined by the presence or absence of a viable foetus using transrectal ultrasonography at the final pregnancy testing on the 12th January 2012. Conception dates were assigned to the corresponding insemination date for the AI bred cows. For the naturally mated cows, the fetal age was used to calculate conception date. Conception dates were used to calculate the proportion pregnant by 6 (6-week in-calf rate) and 10 weeks (10-week in-calf rate) after the planned start of mating (start of the seasonal breeding period). Those diagnosed as non-pregnant by 10 weeks were used to calculate the non-pregnancy rate. The 3-week submission rate and conception rate was not calculated as half of the cows were naturally mated for the full 10 weeks of the mating period. The association between day 42 PMN % and pregnancy status was not investigated as there was only 12 of 213 (8 control, 4 NSAID) cows with SCE (PMN % ≥ 7%) by day 42. Pregnancy rates were analysed using generalized linear models with a binomial error distribution. The model included breed (Holstein-Friesian vs Holstein-Friesian cross), age (3 yr. old vs ≥ 4 yr. old), their interaction, PMN group (described above), and mating (AI vs natural) as fixed effects. When estimating the effect of NSAID treatment, treatment and the interaction of the PMN groups with treatment were also included as fixed effects. Cow was included as a random effect. Metricheck^{TM} group (score ≤ 2 vs scores ≥ 3) was initially included in the model, but excluded when not significant.

Metabolites, milk production, and body condition score

The metabolite, milk production, and BCS data were analysed using mixed models. The metabolite data were divided into five periods: pre-calving period (-28 to -1 days postpartum), D 0, D 4, pre-treatment (6 to 19 days postpartum), and treatment (20 to 47 days postpartum). The BCS data were divided into three periods: pre-calving period (-28 to -1 day postpartum), pre-treatment (6 to 19 days postpartum), and treatment (20 to 47 days postpartum). The milk production data were divided into two periods: pre-treatment (1 to 21 days postpartum) and treatment (3 weeks from four days after the start of treatment). Metabolite (ASAT, GDH, and NEFA) and somatic cell count data were $\log_{10}$ transformed prior to analysis; the raw means are presented to aid interpretation. The model for the pre-calving and pre-treatment period analyses included breed (Holstein-Friesian vs Holstein-Friesian cross), age (3 yr. old vs ≥ 4 yr. old), their interaction, and PMN group (described above) as fixed

effects. For the treatment period model, treatment and the interaction of the PMN groups with treatment were also included as fixed effects. Cow was included as a random effect. When estimating the effect of NSAID treatment, a covariate was also included in the model as pre-treatment data may be associated with PMN group. The covariate period for the metabolite and BCS data was the pre-treatment data. For the milk data, the covariate period was the week prior to treatment. In order to estimate the interaction between PMN group with NSAID treatment, without the confounding of PMN group and pre-treatment data, the covariate for each variable was calculated by using the residuals from a linear model with the pre-treatment data as stated above as the dependent variable, and age, breed, PMN group, and their interactions as the fixed effects. No covariate was included in analyses when estimating PMN group differences for the treatment period.

Alternative models using PMN % as a continuous variable in place of the categorical PMN group were also tested. These alternative models did not alter any of the statistical outcomes or interpretations, and the results from these are not presented. For all analyses, a significant effect was declared at $P < 0.05$, and a trend at $P < 0.1$.

3.4 Results

Average day 14 and day 42 PMN % were 9.9% and 1.5%, respectively (Figure 6), with no effect of NSAID treatment on day 42 PMN % (1.4% and 1.7% for the control and NSAID groups, respectively; SED = 0.5). The SCE cure rate for the control and NSAID group was 92.3% and 96.3%, respectively.

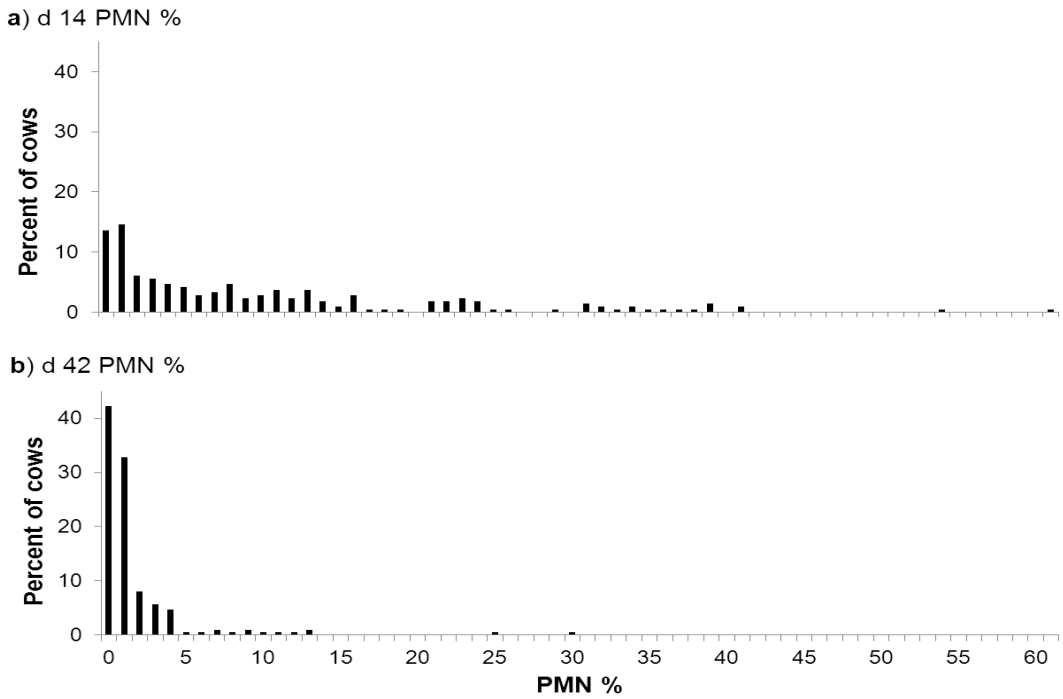

Figure 6: Polymorphonuclear cell (PMN) percentage distribution in uterine cytology samples from dairy cows at a) day 14, and b) day 42 postpartum.

3.4.1 Reproduction

The NSAID treatment did not affect postpartum ovulation rates (46% and 56% ovulated by day 45 postpartum for the control and NSAID groups, respectively; $P > 0.15$), and there were no interactions ($P = 0.98$) between treatment and PMN group. A trend ($P = 0.09$) was found for the Low PMN group to have a higher proportion of cows ovulated by 28 days postpartum than the Medium or High PMN groups, but not at other times (Figure 7).

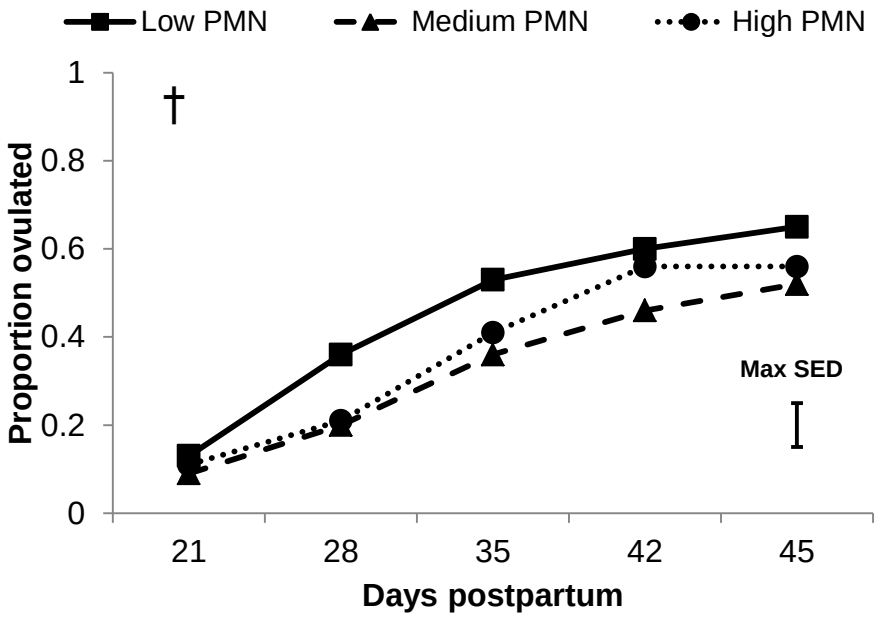

Figure 7: Association between polymorphonuclear cell (PMN) group and the proportion of cows ovulated by a specified day postpartum. The PMN groups are based on uterine cytology results from samples collected on day 14 postpartum: Low PMN (≤ 1% PMN); Medium PMN (2 to 13% PMN); High PMN (≥ 14% PMN). Ovulation was defined as the first sample day postpartum that progesterone concentration was > 1 ng/mL. Raw means and the maximum standard error of the difference (Max SED) are presented. † - There was a trend ($P = 0.09$) for the Low PMN group to have a higher proportion of cows ovulated by 28 days postpartum than the Medium or High PMN groups, but not at other times.

Pregnancy rates were not different between the control and NSAID treatment groups ($P > 0.28$), however, there was an interaction ($P = 0.04$) between NSAID treatment and PMN group 4 weeks after the planned start of mating and there was a trend for an interaction at week 5 ($P = 0.06$), 8 ($P = 0.07$), 9 and 10 ($P = 0.09$) (Figure 8). The interaction reflects an increase in pregnancy rate in the High PMN group treated with NSAID, but not the Low or Medium PMN groups. No associations were determined between PMN category and pregnancy rates ($P > 0.24$). The 6-week in-calf rate was 63% and 70% for the control and NSAID groups, respectively ($P = 0.32$). The non-pregnancy rate after 10 weeks of mating was not affected by treatment: 18% for the control group and 20% for the NSAID group ($P = 0.72$).

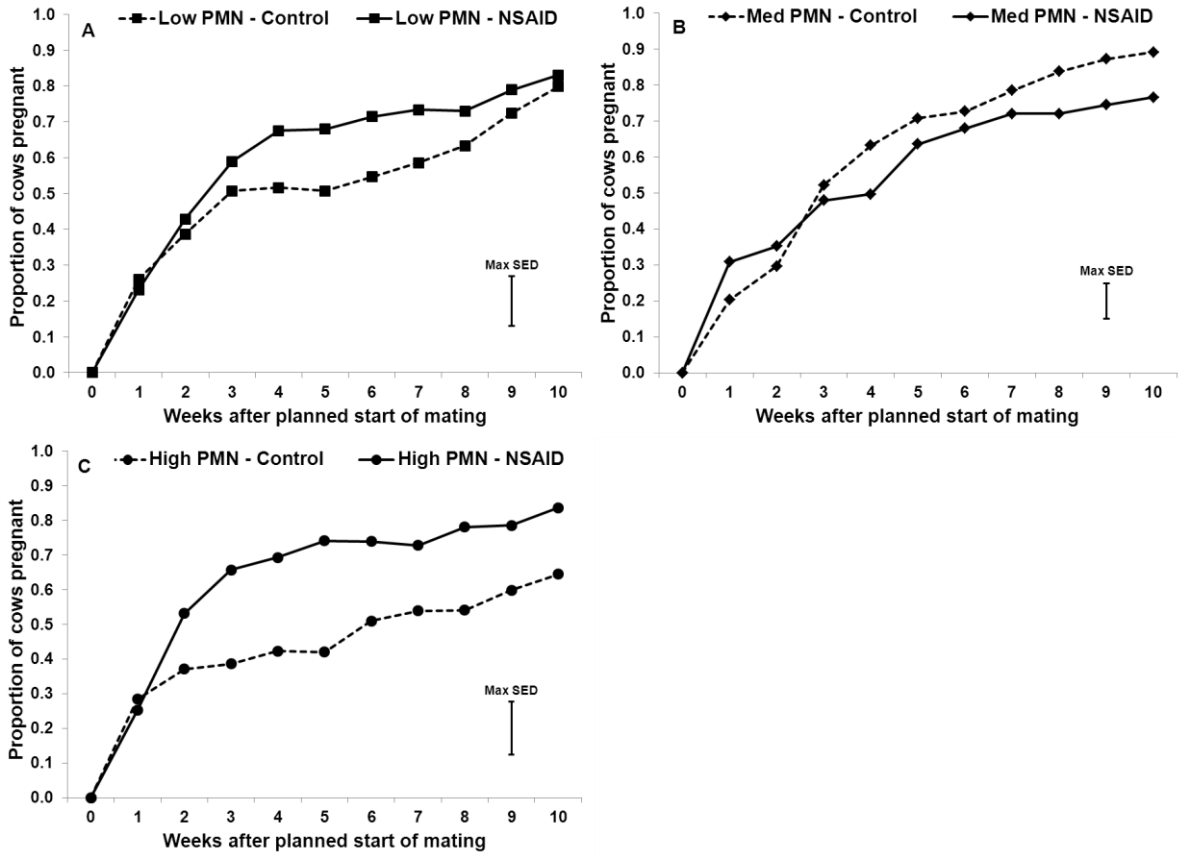

Figure 8: Effect of a non-steroidal anti-inflammatory drug (NSAID) treatment, and the interaction with (A) Low, (B) Medium (MED), and (C) High polymorphonuclear cell (PMN) groups, on the proportion of cows pregnant by a specified week after the planned start of mating. Uterine cytology results from samples collected on day 14 postpartum were used to retrospectively create three PMN groups: Low PMN (≤ 1% PMN); Medium PMN (2 to 13% PMN); High PMN (≥ 14% PMN). The weekly pregnancy proportions have been calculated using the estimated conception date, which was calculated using the final pregnancy test results and mating data. There was an interaction (*P* = 0.04) between NSAID treatment and PMN group 4 weeks after the planned start of mating and there was a trend for an interaction at week 5 (*P* = 0.06), 8 (*P* = 0.07), 9 and 10 (*P* = 0.09); the interaction reflects an increase in pregnancy rate in the High PMN group treated with NSAID, but not the Low or Medium PMN groups.

3.4.2 Metabolites

Following treatment with NSAID, cows had lower plasma concentration of ASAT (*P* = 0.04) and GDH (*P* = 0.18) than untreated cows. The NSAID treatment did not affect the plasma concentrations of the other metabolites measured (*P* > 0.27*)*. No interaction was determined between PMN group and treatment for the metabolic measures.

Plasma albumin concentrations declined pre- ($P = 0.08$) and postpartum ($P < 0.001$) and plasma NEFA increased ($P = 0.03$) on the day of calving with increasing PMN % (Figure 9 and Figure 10). The albumin: globulin ratio tended ($P = 0.07$) to be lower in the High PMN group before the NSAID treatment. Prevalence of SCE was associated with indicators of liver function, with High PMN cows tending ($P > 0.12$) towards greater ASAT and GDH concentrations in blood than Low PMN cows pre- and postpartum. No associations were determined between PMN group and plasma concentrations of total protein and Mg at any time point, but plasma calcium concentration tended to be ($P = 0.09$) lower in the High PMN group on the day of calving.

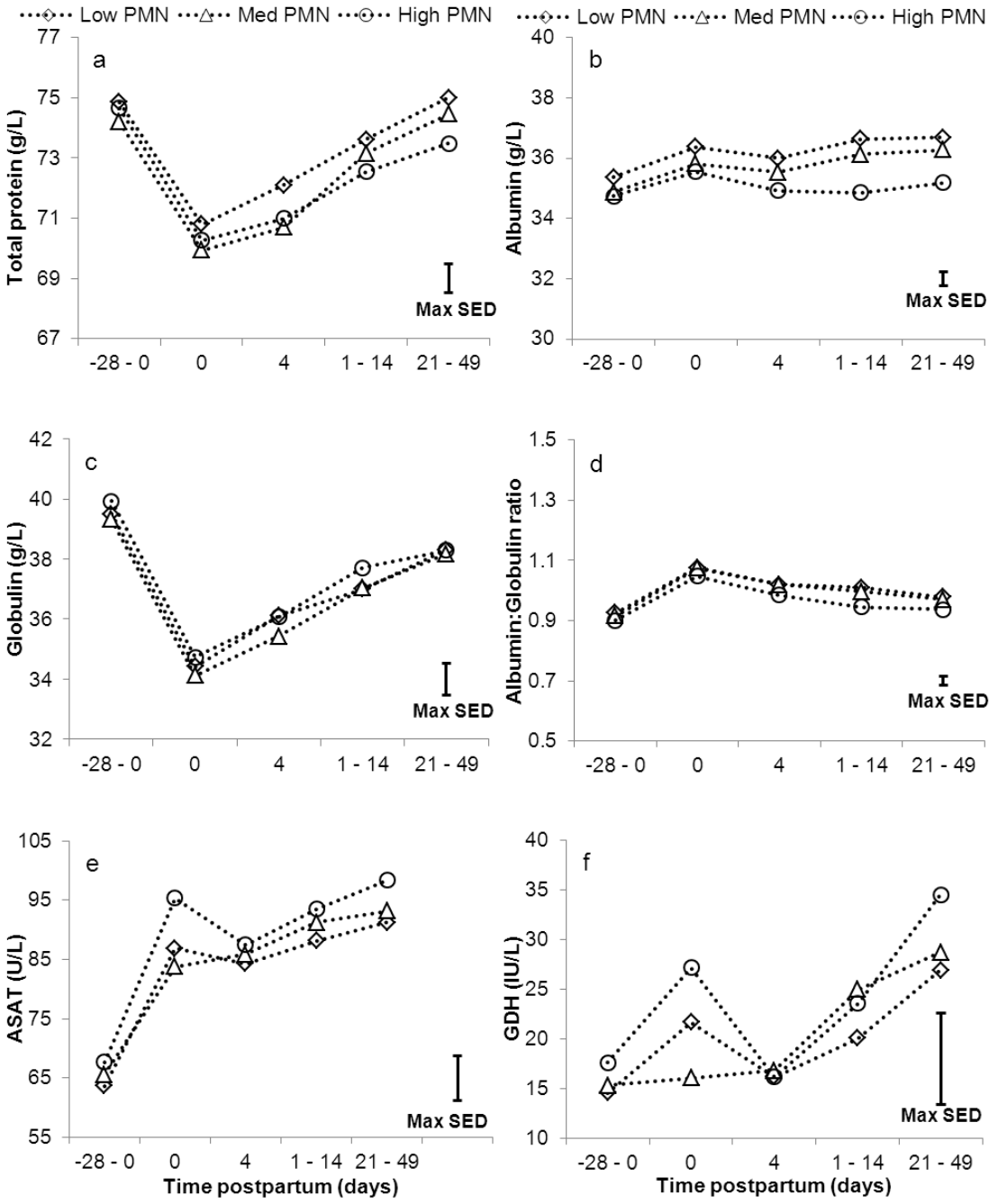

Figure 9: Plasma concentrations of (a) total protein (b) albumin (c) globulin (d) the albumin:globulin ratio (e) aspartate aminotransferase (ASAT) and (f) glutamate

dehydrogenase (GDH) for the polymorphonuclear cell (PMN) groups based on uterine cytology results from samples collected on day 14 postpartum: Low PMN (≤ 1% PMN); Medium PMN (2 to 13% PMN); High PMN (≥ 14% PMN). Raw means and the maximum standard error of the difference (Max SED) are presented.

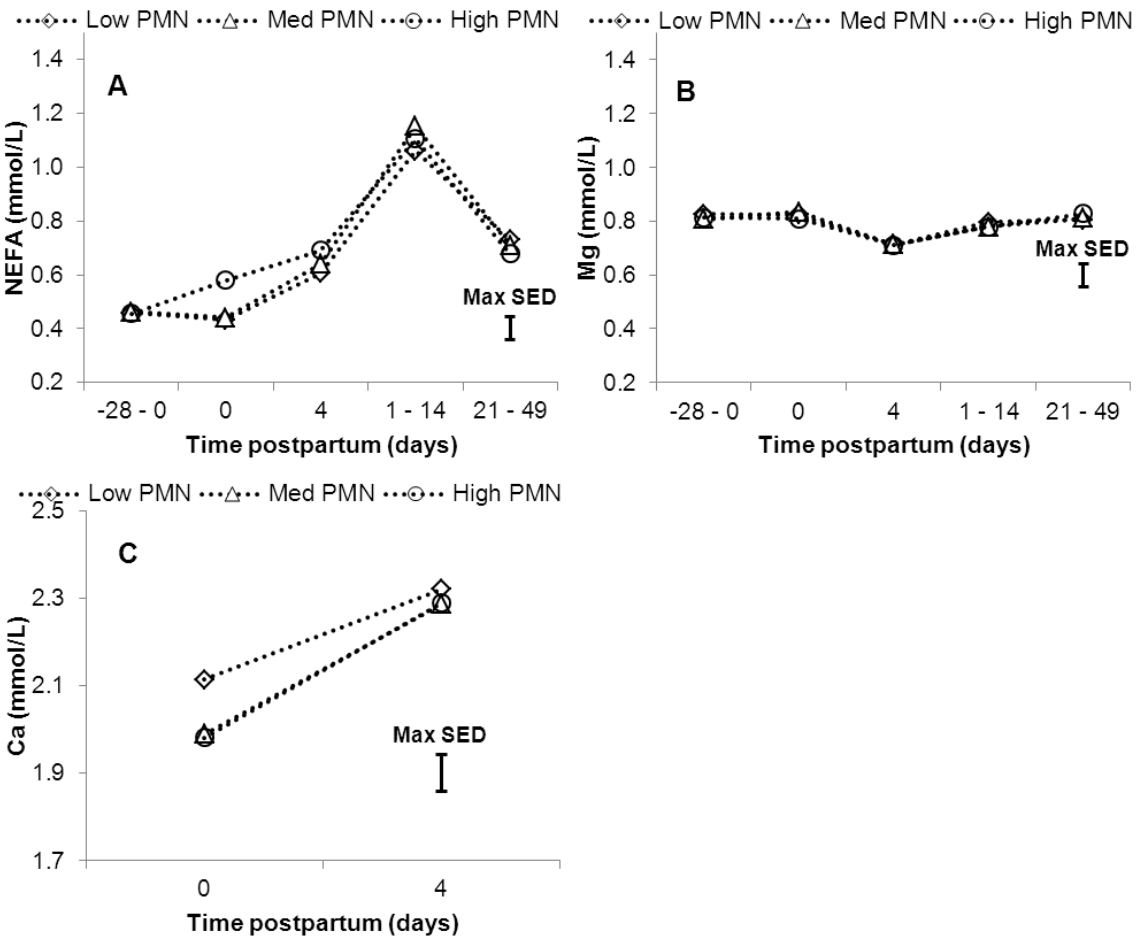

Figure 10: Plasma concentrations of (A) NEFA (B) Mg and (C) Ca for the polymorphonuclear cell (PMN) groups based on uterine cytology results from samples collected on day 14 postpartum: Low PMN (≤ 1% PMN); Medium PMN (2 to 13% PMN); High PMN (≥ 14% PMN). Raw means and the maximum standard error of the difference (Max SED) are presented.

3.4.3 Milk production and body condition score

The mean milk production and composition values for the first 6 weeks of lactation were milk yield = 18.9 kg/day, milk fat = 0.86 kg/day, milk protein = 0.66 kg/day, lactose = 0.93 kg/day, SCC = 66,100 cells/mL. The NSAID treatment did not affect ($P > 0.74$) any of the milk production variables measured, nor was there an effect on BCS ($P = 0.54$, NSAID = 4.1, control = 4.1, SED = 0.03). No associations were determined between PMN category and any of the milk composition variables measured. There was no difference ($P = 0.18$; SED = 0.52) in milk yield between the Low (18.9 kg/d)

and High PMN groups (18.0 kg/d). The High PMN group were thinner (0.2 BCS units) than both the Medium and Low PMN groups pre- ($P = 0.09$; 4.6, 4.8 and 4.8, respectively) and postpartum ($P < 0.05$; 4.0, 4.2 and 4.2, respectively). No interactions were detected between PMN group and NSAID treatment for BCS. There was a tendency ($P = 0.08$) for an interaction between PMN group and treatment for milk fat, with milk fat decreasing from the Low to High PMN groups in the NSAID treated cows (0.87 kg/d, 0.83 kg/day, 0.82 kg/day for the Low, Medium and High PMN groups respectively).

3.5 Discussion

The objective of this study was to determine if reducing inflammation associated with SCE through treatment with a NSAID would reduce the severity (average PMN %) of uterine disease at 42 days postpartum, improve reproductive performance and increase milk production in cows with SCE. In spite of a lower than expected average day 14 PMN % (9.9 PMN % on day 14 postpartum), coupled with a > 90% self-cure rate by day 42 postpartum, some beneficial effects of NSAID were observed on indicators of health and reproductive performance of dairy cows in a pasture-grazed, seasonal calving system.

3.5.1 Effect of NSAID on reproduction, metabolic indicators, and milk production

An interaction was detected between PMN % and NSAID treatment for pregnancy rate 4 to 10 weeks after onset of breeding, indicating a beneficial effect of treatment on pregnancy rate in the High PMN cows. In addition, treatment with a NSAID appeared to improve liver function, with reductions in plasma ASAT and GDH relative to untreated cows; this indicates that the NSAID treatment had some of the intended anti-inflammatory effect. These results confirm the efficacy of treatment with NSAID in moderately improving liver function in all NSAID treated cows and pregnancy rate in High PMN cows. Data also indicate that NSAID treatment had no negative effect on these parameters in Low PMN cows.

There was, however, no effect of NSAID treatment on day 42 PMN %, the proportion of cows ovulated by a specified date, PPAI, or milk production. The reason for the lack of treatment effect on these variables is not clear. One possible explanation is the lower than expected day 14 PMN % (average 9.9%) among the experimental animals, coupled with a high (> 90%) self-cure rate among cows defined as having SCE at day 14. This outcome potentially resulted in a lack of statistical power to detect a treatment response. Alternatively, sufficient concentrations of carprofen, to effect a beneficial reduction of uterine inflammation, may not have reached the uterine tissues due to the route of drug administration used (subcutaneous); a more direct method of administration, such as intra-uterine application, may produce a different result to that observed in the current study.

Another potential reason for the lack of treatment effect may have been because initiation of treatment was delayed until 21 days postpartum to avoid the risk of impeding the prostaglandin-mediated process of uterine involution. However, since the metabolite results indicated that SCE cows were experiencing a degree of liver dysfunction even before calving, delaying treatment to 21 days postpartum may have reduced the potential benefit of treatment on reproduction and milk production in High PMN cows. To support this hypothesis, high-yielding, TMR-fed cows treated with aspirin (a NSAID) during the first five days postpartum in the Trevisi and Bertoni (2008) study had a higher milk production and improved reproductive performance compared with untreated cows. An earlier timing of NSAID treatment (i.e. from calving) in pasture-grazing cows may therefore be worthy of future investigation.

3.5.2 Associations between PMN % and reproduction, metabolic indicators, and milk production

The cut-point of ≥ 14% PMN as the definition of SCE in the current study was based on the lowest value of the upper quartile. The advantage in using this approach is that numbers of animals assigned to PMN groups (Low, Medium and High) are balanced for statistical power; the same approach used by Burke et al. (2010). The limitation, however, is that the cut-point is established by the distribution of PMN % values within the experimental population, and not by a pre-defined level that is necessarily validated as having a significant biological effect. It is therefore possible that SCE as defined in this study is of a less severe condition compared with similarly classified animals in other reports (Kasimanickam et al. 2004; Barlund et al. 2008; Dubuc et al. 2010). This fact may have limited the ability to detect an effect of the NSAID treatment and any interactions between PMN group and treatment. There were, however, some significant biological differences relating to the High PMN group; a trend for a lower proportion of cows ovulated by day 28 postpartum, lower plasma concentrations of albumin and Ca, lower albumin:globulin ratio, elevated concentrations of ASAT, GDH and NEFA, and High PMN cows were thinner than cows in the other two PMN categories. The indications are that the cut-point used in this study is biologically relevant.

The associations between PMN group and reproduction and indicators of metabolic/inflammatory challenge found in the current study are generally consistent with previous reports in pasture-fed dairy cows (Burke et al., 2010; McDougall et al., 2011). These associations indicate a physiological dysfunction, including lower body condition, liver dysfunction and greater metabolic challenge, during the periparturient period among cows with elevated PMN % in the uterus postpartum.

The negative association between PMN group and albumin concentrations and the albumin: globulin ratio is similar to that reported previously (Green et al. 2009; Burke et al. 2010). Albumin is a liver-derived negative acute-phase protein (Fleck 1989), and is commonly regarded as a marker of

impaired liver function (Bertoni et al. 2008). Together with a tendency for plasma concentrations of ASAT and GDH to be higher in the High PMN cows, the indications are that liver dysfunction is a characteristic of cows with elevated PMN %.

Cows in the High PMN group were thinner than cows in the other PMN categories pre- and postpartum and had higher plasma NEFA concentrations on the day of calving. Low pre-calving BCS has previously been reported as a risk factor for SCE in pasture-grazed cows (McDougall et al. 2011), and increased body fat mobilization, as evidenced by elevated NEFA concentrations, was associated with lower DMI and an increased risk of metritis (Hammon et al. 2006; Duffield et al. 2009). Furthermore, the High PMN group had a lower plasma concentration of Ca on the day of calving; a key time that can reveal the extent of hypocalcaemia experienced by a dairy herd (Goff 2008). Low Ca during the transition period reduces immune function and is a risk factor for endometritis in TMR-fed dairy cows (Mateus and da Costa 2002; Kimura et al. 2006). Additionally, cows with reduced liver function have lower plasma Ca concentrations compared with unaffected cows (Bertoni et al. 2008). Further studies are required to better understand the underlying reasons for the links between SCE and indicators of physiological dysfunction in SCE cows pre-calving.

This study found no statistically significant differences in milk yield between the PMN groups which is consistent with the results reported by Dubuc et al. (2011), but is not consistent with other studies in both pasture-grazed and TMR-based systems that have reported a negative association between PMN % and milk yield (Senosy et al. 2009; Burke et al. 2010; McDougall et al. 2011). The 0.9 kg/day reduction in milk yield for the High PMN cows reported in this study is similar to the 1 kg/day and 0.6 kg/day reduction in milk yield reported by McDougall et al. (2011) and Burke et al. (2010), respectively, indicating that a degree of caution is required with this statistical interpretation.

3.6 Conclusion

Although NSAID treatment did not affect uterine PMN % at day 42 postpartum or milk production, treatment increased pregnancy rates in High PMN cows and improved liver functionality in all cows. Results confirm the earlier findings that cows with SCE present with a degree of physiological dysfunction during the periparturient period, with particular evidence for liver dysfunction. Further studies need to be conducted to elucidate the causes of the pre-calving physiological dysfunction associated with SCE and to evaluate the effect of timing of NSAID treatment on milk production and reproduction variables.

Chapter 4

Predicting cows at risk of subclinical endometritis

4.1 Declaration

The content in this experimental chapter has been submitted (manuscript number JDR3822) to the Journal of Dairy Research as a manuscript entitled 'Peripartum serum metabolites and cow body condition are poor predictors of subclinical endometritis in dairy cows' authored by: N.V. Priest, K. L. McLeod, C. R. Burke, S. McDougall, J. R. Roche, M. D. Mitchell, S. L. Greenwood, and S. Meier. I was involved in all aspects of the design, execution, and analysis of the research reported in this chapter. The writing of this manuscript was completed with contributions from my co-authors, and has benefited from the comments and suggestions of anonymous reviewers.

4.2 Introduction

Subclinical endometritis is a uterine disease characterised by an increased proportion of PMN present in the uterus after calving (Barlund et al. 2008; Sheldon et al. 2009; Dubuc et al. 2010), with prevalence ranging from 9 to 55% (LeBlanc 2008; Green et al. 2011). Negative effects of SCE on reproduction include increased postpartum anovulatory interval, reduced first-service conception rate and overall pregnancy rate, and increased number of services per conception (Gilbert et al. 2005; Barlund et al. 2008; Burke et al. 2010). Despite the recognised negative effects of SCE on reproduction, there is no practical on-farm method to diagnose SCE, and it is, therefore, not routinely monitored or managed in commercial herds (Cheong et al. 2011b).

Under research conditions, SCE is diagnosed by uterine cytology, which involves the collection of cells from within the uterus and the manual counting of PMN cells (Kasimanickam et al. 2004; Barlund et al. 2008). This method is invasive and time consuming, requires specialist equipment and technical expertise (Couto et al. 2012), and is impractical and expensive when examining the whole herd. Other more practical methods for SCE diagnosis have been investigated, such as uterine ultrasonography or the leukocyte esterase test (Cheong et al. 2011b; Couto et al. 2012; Meira Jr et al. 2012), but these methods have poor sensitivity and specificity, resulting in either too few cows with SCE being detected or too many cows being submitted for cytological examination. Therefore, a practical on-farm method is required to diagnose SCE or to reduce the number of cows to be submitted for uterine cytology. Such a method should have both high sensitivity and specificity.

Associations between SCE and various biological markers have been reported including: serum metabolites (Burke et al. 2010; Lopdell et al. 2011; Priest et al. 2013), cow BCS (McDougall et al.

2011; Priest et al. 2013), cytokine gene expression (Gabler et al. 2009; Galvão et al. 2011; Ghasemi et al. 2012), uterine gene expression (Gabler et al. 2009), milk yield and composition (Burke et al. 2010; McDougall et al. 2011), and follicular fluid composition (Back et al. 2011). Therefore, it was hypothesised that metabolic and physical characteristics could be used to predict cows at risk of SCE and enable the identification of a subset of cows for cytological examination or precautionary treatment. The objective of this study was to develop a method to predict cows at risk of SCE, based on serum metabolites and BCS, and reduce the number of cows to be submitted for cytological examination or proactive treatment to a manageable level.

4.3 Materials and methods

4.3.1 Dataset 1

Dataset 1 was obtained from a previous experiment reported by Priest et al. (2013), who used 213 multiparous Holstein-Friesian and Friesian-Jersey cross dairy cows, grazed on pasture, in an investigation of NSAID treatment for reducing inflammation associated with SCE. The study involved the collection of uterine samples for cytology at day 14 postpartum as well as weekly blood samples from each cow 25 ± 3 days before predicted calving date to 50 ± 4 days after calving. Serum harvested from the blood samples were analysed for several parameters including NEFA, albumin, globulin, albumin:globulin ratio (AGR), Mg, ASAT and GDH. Body condition score was assessed weekly using the 10 point scale described by Roche et al. (2004).

4.3.2 Dataset 2

This dataset, used for validation, was obtained from a study (Roche et al. 2013) investigating the effect of BCS on cow health during the transition period, in 60 Holstein-Friesian and Friesian-Jersey cross dairy cows that had been managed to generate different BCS at calving (3.5, 4.5 and 5.5; 10-point scale). Cow BCS and blood samples were collected weekly from two weeks prepartum to six weeks postpartum, and uterine samples were collected for cytology on day 14 postpartum. Serum harvested from the blood samples was analysed for several parameters including NEFA, albumin, globulin, AGR, Mg, ASAT, and GDH.

4.3.3 Data used

Two approaches were used to generate models from the serum metabolite and BCS data in Dataset 1. The first approach was to generate models based on week relative to calving, where a single week's data relative to cow calving date were analysed in each model. A single week's data were used because it would be impractical to collect a blood sample at more than one time point from an individual cow on a dairy farm. The second approach was to generate models based on the herd planned start of calving date. The second approach is a feasible option for farmers because they

could sample all cows on one day, rather than on different days in relation to individual cow calving date. Models were generated for weeks -4, -3, -2, -1, +1 and +2 relative to calving, and two set dates relative to the herd planned start of calving: 27 June (week of planned start of calving), 4 July (the week after the planned start of calving). The models using week -4 and -3 data and herd planned start of calving options (27 June and 4 July) could not be validated because equivalent, or insufficient, data were available in Dataset 2.

Serum data from the two datasets included a range of metabolites (8 in Dataset 1, and 13 in Dataset 2) but only 7 parameters were common to both datasets (NEFA, albumin, globulin, AGR, Mg, ASAT and GDH), and therefore used to generate the models. The number of cows included in each model varied due to missing metabolite data.

4.3.4 Statistical analyses

All-subsets regression analysis (GenStat 14.1; VSN-International (2011)) was used to determine which metabolites and BCS were to be included in the final model for each week or date for Dataset 1. For the all-subsets regression analysis, the percentage of PMN cells in the uterine cytology sample was analysed using generalized linear models with a binomial error distribution and the logit link function; ASAT, GDH, NEFA, and Mg were $\log_{10}$ transformed prior to analysis. Initially, for each week or date, the linear and quadratic terms for each metabolite were included in the model, but the quadratic terms were excluded when not significant. Only terms that were significant ($P < 0.05$) were included in the final model for each week or date. The equations from the final models were used to calculate the fitted values for PMN % for each week, or date, analysed in Dataset 1 and were also used to calculate the predicted values for PMN % for Dataset 2. The fitted and predicted PMN % values were back-transformed to aid interpretation and, using simple correlations, the fitted and predicted values for each model were compared with actual PMN %, and an R^2 obtained. A receiver operating characteristic (ROC) curve was plotted using the sensitivity (percentage of at-risk cows correctly identified) and specificity (percentage of healthy cows identified as healthy) for every fitted PMN % obtained for the model with the highest predictive value (R^2). Sensitivity and specificity were calculated based on cows at risk of SCE being defined as having > 18% PMN at day 14 postpartum. This threshold was based on the reproductive-outcome defined PMN % threshold previously reported for uterine cytology examinations conducted around this time postpartum (Kasimanickam et al. 2004). The optimum PMN % threshold for the fitted values obtained from the model with the highest predictive value was calculated from the ROC curve using the Youden index, which is the maximum difference between sensitivity and 1 – specificity (Schisterman and Perkins 2007).

4.4 Results and discussion

The ability to predict the cows at risk of SCE was tested by generating models based on serum metabolite and BCS data from Dataset 1 and validating the results with Dataset 2. The results indicate that a model based on serum metabolite and BCS data can predict the cows at risk of SCE. However, the high percentage of false positives obtained for this model, which would result in 95% of the herd still being submitted for cytology, means that the objective of reducing the number of cows to be submitted for cytological examination or proactive treatment to a manageable level was not achieved.

Table 7: Regression equations for the models generated to predict polymorphonuclear cell % in uterine cytology samples. Models were derived from serum metabolite concentration and body condition score data collected from 4 weeks pre to 2 weeks postpartum and at two set dates (planned start of calving and one week later). Models names including 'V' were validated against a separate data set (results reported in the body of the paper), but suitable data were not available for validating the remaining equations.

Model[†]	Equation	#cows[‡]	P-value	R^2
Week -1V	1.17 – 0.0965 * albumin	203	0.04	0.01
Week +1V	3.90 – 0.1739 * albumin + 0.927 * $\log_{10}$ NEFA	210	<0.001	0.14
Week +2V	5.45 – 0.0858 * globulin – 4.57 * AGR	211	<0.001	0.10
Week -4	-3.818 + 1.540 * $\log_{10}$ GDH	168	<0.001	0.09
Week -3	1.54 – 0.1084 * albumin	210	0.03	0.03
27 June	-0.730 – 1.806 * AGR	144	0.01	0.04
4 July	-4.00 – 0.1846 * albumin + 0.1143 * globulin + 4.09 * AGR	146	0.04	0.05

[†]Each model was generated from either one week's serum metabolite and body condition score data (week relative to calving) or data collected on a date relative to the herd's planned start of calving (planned start of calving and one week later)

[‡]#cows = number of cows included in each model

The significant ($P < 0.05$) models generated from Dataset 1 are presented in Table 7. No model was generated for week -2 as none of the evaluated parameters were significant for this week ($P > 0.05$). There were significant ($P < 0.05$) correlations between fitted and actual PMN % values for all weeks except for week -1 ($P = 0.13$). Despite the significant correlations, the predictive value of the models was relatively poor, with R^2 values that ranged from 0.01 to 0.14. The model with the highest predictive value was week +1 ($R^2 = 0.14$; Figure 11). The poor predictive value of the models was confirmed with Dataset 2. When using the models to predict PMN % for Dataset 2, the only model that had a significant ($P = 0.05$) correlation between the actual and predicted PMN % was week +1, and the R^2 values for the regression of predicted against actual PMN % ranged from 0.03 to 0.11

(Figure 12). The R^2 value for week +1 was lower for the correlation between the predicted and actual PMN % (R^2 = 0.11) than for the correlation between the fitted and actual PMN % (R^2 = 0.14).

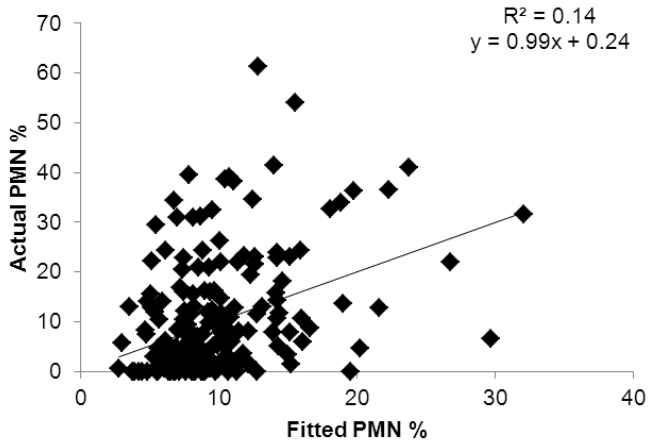

Figure 11: Regression of actual polymorphonuclear cell (PMN) % values against fitted PMN % for the week +1 model. The actual PMN % values were obtained from Dataset 1, and the fitted PMN % values were calculated from Dataset 1 using the equation for the week +1 model. The week +1 model was generated from serum metabolite and cow body condition score data collected one week after calving.

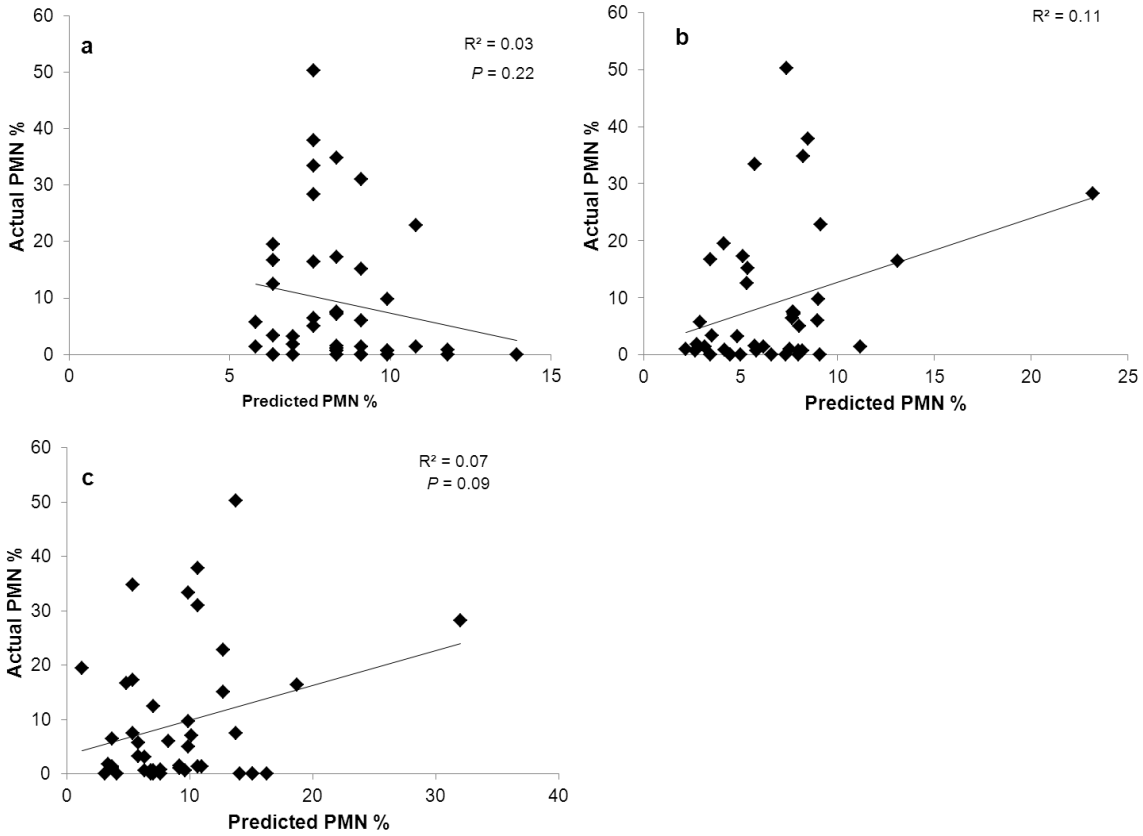

Figure 12: Regression of actual polymorphonuclear cell (PMN) % values against predicted PMN % for models (week relative to calving): a) week -1, b) week +1, and c) week +2. The actual PMN % values were obtained from Dataset 2, and the predicted PMN % values were

calculated from Dataset 2 using the Dataset 1 equations. The models were generated from a single week's serum metabolite and cow body condition score data that was collected weekly relative to calving. The *P*-value displayed is for the correlation between the actual and predicted PMN % values.

In addition to the relatively low R^2 value, the combinations of sensitivity and specificity obtained for the model with the highest predictive value (week +1) were unable to achieve the objectives of the study (Figure 13). From the ROC curve and Youden index, the optimum PMN % threshold for the fitted values of the week +1 model was 10.7%, which had a sensitivity of 58% and a specificity of 81%. This sensitivity would result in a high percentage of the affected cows not being identified. In an attempt to improve sensitivity, the fitted value threshold for PMN % was reduced to 5%. This resulted in an acceptable sensitivity of 100%, but an unacceptably low specificity (7%) would mean that 95% of the herd would be submitted for cytology. Furthermore, the sensitivities and specificities reported here were less than those reported for the leukocyte esterase test, which has already been deemed inaccurate for diagnosing SCE (Cheong et al. 2011b; Couto et al. 2012). Therefore, the results indicate that the combination of metabolites and physical parameters considered here cannot be used to achieve the dual aim of predicting cows at risk of SCE and enabling only a subset of cows to be submitted for cytological examination.

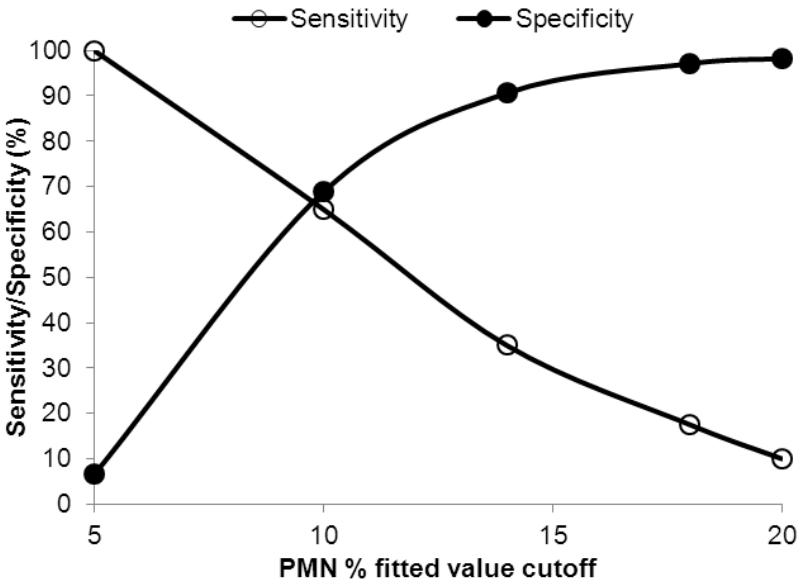

Figure 13: Sensitivity and specificity for predicting cows at risk of subclinical endometritis using five polymorphonuclear cell (PMN) % fitted value thresholds obtained from a receiver operating characteristic curve. The fitted values for PMN % were calculated from a model based on serum metabolite and cow body condition score data obtained one week after calving (week +1 model).

A possible reason for the unacceptable sensitivity and specificity of the model relates to the markers chosen. The markers used were chosen because they are physiological measures that have been reported to be associated with SCE (Burke et al. 2010; Lopdell et al. 2011; McDougall et al. 2011; Priest et al. 2013). However, these markers are indirect physiological measures at the animal level, such as indicators of energy status and liver function, rather than more direct markers of uterine inflammation, such as uterine PMN % or uterine cytokine gene expression (Barlund et al. 2008; Fischer et al. 2010). In addition, the markers evaluated here will undergo significant changes throughout the transition period, for example significant increases in NEFA after calving, which are driven by processes not necessarily related to SCE, such as calving stress and homeorhetic processes facilitating significant increases in milk production (Drackley et al. 2005). Thus, although the markers evaluated here are associated with SCE, there are many other factors that influence their concentrations in serum, which challenges their use for predicting the cows at risk of SCE. However, the negative impact of SCE on reproductive performance (Gilbert et al. 2005; Barlund et al. 2008; Burke et al. 2010) indicates a strong need to identify cows at risk of SCE. Moyes et al. (2013) reported that a physiological imbalance index based on pre-calving blood metabolite (NEFA, beta-hydroxybutyrate, and glucose) concentrations was able to predict the cows at risk of developing clinical postpartum diseases such as mastitis, metritis, milk fever, and lameness. This indicates that indirect markers, e.g. serum metabolites, can be used to predict clinical postpartum diseases. However, although indirect markers could be used to predict cows at risk of clinical disease (Moyes et al. 2013), other, or more direct, biological markers may be required for the prediction of subclinical diseases, such as SCE.

4.5 Conclusion

In conclusion, a model was generated that correctly identified 100% of the cows with SCE, but the specificity associated with this model was low. Therefore, the results indicate that a model based on the parameters used in this study is unable to achieve the dual aim of predicting cows at risk of SCE and enabling only a subset of cows to be submitted for cytological examination. However, the benefit of identifying all cows with SCE may outweigh the costs of still having to examine/treat most of the herd, so the feasibility of a model based on serum metabolites and BCS on-farm still requires further investigation.

Chapter 5

General discussion

The over-arching hypothesis of the first experiment (Priest et al. 2013) was that the inflammation associated with SCE, both uterine and systemic, is a part of the mechanism by which reproductive performance is reduced in cows with SCE. To test this hypothesis, cows were treated with a systemic NSAID, and the effects of the NSAID treatment on uterine PMN %, reproduction, and milk production were measured. In summary, the NSAID treatment did not reduce the severity of uterine pathology at day 42 (average PMN %) or mitigate the negative association between SCE and milk production, but the treatment did increase pregnancy rate in cows with SCE, and it reduced the concentrations of indicators of systemic inflammation and improved liver function in treated cows. This improvement in pregnancy rate in the cows with SCE was a positive outcome for the experiment, and the result is supported by the improvement in pregnancy rate in acetylsalicylate (a NSAID) treated cows reported by Trevisi and Bertoni (2008). One question raised by this result was, what is the mechanism by which NSAID treatment improves pregnancy rate in SCE cows? Was the improvement due to (1) a reduction in uterine inflammation (measured as uterine PMN %) as a result of the NSAID treatment which we were unable to detect in this experiment, (2) the observed reduction in systemic inflammation (ASAT and GDH concentrations), or (3) was the improvement due to the NSAID having an effect on reproduction through other unidentified pathways?

5.1 Mechanisms by which reproduction was improved

For the three scenarios outlined in the previous paragraph, three of the potential pathways by which the NSAID treatment improved pregnancy rate in SCE cows are (Figure 14): (1) that the NSAID reduced uterine prostaglandin synthesis, which in turn reduced uterine cytokine concentration and resulted in the reduction of uterine PMN %, which is the measure used in this experiment to quantify the level of uterine inflammation (scenario 1, blue arrows); (2) that the NSAID reduced systemic prostaglandin synthesis and thus systemic cytokine concentrations, which had flow on effects through two different pathways to the reproductive axis (scenario 2, red arrows); and (3) that the NSAID altered the systemic prostaglandin/eicosanoid profiles which may have had beneficial effects on the reproductive axis (scenario 3, dashed black and purple arrows). Answering the question of how the NSAID treatment increased pregnancy rate in SCE cows is important as the outcome may have implications for how SCE is detected and treated in the future. These three scenarios are discussed in greater detail below - sections 5.1.1, 5.1.2, and 5.1.3.

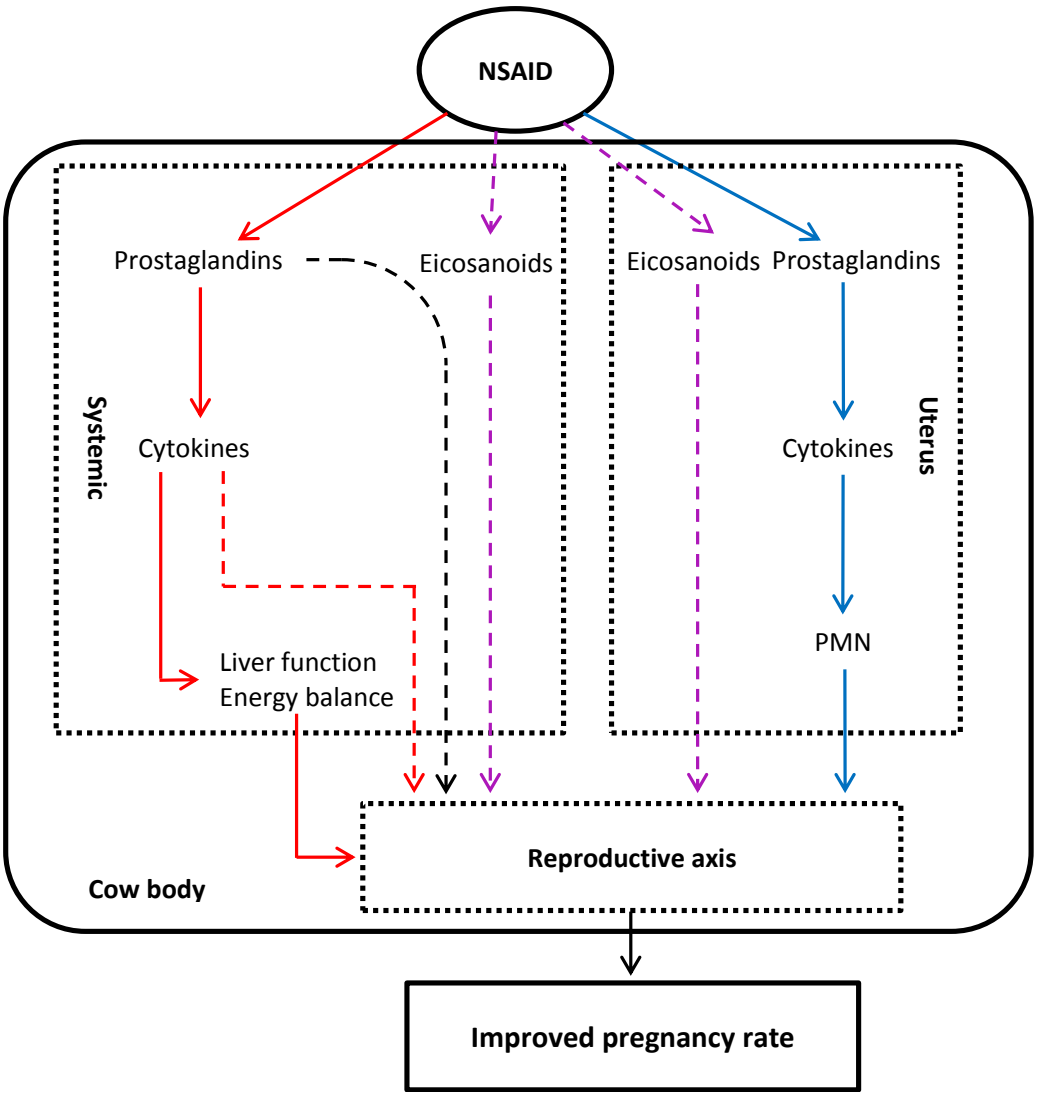

Figure 14: The potential pathways by which a non-steroidal anti-inflammatory drug (NSAID) treatment increased pregnancy rate in cows with subclinical endometritis. The blue arrows represent the pathway discussed for scenario 1, the red arrows represent the pathways discussed for scenario 2, and the dashed black and purple arrows represent the pathways discussed for scenario 3. The box labelled 'reproductive axis' represents components of the reproductive axis such as the hypothalamus and the ovaries. PMN = polymorphonuclear cells.

5.1.1 Scenario 1

Uterine cytology samples were collected at days 14 and 42 postpartum to determine uterine PMN % and the effect of the NSAID treatment on PMN %. The results demonstrated that there was no difference in PMN % between the control and NSAID groups at day 42, and that there was a high self-cure rate (> 90%) of SCE from day 14 to day 42 postpartum in the control cows. However, due to the reasons listed below, it is possible that the NSAID treatment did have an effect on uterine PMN %, and therefore uterine inflammation, which was unable to be detected in this experiment.

- Samples not being taken (because of logistical constraints) during the time period of expected treatment effect (approximately days 21 to 32 postpartum)
- The long period of time between the uterine samples that were collected
- The high SCE self-cure rate in the control cows

In other words, although the change in PMN % from day 14 to day 42 was similar between the control and NSAID groups, if samples had been taken between days 14 and 42, they may have shown a steeper slope of decline in PMN % in the NSAID treated cows (Figure 15). A faster reduction in PMN % and uterine inflammation would mean that the NSAID-treated SCE cows may return to a healthy uterine status faster than the untreated (control) cows with SCE, which would be beneficial to their reproductive performance. Therefore, a faster reduction of uterine inflammation (PMN %) may have been the mechanism by which pregnancy rate was improved in the SCE cows.

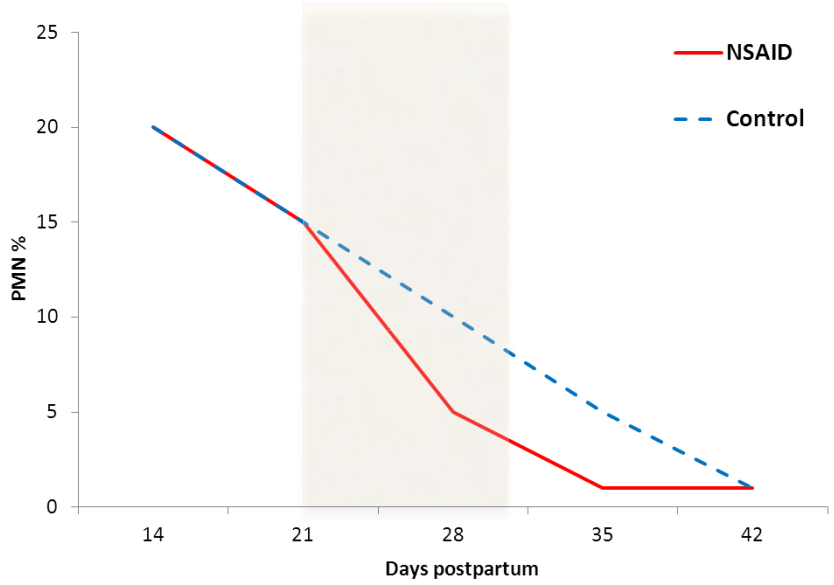

Figure 15: Hypothetical example of potential differences in polymorphonuclear cell (PMN) % rate of decline from 14 to 42 days postpartum between cows that were treated with a non-steroidal anti-inflammatory drug (NSAID) and those that were not (Control). The shaded area is the period during which the NSAID treatment was administered.

This potential faster reduction of PMN % could have an implication at the farm system level. As discussed in the literature review (Chapter 2, section 2.8.2), Hill and Gilbert (2008) reported that embryos cultured in the products of an inflamed endometrium are of poorer quality, which may lead to reduced embryo survival. This means that reducing uterine inflammation with a NSAID may be beneficial to the pregnancy rate of late calving cows. This is because late calving cows will have had a shorter time, compared with early calving cows, from calving to when they are mated in which to resolve their uterine inflammation. This shorter interval from calving to mating means that the late calving cows could still have some uterine inflammation at the time of breeding, which could reduce

their conception and pregnancy rates. If the conception rate of late calving cows were improved, this would help to improve a farm's 6-week in-calf rate, which would increase days in milk and reduce culling due to poor reproductive performance (Burke et al. 2007).

On the other hand, it has never been shown that a systemic NSAID treatment, such as that used in the first experiment, actually affects uterine inflammation (PMN %) or the uterine prostaglandin profile; it has just always been assumed that it does. Several studies have investigated the use of a NSAID to reduce uterine and reproductive tract inflammation around the time of insemination or embryo transfer (Guzeloglu et al. 2007; Erdem and Guzeloglu 2010; von Krueger and Heuwieser 2010; Heuwieser et al. 2011). None of these studies, or any other studies as far as I am aware, have tested or reported whether a systemic NSAID (i.e. a NSAID administered s.c. or i.m., not inter-uterine) actually alters/reduces uterine prostaglandin concentrations, which is the expected physiological response to a NSAID, or indeed reduces uterine inflammation (e.g. uterine PMN % or cytokine concentrations). Therefore it is just as likely that the NSAID treatment did not affect uterine PMN % at all, as the results for this experiment suggest. An experiment needs to be conducted to determine whether a systemic NSAID does actually have any effect on uterine prostaglandin and therefore uterine inflammation profiles.

5.1.2 Scenario 2

The metabolite results indicate that the NSAID treatment reduced the level of systemic inflammation (ASAT and GDH concentrations) in all cows treated. It has previously been reported by Bertoni et al. (2008) that cows with lower levels of inflammation during the periparturient period have greater reproductive performance. In addition, the reduction of inflammation during the early postpartum period through the use of a NSAID resulted in higher pregnancy rates in NSAID treated cows (Trevisi and Bertoni 2008). The combination of these results indicates that it is possible that the reduction in systemic inflammation could be the mechanism by which the pregnancy rate was improved in the SCE cows.

Two potential mechanisms by which systemic inflammation during the periparturient period could reduce reproductive performance have been proposed by Bertoni et al. (2008). Both of these mechanisms are associated with the effects of cytokines, whose production is stimulated by prostaglandins (Bos et al. 2004). The first potential mechanism is liver function impairment, which involves the diversion of liver protein synthesis from negative acute phase proteins (-APP) to positive acute phase proteins (+APP). When an inflammatory event occurs, pro-inflammatory cytokines promote the production of +APP proteins by the liver, such as globulin and haptoglobin, at the expense of the proteins normally produced by the liver, such as albumin and retinol binding protein (-APP), apolipoproteins, and the proteins involved in antioxidant activity or bilirubin clearance (Fleck

1989; Bertoni et al. 2008; Trevisi and Bertoni 2008). This reduction in liver function increases the risk of liver lipidosis and the risk of exposure to the negative effects of oxidative stress (Drackley et al. 2005; Trevisi and Bertoni 2008), both of which have negative effects on the ovary and the embryo, and therefore reproduction (Rukkwamsuk et al. 1999; Guérin et al. 2001; Bobe et al. 2004). The second potential mechanism is the exacerbation of negative energy balance. Pro-inflammatory cytokines reduce feed intake (Johnson and Finck 2001) and increase energy expenditure (Elsasser et al. 2000). Combined, these two changes result in the exacerbation of negative energy balance, which has been reported to have detrimental effects on reproductive performance (Butler 2003; Friggens et al. 2010). These changes in energy balance and liver function could result in homeorhetic responses that divert biological resources from reproduction (Drackley et al. 2005), which would result in reduced reproductive performance in cows with systemic inflammation.

In addition to the two mechanisms proposed by Bertoni et al. (2008), in the literature review (Chapter 2, section 2.8.2) it is reported that cytokines have negative effects on the reproductive axis which include inhibiting the release of hypothalamic GnRH (Rivest et al. 1993), reduction of oestradiol production by the granulosa cells of the ovary (Spicer and Alpizar 1994; Sheldon et al. 2002; Sheldon et al. 2009), and the reduction of progesterone production by the luteal cells of the ovary (Petroff et al. 2001; Okuda and Sakumoto 2003; Sheldon et al. 2009). Therefore, the reduction in systemic inflammation, and thus systemic concentrations of cytokines, may have ameliorated these negative effects of cytokines on liver function, energy balance, and the reproductive axis, resulting in improved reproductive performance.

5.1.3 Scenario 3

While it is possible that either scenario 1 or 2, or a combination of the two scenarios, was the mechanism by which the NSAID treatment improved pregnancy rate in cows with SCE, it is also possible that the NSAID improved pregnancy rate through alternative pathways. One potential pathway is that the NSAID treatment altered the systemic prostaglandin profile (dashed black arrows), for example the ratio of $PGF_{2\alpha}$/PGE_2 (luteolytic and luteotrophic, respectively, Arosh et al. (2004)), which may have had beneficial effects on the ovary and, therefore, reproductive performance. Alternatively, as cyclooxygenase not only catalyses the reaction for prostaglandin synthesis but also catalyses those for other eicosanoids such as thromboxane (Reilly and Fitzgerald 1993), the NSAID treatment may have altered the systemic or uterine concentrations of these other eicosanoids (dashed purple arrows) in a way that was beneficial to reproductive performance. For example, thromboxane is a vasoconstrictor (Reilly and Fitzgerald 1993), thus, perhaps reducing the concentration of thromboxane could be beneficial to reproduction by allowing greater blood flow to the ovaries and/or the uterus, and therefore allowing a greater influx of beneficial factors to these organs.

5.2 Hypotheses generated

Based on the scenarios discussed in the previous section, there are several hypotheses that can be generated. Testing these hypotheses could potentially result in the determination of the mechanism by which the pregnancy rate was improved in NSAID-treated SCE cows.

To determine whether the pathway described in scenario 1 was the mechanism by which the NSAID treatment improved pregnancy rate in cows with SCE, the three key hypotheses to be tested are:

- That a systemic NSAID treatment alters uterine prostaglandin profiles and reduces uterine inflammation (PMN %).
- That a systemic NSAID results in a faster rate of decline of uterine inflammation (PMN %) in NSAID-treated cows compared with untreated cows.
- Cows that have a faster reduction of uterine inflammation (PMN %) have higher pregnancy rates.

The three key hypotheses for testing whether it was the pathways described in scenario 2 that was the mechanism by which the pregnancy rate was improved in cows with SCE are:

- That treatment with a NSAID reduces systemic concentrations of pro-inflammatory cytokines.
- That the reduction of systemic inflammation, specifically systemic concentrations of pro-inflammatory cytokines, by a systemic NSAID treatment is associated with improved reproductive performance in treated cows compared with untreated cows.
- That a systemic NSAID treatment, by reducing systemic inflammation and cytokine concentrations, improves liver function (increases –APP and decreases +APP concentrations) and energy balance (increases dry matter intake and reduces energy expenditure), and mitigates the negative effects of cytokines on follicle development, ovulation, and luteal function.

For the pathways described in scenario 3, the three key hypotheses to test are:

- That a systemic NSAID treatment alters the systemic prostaglandin profiles and reduces the systemic concentrations of the other eicosanoids (e.g. thromboxane).
- That the alteration of systemic prostaglandin profiles by a NSAID treatment is beneficial to the pregnancy rate of treated cows.
- That the reduction of systemic concentrations of thromboxane is beneficial to the pregnancy rate of treated cows.

To test these hypotheses and determine the mechanism by which the NSAID treatment improved the pregnancy rate of cows with SCE would require a large program of work and several targeted studies.

Given the interrelated nature of these hypotheses, there is the potential for more than one hypothesis to be tested at the same time. The experimental model that is the most appropriate to test each hypothesis, e.g. SCE cows or cells in culture, will depend on where the priorities lie for the programme of work. For example, the programme may be focused on acquiring knowledge at the cellular level or at the whole cow level. However, whilst answering the question of how the NSAID treatment increased pregnancy rate in the SCE cows is important, it would take several studies across many years. This timeframe is not ideal for farmers who are dealing with the disease now. Therefore, it could be argued that it is not important to know how the NSAID treatment improved pregnancy rate, but simply to confirm that it does. In other words, can the result obtained in this experiment be replicated and validated? Further studies need to be conducted to validate whether the NSAID treatment does improve pregnancy rate in SCE cows and to optimise the treatment protocol (e.g. when to administer, how many injections). If the improvement in pregnancy rate obtained in the current experiment is validated, this would provide a usable result to farmers faster than any investigation of the mechanism by which the NSAID treatment improved pregnancy rate. However, as (1) blanket/mass treatment of all cows with a NSAID would be unacceptable, and (2) the NSAID treatment was not beneficial to any group of cows except for the SCE cows, the cows with or at risk of SCE would still need to be identified for treatment. This is where the use of a model to identify the cows at risk of SCE would come in.

5.3 Identifying the cows with subclinical endometritis

The hypothesis for the second experiment was that differences in serum metabolite concentrations and BCS between cows with and without SCE could provide information accurate enough to achieve the dual aim of predicting those cows at risk of SCE and reducing the number of cows undergoing cytology or proactive treatment to a manageable level. Although a model was developed that could identify 100% of the cows at risk of SCE, the model was unable to reduce the number requiring diagnosis or treatment to a manageable level. This result is in agreement with those reported by Kaufmann et al. (2010a), who was also unable to use serum metabolites to predict the cows with SCE. However, the importance of identifying cows at risk of SCE means that other biological markers or methods of detection need to be investigated.

The markers used in the second experiment, e.g. ASAT, albumin, and NEFA, are indirect markers. In other words, the markers used are compounds that are not directly associated with uterine inflammation but are associated with a part of the physiological processes involved with SCE. Thus, these markers are only measures of physiological processes that occur at the systemic level, e.g. energy status, and they also undergo significant changes throughout the postpartum transition period. This means that finding an indirect/systemic marker(s) that is able to predict the cows at risk of SCE will be challenging. Therefore, markers that are directly affected by, or are part of the process

of uterine inflammation need to be found, as these may be the best candidates to use for the prediction of at-risk cows or the rapid diagnosis of cows with SCE. To identify direct markers for SCE, further research into the aetiology of SCE is needed, so that cause and effect for SCE can be determined and potential markers identified.

Possibilities for research into the aetiology of SCE include investigating the causes of SCE uterine inflammation, e.g. the role of the pre-calving physiological dysfunction reported by Burke et al. (2010) and Priest et al. (2013), and investigating the role of the neutrophil and potential immune system dysfunction reported by Hammon et al. (2006), Galvão et al. (2010), and Hansen (2013). However, in order to conduct an experiment that is more than just an association study, an experiment would need to be conducted on a small population (a large population would be logistically impractical) that was intensively measured. The study population would need to be comprised of cows that had been selected to generate an experimental cow model. For example, to investigate causes of the inflammation associated with SCE, and knowing that cows with SCE have both systemic and uterine inflammation, the experimental population should include the following four groups of cows: cows with only systemic inflammation, cows with only uterine inflammation, cows with both systemic and uterine inflammation, and cows that do not have any inflammation at all. After collecting detailed measurements from these cows, markers for each group of cows could potentially be identified. If markers were identified for each group, to try to figure out the causes of SCE inflammation, a group of cows that do not have any systemic or uterine inflammation would need to be manipulated in some way, e.g. alter BCS, to see if the manipulation induces expression of the identified markers. If the manipulation does induce expression of the identified markers, then the effect of the manipulation is a part of the cause of SCE. Markers specific to this effect, and therefore to SCE, could then be investigated to provide candidates for either predicting or detecting SCE.

Although further research may identify direct markers, their value must be assessed in relation to the cost and practicality of collecting these data. In other words, although a direct marker may be identified that can be used to predict cows at risk of SCE, or to diagnose cows with SCE, the practicality and cost of collecting and measuring the direct marker must be taken into account when determining whether the marker is appropriate for on-farm SCE management. However, if a direct marker is identified that is initially impractical or too costly to collect or measure, advances in technology may result in the subsequent development of more practical and cost effective ways to either collect or measure the marker. Additionally, advances in technology, such as mass spectrometry, may allow more in-depth and faster analyses of blood, milk, and uterine mucus, which may result in the identification of direct markers in these easy-to-collect body fluids.

Alternatively, perhaps we should focus on the herd rather than on individual cows for management of SCE. This approach would mean that instead of trying to determine if individual cows have the disease in the first instance, we would start by investigating whether the herd is at risk of SCE. A herd at risk of SCE could be defined as having a high proportion of cows that had experienced assisted calving, twinning, dystocia, etc. (the risk factors for SCE listed in section 2.4, Table 2). If the herd is at-risk, then submit the whole herd for cytology. If the herd is assessed to be not-at-risk, then do not test the herd at all. This herd-risk assessment approach would reduce the costs of determining whether a cow should be submitted for cytology or not, but introduces the possibility of overlooking a potential SCE problem in herds that have been assessed to be at low risk. This is because over half of the cows with endometritis do not have any of the risk factors listed in section 2.4, Table 2 (LeBlanc et al. 2002a). This would potentially result in herds being assessed as having a low risk of SCE when the actual prevalence of SCE in the herd is much higher. Based on a SCE prevalence ranging from 5 to 50% (Table 8), not identifying and treating cows with SCE could potentially be reducing the herd's 6-week in-calf rate by 1 to 14%, increasing the herd's empty rate by 2 to 15%, and reducing potential farm profit by $20 to 204/cow/year. This negative impact on reproduction and farm profit if SCE cows are not identified and treated means that until more accurate herd risk factors are identified, individual cow assessment for either risk of SCE, or diagnosis of SCE, is the best option for reducing the negative effect of SCE on reproduction and farm profit. However, although there are obvious negative impacts if the cows with SCE within a herd are not treated, it is acknowledged that the cost of identifying and treating the cows with SCE must be taken into account when calculating the cost/benefit for SCE management.

Table 8: The impact[1] of varying levels of subclinical endometritis on the reproductive performance and farm profitability of a 400 cow herd with a 12 week mating period

	Herd subclinical endometritis prevalence						
	0%	5%	10%	20%	30%	40%	50%
6-week in-calf rate	70%	68.75%	67.5%	65.0%	62.5%	60.0%	57.5%
Empty rate[2]	10%	11.5%	13%	16%	19%	22%	25%
Economic loss/year	$0	$8000	$16000	$32000	$48000	$64000	$80000
Economic loss/cow/year	$0	$20	$40	$81	$122	$163	$204

[1]For the assumptions used and calculations made to obtain these values, see Appendix A.

[2]Empty rate is the percentage of cows in the herd that are diagnosed as not pregnant at the final pregnancy testing after 12 weeks of mating.

5.4 Conclusions and implications

If either scenario 1 or 2 was the mechanism by which pregnancy rate was improved, or if both contributed to increasing the pregnancy rate of SCE cows, this would suggest that the inflammation

associated with SCE is a part of the mechanism by which the reproductive performance of SCE cows is reduced. This conclusion is in contrast to those studies which report (or base their hypotheses on the assumption) that reproductive performance of cows with endometritis/SCE is reduced by bacteria (Williams et al. 2005; Galvão et al. 2009b; Herath et al. 2009; Sheldon et al. 2009; LeBlanc et al. 2011), but is congruent with studies reporting a stronger association of poor reproductive performance with uterine inflammation (as assessed by PMN %) than with the presence of bacteria in the uterus postpartum (McDougall et al. 2011; Barański et al. 2012). One implication of this conclusion is that treatment of SCE should be focusing on reducing the inflammation associated with SCE, both pre- and postpartum, instead of simply trying to eliminate bacteria from the uterus. Another implication is that research into markers for SCE prediction or diagnosis should focus on markers that are associated with inflammation, both the causes of and the products of inflammation. However, as identified in the literature review, the question still remains, where does this inflammation, both systemic and uterine, come from? If the source of the inflammation could be identified, then it may be possible to develop management strategies to prevent or at least reduce the prevalence of SCE, and thus reduce the need to treat cows for this disease in the first place, or provide better candidate markers for SCE prediction or detection. Therefore, future studies need to investigate what the causes of the systemic inflammation and uterine inflammation are, and whether the systemic inflammation is a result of whatever is causing SCE or a part of the cause of SCE itself, or whether the systemic inflammation is a part of a presently unidentified disease that is occurring concurrently with SCE. It must be noted though, that the costs of making herd management changes, identifying the cows at risk of SCE or diagnosing those with SCE, and treating the affected cows, must be taken into account when considering whether a particular approach to reducing the prevalence of or identifying/treating cows with SCE will beneficial or not.

Appendix A

Calculating the cost of subclinical endometritis

The values reported in Table 8 of the General Discussion (Chapter 5) were calculated using the 'gap calculator' from the InCalf Economics of Reproductive Performance Tool (InCalf 2013) and data from McDougall et al. (2011). The assumptions for these calculations were that the herd comprised of 400 cows and had a 12 week mating period.

The first step for calculating the values in Table 8 was to identify the difference in 6-week in-calf rate and empty rate between cows with and without SCE. The 6-week in-calf rate was defined as the percentage of cows pregnant by six weeks after the start of the mating period. The empty rate was the percentage of cows that were not diagnosed as pregnant at the final pregnancy diagnosis after the herd had been submitted for 12 weeks of mating. I estimated the 6-week in-calf and empty rates for the cows with and without SCE from the percentages displayed in the figure reported by McDougall et al. (2011) (Figure A.1). The blue lines in Figure A.1 indicate the approximate 6-week in-calf rates for cows with (45%) and without SCE (70%). The purple lines indicate the approximate empty rates for the cows with (40%) and without SCE (10%).

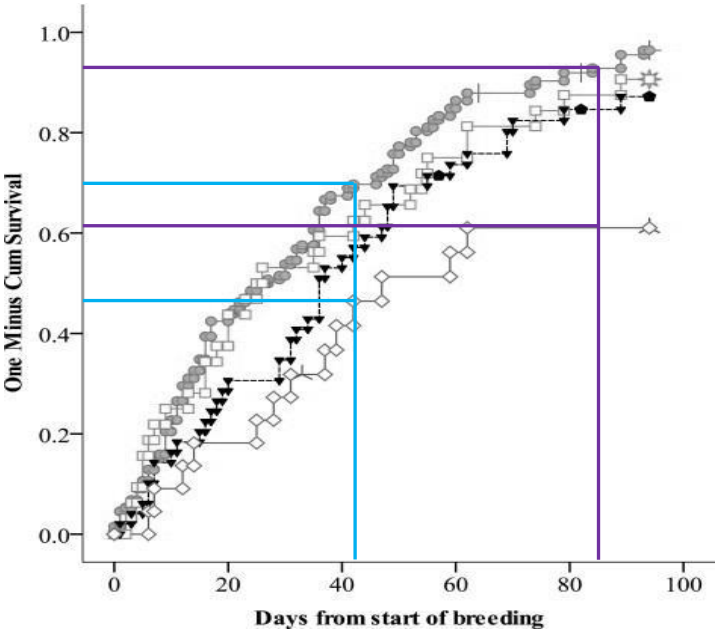

Figure A.1: Cumulative proportion of cows pregnant for cows with (◊) and without (●) subclinical endometritis. McDougall et al. (2011), reproduced with permission from Elsevier.

Using the estimated 6-week in-calf rate and empty rate percentages, I calculated what the herds 6-week in-calf rate and empty rate would be, based on a prevalence of SCE ranging from 5 to 50%

(Table A.1). This range of prevalences was used as they span the range reported in the literature (Gilbert et al. 2005; Green et al. 2009).

Table A.1: The impact of varying levels of subclinical endometritis on herd reproductive performance

	Herd subclinical endometritis prevalence						
	0%	5%	10%	20%	30%	40%	50%
6-week in-calf rate	70%	68.75%	67.5%	65.0%	62.5%	60.0%	57.5%
Empty rate	10%	11.5%	13%	16%	19%	22%	25%

The second step was to calculate the potential economic loss due to the reduced reproductive performance of the SCE cows within the herd across the various prevalences. Firstly I calculated the dollar value of the difference between the obtained 6-week in-calf rate (e.g. 68.75% for a herd with a SCE prevalence of 5%) and desired 6-week in-calf rate (i.e. the 6-week in-calf rate for a herd without SCE, 70%). The equation for this calculation is Equation 1 (below), where A is the economic loss, B is the observed 6-week in-calf rate and C is the desired 6-week in-calf rate. The dollar value ($4) in the equation is an economic multiplier (supplied in the tool) that was estimated through modelling assuming a $5.50 per kg milksolids pay-out.

$$A = \ C - B \ * \$4 * number\ of\ cows\ in\ herd$$

Equation 1: Equation used to calculate the dollar value for the difference between the obtained 6-week in-calf rate and the desired 6-week in-calf rate. The letters used in this equation are explained in the text above.

Next, I calculated the dollar value for the difference between the obtained empty rate (e.g. 11.5% for a herd with a SCE prevalence of 5%) and desired empty rate (i.e. the empty rate for a herd without SCE, 10%). The equation for this calculation is Equation 2 (below), where D is the economic loss, E is the obtained empty rate and F is the desired empty rate. The dollar value ($10) in the equation is an economic multiplier (supplied in the tool) that assumes a $1000 value differential between an empty and in-calf cow.

$$D = \ E - F \ * \$10 * number\ of\ cows\ in\ herd$$

Equation 2: Equation used to calculate the dollar value for the difference between the obtained empty rate and the desired empty rate. The letters used in this equation are explained in the text above.

The dollar value for the overall reduction in herd reproductive performance was calculated by adding A from Equation 1 (the dollar value for the difference between the obtained 6-week in-calf rate and the desired 6-week in-calf rate) to D from Equation 2 (the dollar value for the difference between the

obtained empty rate and the desired empty rate). The values calculated for A, D, and their combined total, are displayed in Table A.2.

Table A.2: Economic loss for the differences between obtained and desired 6-week in-calf rates and empty rates for a herd with varying prevalence of subclinical endometritis.

	Herd subclinical endometritis prevalence					
	5%	10%	20%	30%	40%	50%
6-week in-calf rate loss (A)	$2,000	$4,000	$8,000	$12,000	$16,000	$20,000
Empty rate loss (D)	$6,000	$12,000	$24,000	$36,000	$48,000	$60,000
Total $ loss per year (A + D)	$8,000	$16,000	$32,000	$48,000	$64,000	$80,000
Economic loss/cow/year	$20	$40	$81	$122	$163	$204

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