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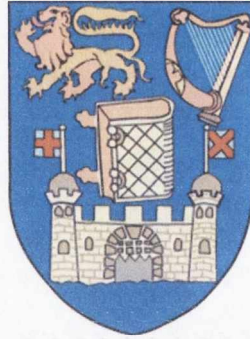
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**Towards an improved understanding of local uterine
innate immunity and histological change in postpartum
cows: relevance to fertility?**

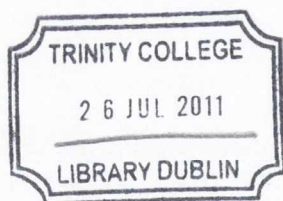


This thesis is submitted to the University of Dublin for the degree of Doctor of
Philosophy

September 2009

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THESIS

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This thesis is submitted by the undersigned for the degree of Doctor in Philosophy at the University of Dublin. It has not been submitted as an exercise for a degree at any other university.

Apart from the advice, assistance, and joint effort mentioned in the acknowledgements and in the text, this thesis is entirely my own work.

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Dedication

This thesis is dedicated to my mother and father, not only for their genes, but also their vision and guidance.

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Publications arising from this thesis

1. **Chapwanya, A.**, Meade, K.G., Doherty, M.L., Callanan, J.J., Mee, J.F. and O'Farrelly, C. 2009. Histopathological and molecular evaluation of Holstein-Friesian cows postpartum: Toward an improved understanding of uterine innate immunity. *Theriogenology* **71** (9): 1396–1407.
2. **Chapwanya, A.**, Meade, K.G., Narciandi, F., Stanley, P., Mee, J.F., Doherty, M.L., Callanan, J.J. and O'Farrelly, C. 2009. Endometrial biopsy – a useful clinical and research tool in bovine reproduction. *Theriogenology*
3. **Chapwanya, A.** 2008. Uterine disease in dairy cows: classification, diagnosis and key roles for veterinarians. *Irish Veterinary Journal* **61** (3): 183-186.

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1. **A Chapwanya**, KG Meade, ML Doherty, JJ Callanan & C O'Farrelly. 2009. Characterisation of local uterine innate immunity and histological change in postpartum cows: relevance to fertility. *Veterinary Research*.
2. **A Chapwanya**, KG Meade, J Cronin, ML Doherty, JJ Callanan, IM Sheldon & C O'Farrelly. 2009. *Escherichia coli* induce acute phase proteins (APPs) production by bovine endometrial epithelial cells *in vitro*. *Journal of Veterinary Immunology*.

Other Publications

1. Cormican, P., Meade, K.G., Cahalane, S., Narciandi, F., **Chapwanya, A.**, Lloyd, A.T. and O'Farrelly, C. 2008. Evolution, expression and effectiveness in a cluster of novel bovine beta-defensins. *Journal of Immunogenetics* **60** (3-4): 147-156.

Abbreviations

ACTB	Actin β
AI	Artificial insemination
AMP	Antimicrobial peptide
APC	Antigen presenting cells
APP	Acute phase protein
APR	Acute phase response
BNBD4	Bovine neutrophil beta defensin 4
BEND	Bovine endometrial epithelial cells
bp	Base pairs
CBA	Columbia Blood Agar
CD	Cluster of differentiation
CRP	C-reactive protein
DC	Dendritic cells
cDNA	Complementary deoxyribonucleic acid
DEFB5	Bovine defensin 5
DMI	Dry matter intake
EPP	Early postpartum
EtOH	Ethanol
μg	Microgram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GH	Growth hormone
H&E	Haematoxylin and eosin
Hb	Haemoglobin
HBD	Human beta defensins
HP	Haptoglobin
IFNG	Interferon- γ
IL	Interleukin
IRF	Interferon- γ regulatory factor
LAP	Lingual antimicrobial peptide
LPP	Late postpartum
LBP	LPS binding protein
LPS	Lipopolysaccharide

LRR	Leucine-rich regions
LTA	Lipoteichoic acid
nM	Nano molar
μM	Micrometer
MRPS	Mitochondrial ribosomal protein subunit
MyD88	Myeloid differentiation primary response gene 88
NCT	Non-template control
NFκB1	Nuclear factor KappaB1
NK	Natural killer cells
NOD	Nucleotide-binding oligomerization domain
PAMP	Pathogen-associated molecular patterns
PBL	Peripheral blood leucocytes
PBS	Phosphate buffer saline
PMN	Polymorphonuclear neutrophils
PPR	Pathogen pattern receptor
PRR	Pattern recognition receptor
qRT-PCR	Quantitative real time polymerase chain reaction
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing technology
SAA3	Serum amyloid A3
SEM	Standard error of the mean
SNP	Single-nucleotide polymorphism
TAP	Tracheal antimicrobial peptides
TIR	Toll/Interleukin 1 receptor domain
TLR	Toll-like receptors
TNF	Tumour necrosis factor
TRIF	TIR-domain-containing adapter-inducing interferon-β

Summary

Uterine involution in postpartum (pp) cows is necessary for establishment of the next pregnancy. Endometritis perturbs involution, impairs uterine function and causes systemic illness leading to significant involuntary cow culling. This costs dairy farmers and national exchequers €2.5 billion annually world-wide. At calving, bacteria enter the uterus *via* the open cervix presumably stimulating a cascade of innate immune defence events that culminate in transient uterine inflammation. In most cows this is followed by the clearance of infection, resolution of inflammation and resumption of cyclic activity. The local uterine immune mechanisms that are critical for pathogen elimination and resolution of inflammation post calving have not yet been characterised. Both the expression and regulation of innate immune genes are likely to hold clues regarding the role of the local uterine innate immunity in pp cows. The focus of this thesis was to characterise the local uterine involutionary response in postpartum cows with a broader view to unlocking the key factors to improving fertility in cattle.

To examine whether the postpartum uterine environment is inflammatory, a pilot study of 13 postpartum dairy cows was performed. Histological assessment of the uterine tissue revealed variable influxes of inflammatory cells at two weeks pp. qRT-PCR analysis also revealed that observed histological cellular events occurred concurrent with alterations in expression of innate immune genes, yielding evidence of a proinflammatory uterine environment.

Analysis of the innate uterine immune gene expression was performed in tissues obtained from 13 recently calved beef cows. qRT-PCR profiling revealed a unique finding where innate immunity corresponded to histological change locally *in utero* during the postpartum period.

The *in vitro* study was used to investigate the response of primary bovine endometrial epithelial cells to *E. coli* stimulation. Results showed that endometrial cells produce effector immune molecules suggesting that their expression in the uterus is driven, at least in part, by endometrial epithelial cells.

The work in this thesis goes some way to defining uterine molecular mechanisms underlying clearance of infection and resolution of inflammation postpartum. The information will facilitate the identification of biomarkers for the early and accurate detection of cows likely to conceive. Furthermore, characterisation of expression patterns and variations in these genes will provide important tools for devising breeding programmes to improve cattle fertility.

Chapter 1

Introduction

1. Introduction

A key challenge to be met by scientists, veterinarians and farmers is cattle sub-fertility which is a major impediment to profitable beef and dairy production worldwide. There is need to understand which genes control uterine involution and how these genes can be manipulated to enhance uterine health. For optimum reproductive performance, every cow should conceive and deliver a viable calf each year, and the calving to conception interval should be less than 80 days. The key to achieving this goal is to compliment genetic selection with optimum nutrition and herd health management in the transition cow (Mulligan & Doherty, 2008). The postpartum period is therefore critical and warrants particular attention in order to avoid uterine disease and perturbed fertility. Mammalian uterine involution during the postpartum period is characterised by microbial invasion, physical reduction in size, morphological and histological changes (Azawi, 2008; Balbin *et al.*, 1998; Gier & Marion, 1968; Ogawa *et al.*, 2001; Paisley *et al.*, 1986). Postpartum, all cows experience an influx of microorganisms consisting predominantly of bacteria (Azawi *et al.*, 2008c; Paisley *et al.*, 1986; Williams *et al.*, 2007). The microbial invasion is accompanied by the innate immune response orchestrated to eliminate the bacteria in the first instance, and then resume normal oestrus cyclicity within 4-6 weeks of parturition (LeBlanc *et al.*, 2002; Singh *et al.*, 2008). Even though the process controlling postpartum recovery is complex, it is possible that the local innate immune response of the uterus is predominantly inflammatory during this period. Innate uterine immune responses after calving therefore warrants further studies in the context of improved cattle fertility.

The uterus is a fascinating organ which accepts substances foreign to it (spermatozoa or calf) by blocking the normal body defence mechanisms designed to destroy the foreign 'invaders', but reacts to eliminate harmful pathogens. After calving, the uterus requires time to involute and prepare for the next pregnancy. It is that recovery process between calving and the next pregnancy that is so critical to a profitable cattle enterprise — and is poorly understood. A decline in dairy cow fertility is well documented (Chagas *et al.*, 2007; Lucy, 2007; Mee, 2007; Royal *et al.*, 2000; VanRaden *et al.*, 2004), but there is a dearth of current information on pathogenesis of bovine uterine inflammation, or indeed innate immunity and histological change during uterine involution in postpartum cows.

A healthy uterus is a pre-requisite to subsequent conception (Hammon *et al.*, 2006; Murphy & Dobson, 2002; Sheldon *et al.*, 2004), yet it is thought that approximately 20% of cows experience a perturbed postpartum period. It is estimated that only 34 live births occur for every 100 cows served for the first time in the United Kingdom (UK) (Dobson *et al.*, 2007). This is mainly due to unresolved inflammation and infection during the postpartum period that compromises uterine function (LeBlanc, 2008; Miller *et al.*, 2007; Sheldon *et al.*, 2006). In addition, an inverse relationship between milk yield and reproductive performance may be contributing to the declining fertility because of the emphasis on milk production during the selection of dairy cows (Caraviello *et al.*, 2006; McCarthy *et al.*, 2007).

In mammals, particularly cattle, following calving, the uterus must expel the foetal membranes and fluid, eliminate microorganisms, repair the endometrial lining, and shrink to a size ready to accept the next embryo. If undisturbed, these events culminate in resumption of normal oestrous cyclicity, conception and implantation (Blank *et al.*, 2008; Mitko *et al.*, 2008). Therefore, the key to devising management strategies for reversing the declining trends in fertility lies in understanding innate immune responses during uterine involution that underlie clearance of infection, resolution of inflammation and resumption of cyclicity in postpartum cows. This thesis explores the unique innate immune response of the bovine uterus, associated histological changes and their relevance to fertility during the postpartum period.

1.1 Immune system

The immune system is a complex system that enables the body of the host to be protected against or to eliminate pathogens (Janeway *et al.*, 2001b; Medzhitov & Janeway, 2000b; Wira *et al.*, 2005a). It is characterised by the capacity to distinguish between foreign, potentially harmful, agents and harmless 'self' molecules, and comprises of innate, early responding non-specific, and adaptive specific arms (Janeway, 1993; Linde *et al.*, 2008). The immune system determines host protection, particularly on gastro-intestinal, respiratory or reproductive mucosal surfaces (Medzhitov & Janeway, 2002). Further, the innate immune system is able to respond rapidly to potential pathogens it has not previously encountered (Janeway *et al.*, 2001a). In contrast, the adaptive immune defence is *via* recognition and a gradual response

(slow) that is specifically mediated through antibodies and clonal expansion of T-cell lymphocytes (Janeway & Medzhitov, 2002; Wira & Fahey, 2004). Regionally, the uterus is a part of the complex reproductive tract mucosal tissue with unique immune cells that play an important role in the innate immunity host defence (Eriksson *et al.*, 2006). Uterine immunity is important for conception and successful reproduction in mammals.

1.2 Overview of innate immunity

Recognition of invading pathogens is paramount to host survival, and innate immunity is the first line of host defence constitutively present and ready to be mobilised (Medzhitov & Janeway, 2000a; O'Neill, 2001; O'Neill, 2005; Palsson-McDermott & O'Neill, 2007). The innate system is a dynamic interactive network of cells and molecules that reacts efficiently to a variety of potentially harmful organisms (Brikos & O'Neill, 2008; Scott *et al.*, 2007). Innate immunity confers non-specific protection using anatomical barriers, secretory molecules and cellular components in a precarious balance between action and inaction (Lippolis, 2008), and also controls the initiation of the adaptive immune response.

Mechanical anatomical barriers such as epithelial or stromal cells are an integral part of the constitutive immune mechanism. Associated with these barriers are chemical and biological agents such as fatty acids in sweat, lysozyme and phospholipase in tears, saliva and nasal secretions, antimicrobial peptides and normal flora (Andersen *et al.*, 2006). Innate immune responses contribute to the activation of adaptive immunity through antigen recognition, binding and presentation by pathogen recognition receptors (PRRs) (Linde *et al.*, 2008; Medzhitov & Janeway, 2002). Innate cellular responses are mediated by phagocytic cells such as polymorphonuclear neutrophils (PMN), macrophages and dendritic cells (DCs). The phagocytic cells kill invading microorganisms and secrete proinflammatory cytokines important for the regulation of adaptive immune responses. Immature tissue-resident phagocytic cells, especially DCs possess receptors on their surface that recognise common pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), peptidoglycan or lipoteichoic acid (LTA) (Hopkins & Sriskandan, 2005). On recognising danger, the phagocytic cells engulf and degrade the pathogen intra-cellularly, in the process becoming activated into potent antigen presenting cells (APC) (Iwasaki & Medzhitov,

2004; Janeway, 1993). The APC then transport the antigens to nearby lymph nodes for presentation to T-lymphocytes to activate the adaptive response (Janeway *et al.*, 2001a).

1.2.1 Inflammation

Local inflammation plays a key role in the early response to infection (Medzhitov, 2007). Inflammation can be divided into acute and chronic phases. Following tissue injury or infection, acute inflammation consists of immediate vascular response within minutes marked by vasodilatation and increased blood flow (Banks, 1993). Within hours, acute cellular events occur of marked granulocyte influx especially neutrophils via margination and diapedesis at the point of injury or infection (Medzhitov & Janeway, 2000b). If injury or infection persists, the chronic inflammatory response, characterised by the appearance of macrophages and lymphocytes, is orchestrated. Macrophages function to kill pathogens and to clear cellular and tissue debris. Phagocytic dendritic cells mature and travel to lymph nodes, to present antigens to naïve T-cells and initiate adaptive responses (Janeway *et al.*, 2001a). At a molecular level, inflammation is characterised by the up-regulation of PRRs, chemokines and cytokines (Akira *et al.*, 2006). The events of the inflammatory response including production of acute phase proteins (APP) in the liver and systemic immunity initiation as a result of T-cell activation in regional lymph nodes, also occur (Linde *et al.*, 2008).

1.2.2 Immune cells

The innate immune cells required to orchestrate an inflammatory response include PMNs (involved in phagocytosis and intracellular killing) and monocytes (involved in phagocytosis, intracellular and extracellular killing). Progenitor cells also differentiate into macrophages (which act as antigen-presenting cells), eosinophils (involved in defence against parasites), basophils and natural killer cells (involved in non-specific immunity to viral infections and tumour surveillance). A key component of the host innate immune response is the up-regulation of proinflammatory cytokine production such as *TNF- α* and *IL-1* (Koj, 1996). The cytokines trigger an influx of immune cells to the site of injury or inflammation as well as activate adaptive immune responses.

1.2.2.1 Neutrophils

PMNs are the earliest cells to be recruited to the site of tissue injury, infection or inflammation. PMNs are equipped with multiple antibacterial mechanisms that are activated upon contact with the pathogen. The main functions in fighting infection include opsonisation and phagocytosis of the invading microorganisms (Medzhitov & Janeway, 2000b).

1.2.2.2 Macrophages and Monocytes

Macrophages are ubiquitously distributed in tissues where their population is maintained by the influx of monocytes from circulation or local proliferation (Banks, 1993). Macrophages participate in non-specific immunity against microbes via phagocytic uptake of the pathogens, and also adaptive immunity via antigen presentation (Janeway *et al.*, 2001a) and *IL-1* production (Lee & Rikihisa, 1996). Phagocytosis is in part facilitated by opsonins that bind to both microbes and receptors on the phagocytes (Medzhitov, 2007).

1.2.2.3 Eosinophils

Eosinophils are terminally differentiated leukocytes in the sub-mucosa of tissues that are recruited to the site of specific immune responses such as allergic or hypersensitivity reactions against multi-cellular parasites such as helminths (Banks, 1993). Eosinophils are recruited to the site of infection where they produce proinflammatory cytokines such as *IL-1*, *IL-6* and *TNF- α* as well as chemokine *IL-8* (Simpson *et al.*, 2007).

1.2.2.4 Mast cells and Basophils

Mast cells and basophils play a central role during the inflammatory response through the release of potent mediators such as histamine and the cytokines *IL-6* and *TNF- α* (Blanks & Ebert, 2008). Mast cells reside in the mucosal and connective tissues. During bacterial infection, mast cells can be directly activated by toll-like receptors (TLRs) (Galli *et al.*, 2005).

1.2.2.5 Dendritic cells (DCs)

DCs are professional APCs equipped to initiate and modulate the immune response via the stimulation of naïve T-lymphocytes, and thus play an important role in linking the innate to adaptive immunity (Janeway & Medzhitov, 2002). DCs sense pathogens through expression of cell surface toll-like receptors (TLRs), the important proteins crucial for linking innate and adaptive immunity (Pasare & Medzhitov, 2005). Immature DCs are normally resident in peripheral tissue where they take up antigens, mature and migrate to regional lymph nodes for presentation of antigens to naïve T-lymphocytes (Yang *et al.*, 1999). DCs also detect cytokines produced by other immune cells, thus detecting danger indirectly, and then activating the appropriate adaptive immune response (Matzinger, 2002).

1.2.2.6 Natural killer (NK) cells

NK cells are specialised innate immune defence cells against intracellular pathogens such as viruses through inducing apoptosis of infected cells and cytokine production (e.g. IFN- γ) (Lanier, 2005). NK cells distinguish between normal healthy cells and abnormal cells by using cell surface receptors that control their activation and proliferation (Hamerman *et al.*, 2005).

1.2.2.7 Epithelial cells

Endometrial epithelial cells line the entire uterine mucosal surface forming a physical barrier protecting the host from pathogen invasion as well as contributing to local innate immunity (Herath *et al.*, 2006b; Soboll *et al.*, 2006; Wira *et al.*, 2005b). It is important in the context of uterine recovery that there is considerable damage to the epithelial layer after placental separation so that the endometrium does not form a complete physical barrier during the early postpartum period. Endometrial cells in the bovine uterus are the first innate immune cells to contact and recognise pathogens via surface receptors such as TLRs (Davies *et al.*, 2008a). It is now appreciated that endometrial cells are also critical components of local innate immunity; they interact with, recognise and respond to potential pathogens. The endometrial epithelial cells are also thought to present antigens to other immune cells (Wira & Fahey, 2004).

In the female reproductive tract, epithelial cells are modules of innate immunity that express TLRs (Davies *et al.*, 2008a; Schaefer *et al.*, 2004; Wira *et al.*, 2005b) and proinflammatory cytokines (Fahey *et al.*, 2005). Elsewhere, mammary epithelial cells express antimicrobial peptides (AMPs) (Roosen *et al.*, 2004) and APPs (Eckersall *et al.*, 2006). The AMPs and APPs function to kill and/or limit the multiplication of bacterial pathogens. Uterine endometrial epithelial cells secrete chemokines and cytokines that attract and activate other immune cells, thereby providing a link between the innate and adaptive immune responses (Fahey *et al.*, 2005). In humans, previous studies suggest that endometrial cells are capable of presenting antigens to T-lymphocytes (Wira & Rossoll, 1995). Similarly in the rat, together with stromal cells, uterine epithelial cells present antigens to T-cells (Wira *et al.*, 2005c). An additional innate immune role of epithelial cells at the mucosal surface is the production of mucins known to prevent the attachment and entry of bacteria (Medzhitov, 2007).

1.3 Initiation of an immune response

Microbial pathogens possess specific PAMPs that are recognised by germ line-encoded PRRs to elicit immune responses (Lippolis, 2008). PRRs include cell surface TLRs and intracellular nucleotide binding oligomerisation domain-like receptors (NODs) (Creagh & O'Neill, 2006). The PRRs detect and bind specific 'non-self' antigens, then induce the production of proinflammatory cytokines such as *IFN- γ* or *TNF- α* and antigen presentation (Medzhitov & Janeway, 2002).

1.3.1 Pattern recognition

Pathogen molecules are detected by PRRs such as TLRs and NOD-like receptors. TLRs are a well characterised family of cell surface receptors widely encoded in the mammalian genome that recognise PAMPs from bacteria, viruses or fungi (Underhill, 2003). The TLRs are trans-membrane proteins with extracellular leucine-rich regions (LRRs) that recognise microorganisms (Creagh & O'Neill, 2006). In addition, there are cytoplasmic Toll/Interleukin 1 receptor (TIR) domains that activate intracellular signalling cascade pathways through interaction with adapter proteins (Nunez Miguel *et al.*, 2007). PAMPs are conserved motifs unique to microbes that include components of bacterial cell wall LPS or LTA.

TLRs are part of the earliest surveillance mechanism for sensing danger and are germ line-encoded, and therefore restricted in their adaptability (Janeway & Medzhitov, 2002). Reports from knock out studies concluded that TLRs function to recognise danger and that each TLR recognises specific microbial pathogens (Takeda & Akira, 2007). TLRs are also expressed on epithelial cells and cells of haematopoietic lineage (Werling & Jungi, 2003). To date 11 members of the TLR family have been identified in humans (Akira, 2003) where they play a role in defence against both viral and bacterial pathogens. In the murine model 13 TLRs have been identified, and the expression of TLR 1-9 described in epithelial cells in the respiratory (Schutte & McCray, 2002), gastrointestinal (Underhill *et al.*, 1999) and reproductive tracts (Soboll *et al.*, 2006). Ten TLR family members have been described in cows (Davies *et al.*, 2008a). Different TLRs are known to be activated by different PAMPs. TLR 1, 2 and 6 recognise bacterial cell wall lipid component LTA (Aderem & Ulevitch, 2000). *TLR 3*, 7 and 8 recognise single-stranded viral nucleic acids and synthetic nucleotide derivatives (Heil *et al.*, 2004). *TLR9* senses non-methylated viral and bacterial CpG containing DNA (Hemmi *et al.*, 2000). Some CpG molecules such as CpG-B are known to be potent inducers of the proinflammatory cytokines *IL-12* and *TNF- α* (Nakata *et al.*, 2006). *TLR4* was the first TLR to be described in humans (Medzhitov *et al.*, 1997). The commonly studied ligand LPS, a component of the outer membrane of gram-negative bacteria such as *Escherichia coli* (*E. coli*), is recognised by *TLR4* (Girling & Hedger, 2007). *TLR4* is unique among the TLRs in that it requires at least three accessory proteins for the initiation of LPS-induced signalling. LPS is bound by LPS binding protein (LPB) and then transferred to CD14 (Wright *et al.*, 1990). The CD14 then transfers the bound LPS to accessory protein MD-2 thereby offsetting a cascade intracellular signalling pathway (Okamura *et al.*, 2001). *TLR5* detects bacterial flagellin, a protein component of flagella present in both Gram-positive and Gram-negative bacteria (Hayashi *et al.*, 2001). In the same study, *TLR5* induction of *TNF- α* in mouse macrophages in an *NFkB*-dependent manner was observed.

TLRs play an important role in an innate immune response by recognising PAMPs and then activating the induction of inflammatory and immune genes. These include proinflammatory cytokines (e.g. *TNF- α* , *IL-1*, *IL-6*) and chemokines (e.g. *IL-8*). The activation of the TLRs can also induce proteins with direct anti-microbial activity such as AMPs (Hertz *et al.*, 2003). In addition, proteins involved in the acute phase response

(APR), including the APPs serum amyloid A (SAA) and haptoglobin (HP), are induced (Motley *et al.*, 2004). TLR activation recruits different transcription factors such as NF- κ B for eliciting an appropriate immune response to particular pathogens detected (O'Neill, 2005).

Another group of PRRs bind to intracellular microbial molecules. These are known as nucleotide binding oligomerisation domain-like receptors (NODs), which bind to both Gram-negative and Gram-positive bacterial peptidoglycan molecules (Shaw *et al.*, 2008). Following the binding of ligands, NODs oligomerise and then activate NF- κ B downstream (Creagh & O'Neill, 2006).

1.3.2 Cellular signalling

TLR or NOD-like receptors trigger NF- κ B transcription factor, thus initiating a signalling pathway responsible for immune response (Janeway *et al.*, 2001a; O'Neill, 2006). Activation of *NF κ B1* is one of the ultimate steps of TLR signalling cascade leading to induction of genes encoding cytokines and chemokines that are central to an immune response (O'Neill, 2004). As shown in Figure 1.1, a typical feature of TLR stimulation is the production of proinflammatory cytokines such as *IL-1*, *IL-6* and *TNF- α* involved in infection clearance via stimulation of macrophages and lymphocyte maturation (Xing *et al.*, 1998). In addition, TLR activation leads to the production of the chemokine *IL-8* that acts by recruiting neutrophils (Parker *et al.*, 2007). NF- κ B is the main down-stream signalling pathway involved in immune response (Lippolis, 2008). In addition, intracellular signalling through the NF- κ B pathway, activation of alternate kinases regulating the interferon regulatory factor (IRF) family leads to expression of various *IFN- γ* genes (O'Neill, 2006). The pathway activated depends on the TLR stimulated. TLR2 activates NF- κ B to cause the expression of the *TNF- α* gene while TLR3 activates both NF- κ B and the IRF3 factors to express the *IFN- γ* and *TNF- α* genes (Doyle & O'Neill, 2006). Figure 1.1 shows the nuclear factor activation pathways following stimulation of the various TLRs. In the uterus in humans, *IL-1*, *IL-6* and *TNF- α* have been reported to stimulate the translocation of NF- κ B to the nucleus and subsequently triggering the transcription of mediators of inflammation including *IFN- γ* and *IL-10* (Schaefer *et al.*, 2004). Natural killer cells are thought to be the immune cells responsible for the production of these inflammatory mediators (Eriksson *et al.*, 2006).

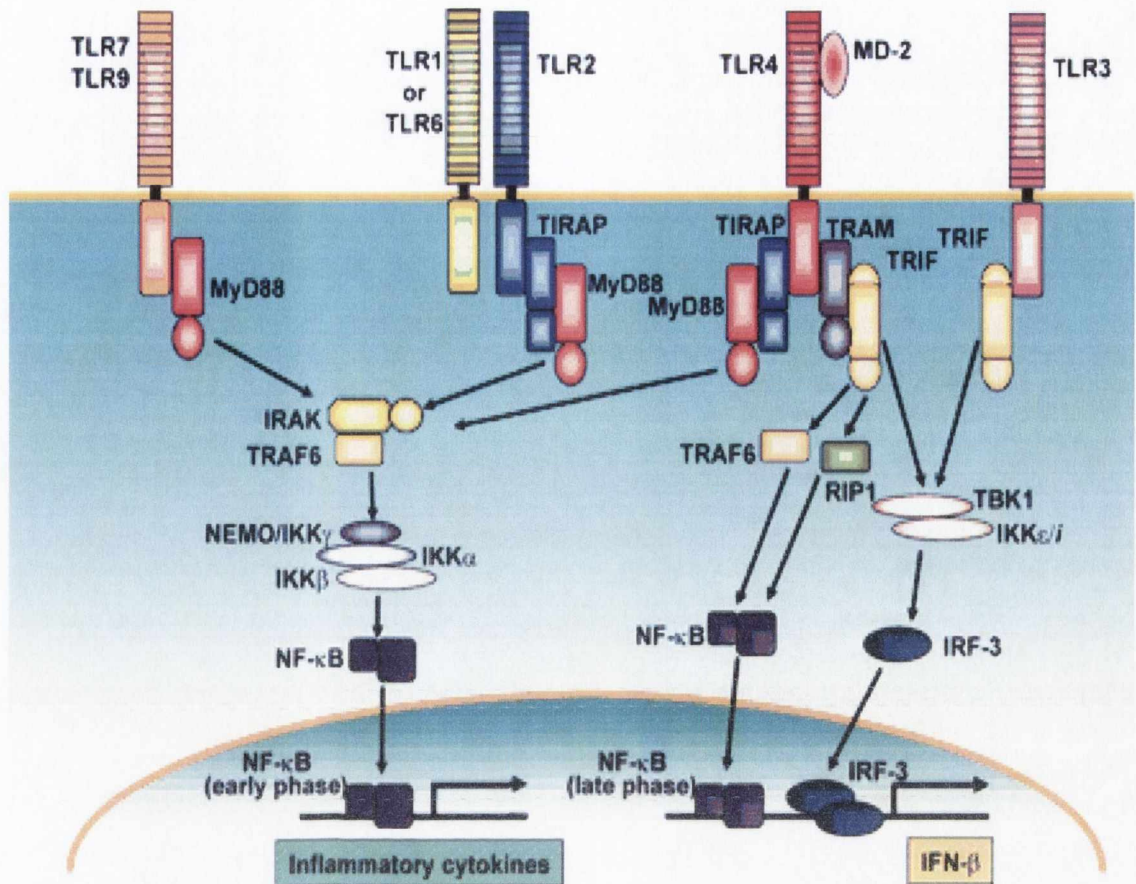


Figure 1.1: Overview of TLR signalling. Myeloid differentiation primary response gene 88 (MyD88) is essential for inflammatory cytokine production in response to all TLR ligands, except for the TLR3 ligand. The TLR signalling through MyD88 results in the activation of NF- κ B and TIR-domain-containing adapter-inducing interferon- β (TRIF), and eventually the production of proinflammatory cytokines. NF- κ B is one of the central regulators of down-stream TLR signalling and immune gene expression. Shown in the figure is typical TLR stimulation and production of proinflammatory cytokines such as IFN.

1.3.3 Nuclear factor kappa B (NF- κ B) transcription factor

A key mediator of innate and adaptive immune responses is the transcription factor NF- κ B, a protein complex that controls the transcription of DNA. NF- κ B is the main proinflammatory transcription factor activated by TLRs. NF- κ B is found in almost all animal cell types and is involved in cellular responses to stimuli such as injury, cytokines, bacteria or viruses (Sen & Baltimore, 1986). NF- κ B plays key roles in the regulation of the immune responses to infection. NF- κ B is normally stored in the cytoplasm as an inactive dimer. The identification of toll-like receptors (TLRs) as specific pattern recognition molecules and the finding that stimulation of TLRs leads to activation of NF- κ B improved our understanding of how different pathogens activate NF- κ B (Doyle & O'Neill, 2006). NF- κ B is activated by various stimuli, both intracellular and extracellular, such as cytokines, bacteria or viruses (Li & Verma, 2002). Following TLR activation, the signal transduction pathway initiated culminates in upregulation of proinflammatory cytokines such as *TNF- α* and *IL-1*, *IL-2* and *IL-6* (Bonizzi & Karin, 2004). The enhanced expression of the cytokines results in the orchestration of the inflammatory response, where NF- κ B is a central regulator of down-stream TLR signalling and immune gene expression. In addition, NF- κ B is widely used by eukaryotic cells as a regulator of genes that control cell proliferation and cell survival, given that NF- κ B regulates the expression of over 150 genes including inflammatory cytokines (Li & Verma, 2002). While physiological activation of NF- κ B is required for cell protection, abnormal induction leads to acute inflammation and development of disease (Sen & Baltimore, 1986). Therefore the production of NF- κ B may be essential for mammalian immunity, including uterine involutionary responses after calving.

1.3.4 Inflammatory cytokines

The expression of cytokines is tightly regulated. In response to tissue injury or infection, TLR signalling through TRIF or NF- κ B leads to the upregulation of proinflammatory cytokines and chemokines (Hopkins & Sriskandan, 2005). Chemokines such as *IL-8* attract immune cells, particularly neutrophils, to the site of infection or injury. Cytokines play important roles in the defence and repair of tissue, as well as cause the functional maturation of immune cells (Medzhitov & Janeway, 2002). Mature immune

cells then respond to, recognise or present the pathogen to T-lymphocytes (Lippolis, 2008). In addition, cytokines activate target cells to secrete proteins, cytokines and chemokines. *IL-1*, *IL-6* and *TNF- α* produced at the site of injury or infection are part of an APR leading to the induction of APPs (Motley *et al.*, 2004).

1.3.4.1 Tumour Necrosis Factor (*TNF- α*)

TNF- α is a cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the host acute phase reaction to injury or infection. In addition, *TNF* is a prominent mediator central to the induction of the innate immune inflammatory response (Hehlhans & Pfeffer, 2005). *TNF- α* is produced by macrophages, monocytes and T-lymphocytes and leads to multiple effects on the host response. The primary role of *TNF* is in the regulation of immune cells. During the inflammatory response, *TNF- α* is involved in the induction of cytokine production, and the expression and activation of adhesion molecules. *TNF- α* is among the early cytokines produced following injury or infection (Papadakis & Targan, 2000; Wira *et al.*, 2005b). Among the potent biological roles of *TNF- α* are the stimulation of NK cells, macrophages and monocytes as well as induction of fever (Notebaert *et al.*, 2008). In clinical practice, the use of anti-*TNF* antibodies has become a strategy in the treatment of human patients with chronic inflammatory diseases (Danese *et al.*, 2006).

1.3.4.2 Interleukin 1 (*IL-1*)

IL-1 is produced by macrophages, monocytes dendritic and epithelial cells. Like *TNF- α* , *IL-1* forms an important part of the inflammatory response of the body against infection by stimulating T-cells and macrophages as well as inducing cytokine secretion and fever (Fahey *et al.*, 2005). *IL-1* is therefore called an endogenous pyrogen. Immune-modulating activity via *IL-1* is clearly of benefit for host protection, as *IL-1* produced upon infection with bacteria or viruses would enhance the immune response against these pathogens. There are 2 main forms of *IL-1*, *IL-1 α* and *IL-1 β* , which are pleiotropic cytokines involved in various immune responses and inflammatory processes (Janeway & Medzhitov, 2002). In addition, *IL-1* induces chemokine and APP secretion, as well as leukocyte recruitment via the induction of neutrophil release from the bone marrow

(Gruys *et al.*, 2005). In the immune system, *IL-1* is known to activate lymphocytes, monocytes, macrophages, and NK cells (Dinarello, 1997).

1.3.4.3 Interleukin 6 (IL-6)

IL-6 is a pro-inflammatory cytokine which is secreted by T cells, macrophages and epithelial cells to stimulate immune response to injury or infection. *IL-6* is one of the most important mediators of fever and of the mammalian acute phase reaction. *IL-6* provokes a broad range of cellular and physiological responses in the host. During the inflammatory response, *IL-6* regulates the growth and differentiation of T- and B-lymphocytes as well as inducing fever (Janeway *et al.*, 2001a). Other key roles of *IL-6* during inflammation include the inhibition of apoptosis of neutrophils and the induction of the hepatic acute phase response (Koj, 1996; Moshage, 1997). The secretion of *IL-6* is also induced by *IL-1* and *TNF- α* (Notebaert *et al.*, 2008).

1.3.4.4 Interferon- gamma (*IFN- γ*)

IFN- γ , or type II interferon, is a cytokine which is critical for innate and adaptive immunity against viral and intracellular bacterial infections, and for tumour control (Schroder *et al.*, 2004). Interferons are a class of glycoprotein cytokines produced by the cells of the immune system in response to challenges such as viruses and bacteria. Interferons are produced by a wide variety of cells in response to the presence of pathogens. *IFN- γ* drives the proinflammatory immune response. Interferons inhibit viral replication within host cells, activate NK cells, increase antigen presentation to lymphocytes, and induce the resistance of host cells to infection (Liu & Whitton, 2005; Vilcek, 2003). In addition, *IFN- γ* activates macrophages and T-lymphocyte proliferation and antigen-presentation, all of which function to eliminate pathogens (Karpuzoglu-Sahin *et al.*, 2001).

1.3.4.5 Interleukin 8 (IL-8)

IL-8 is a prototypic chemokine produced by macrophages, neutrophils and other cell types such as epithelial cells (Baggiolini & Clark-Lewis, 1992). The chemokine *IL-8* is

one of the major mediators of the inflammatory response which functions as a chemoattractant, and also a potent angiogenic factor. During the inflammatory response, *IL-8* plays a key role of controlling immune cell transportation, particularly neutrophils to the site of injury or infection (Quayle, 2002). *IL-8* is a central mediator of inflammation whose secretion is induced by *IL-1*, *TNF- α* and microorganisms (Harada *et al.*, 1994). *IL-1* or *TNF- α* are known to upregulate the expression of *IL-8* 100 fold, while bacterial pathogens induce a 10 fold increase (Hoffmann *et al.*, 2002). *IL-8* also functions as a chemoattractant for neutrophils initially, and then an activator of monocytes and endometrial epithelial cells (Schaefer *et al.*, 2004). In humans, *IL-8* plays a central role in inflammatory diseases (Amoli *et al.*, 2002; Hull *et al.*, 2000). In animals, *IL-8* was reported to be an important component of ovine reproductive physiology where it induced inflammation necessary for normal cervical function (Mitchell *et al.*, 2002). In cattle with acute mastitis, *IL-8* was detected and a role in resolving infection and inflammation was hypothesised (Boudjellab *et al.*, 1998). It can be argued that *IL-8* plays a pivotal role during the involutionary response in the uterus after calving.

1.4 The acute phase response (APR)

The term APR refers to the inflammatory response of the host occurring shortly after tissue injury or infection (Baumann & Gauldie, 1994; Dinarello, 1984; Koj, 1996; Kushner, 1982; Raynes, 1994). The APR is a non-specific immune mechanism aimed at restoring disturbed homeostasis (Emmanuel *et al.*, 2008; Mackiewicz *et al.*, 1987; Nilsen-Hamilton *et al.*, 2003). In general terms, the APR refers to the changes in concentrations of a large number of plasma proteins that are associated with the host response. The APR is initiated at the site of injury or infection. The inflammatory cascade is usually started by mononuclear cells, i.e. tissue macrophages or monocytes (Monshouwer *et al.*, 1996). The functions of the APR are host protection against infection, inflammation or tissue damage. These changes are predominantly the result of alterations in the pattern of protein synthesis in the liver (Pannen & Robotham, 1995). In addition, there are accompanying physiological changes such as fever and leucocytosis (Hirvonen *et al.*, 1999; Ohtsuka *et al.*, 2001). Systemic levels of the hepatically-derived plasma APPs increase rapidly. It is now recognised that localised synthesis of APPs is induced in other tissues (Chapwanya *et al.*, 2009; Eckersall *et al.*, 2006; Pyorala, 2003). The APR provides early non-specific defence against infection or

injury prior to onset of adaptive immunity (Dinarello, 1984; Lomborg *et al.*, 2008). Proinflammatory cytokines such as *IL-1*, *IL-6* and *TNF- α* are pivotal mediators of the APR acting as messengers between the local site of injury and the liver synthesising the APPs (Moshage, 1997).

1.4.1 Acute phase proteins (APPs)

In humans, a group of hepatically-derived glycoproteins called APPs are rapidly induced to restore homeostasis after infection, inflammation or injury (Baumann & Gauldie, 1994; Chan *et al.*, 2004). APPs are produced as a result of altered gene expression in the liver and have been shown to modulate cellular immune responses (Suffredini *et al.*, 1999). The APPs are part of the innate immunity and appear at substantially different concentrations in the peripheral blood where they are classified according to the magnitude of increase (positive APP e.g. Haptoglobin) or decrease (negative APP e.g. Albumin) in serum concentrations (Petersen *et al.*, 2004). The maximum serum concentration of APPs is typically reached within 24 to 48 h after the initiation of the response (Emmanuel *et al.*, 2008; Liu & Nilsen-Hamilton, 1995).

Proinflammatory cytokines, the main mediators of the APR separate into two groups with respect to APP induction, namely *IL-1* type cytokines (*IL-1*, *TNF- α*) and *IL-6* type cytokines acting through different receptors located on the membrane of the hepatocytes (Lehtolainen *et al.*, 2004). *IL-1* and *TNF- α* induce type 1 APPs serum amyloid A (SAA), C-reactive protein (CRP) and fibrinogen while *IL-6* induces type 2 APPs - haptoglobin (HP) (Gabay & Kushner, 1999). There are fundamental differences between the APP groups. Type 1 APP synthesis is synergistically induced by *IL-6* while type 2 APPs are neither induced nor synergistically stimulated by *IL-1* (Badolato *et al.*, 1994). *IL-1* induced type 1 APPs are characterised by a dramatic increase in serum concentration early after the inflammatory stimulus followed by rapid normalisation (Petersen *et al.*, 2004). Generally type 1 APP responses are stronger than type 2 APP (Gabay & Kushner, 1999).

1.4.2 Role of APPs

In cattle, the most sensitive APPs are HP and SAA, whose concentrations increase in peripheral circulation in response to acute inflammatory conditions (Eckersall *et al.*,

2006; Gruys & Snel, 1994; Gruys *et al.*, 2005; Horadagoda *et al.*, 1993; Karreman *et al.*, 2000). Acute phase proteins are thought to act directly and indirectly to protect the host (Bertoni *et al.*, 2008). In cows, HP is one of the most specific APPs which can increase up to 100 fold within 24 hours of induced inflammation (Chan *et al.*, 2004; Huzzey *et al.*, 2009). Apart from distinguishing acute from chronic inflammation, HP functions as a bacteriostatic agent through binding haemoglobin released by damaged erythrocytes thereby depriving microbial pathogens of vital free iron necessary for metabolism (Gabay & Kushner, 1999; Nazifi *et al.*, 2009). SAA on the other hand has a role in bacterial and pyogenic infections and increases in common infectious diseases (Winter *et al.*, 2006). SAA is anti-pyretic and also has a chemotactic effect on leukocytes, particularly neutrophils (Badolato *et al.*, 1994). Furthermore, SAA is known to down-regulate proinflammatory cytokine production and activity in monocytic cells, potentially providing a feedback mechanism by which they affect their own induction as well as other cytokine-driven aspects of the acute phase response *in vivo* (Hari-Dass *et al.*, 2005). In humans, CRP modulates cytokine production by monocytes and macrophages (Ballou & Lozanski, 1992). CRP also binds released bacterial or host DNA (Eckersall *et al.*, 2001). LPB inhibits LPS-mediated TNF release thus preventing death from shock (Schroedl *et al.*, 2001). It was reported that levels of hepatically-derived APPs increased in peripheral circulation of postpartum cows, and suggested that the synthesis of the APPs is a necessary event of normal uterine involution (Sheldon *et al.*, 2001). Although the liver is the primary site of APPs production, there is growing evidence of local synthesis of the APPs in sites distal to the liver (Eckersall *et al.*, 2006; Pyorala, 2003).

1.4.3 Extra-hepatic production of APPs

The recent recognition that APPs are produced locally in other tissues such as mammary tissue in response to infections (Eckersall *et al.*, 2006) has provided potential for use of APPs in the detection of economically important diseases in cattle. Extra-hepatic production of APPs was also described in macrophages and epithelial cells in mice (Zhang *et al.*, 2005). An inflammatory response model in mice inoculated with *E. coli* reported amyloid A production in dermal pouches (Notebaert *et al.*, 2008). In that model, there was production of the cytokines *IL-1*, *IL-6* and *TNF- α* within a few hours after the initiating stimulus. *In vitro*, mammary epithelial cells stimulated with LPS are known to produce SAA protein (Larson *et al.*, 2005). In bovine mammary tissue, the

APR is thought to be part of a local defence-response towards tissue injury or inflammation (Lomborg *et al.*, 2008; Murata *et al.*, 2004). The mammary-derived APPs prevent further injury of mammary gland, isolate and destroy the pathogens, as well as remove any harmful molecules and debris. In addition, the APPs activate the repair processes which are necessary to return the udder to its normal function (Eckersall *et al.*, 2001; Gruys & Snel, 1994). In the bovine uterus there is local production of APPs (Chapwanya *et al.*, 2009), thought to play an important role in restoring homeostasis following the inflammatory response during involution (Sheldon *et al.*, 2001).

1.4.4 APPs: a role in local uterine innate immunity?

The detection and control of metritis are primary goals for cattle producers. There is evidence of APPs locally in the uterus during the postpartum period in metritis-affected cows (Sheldon *et al.*, 2001). The initiation of the APR locally results in the increase in capillary permeability and infiltration of leucocytes to the uterus. As shown in Figure 1.2, a local inflammatory cascade initiated by mononuclear cells such as tissue macrophages seems to be at play in the postpartum uterus. A broad spectrum of inflammatory mediators, such as cytokines is released in the uterus which in turn drives the production of APPs locally. Could locally produced APPs play a central role in direct or indirect uterine protection during the inflammatory immune response to infection during the postpartum period? Could the APPs also be used as diagnostic markers for metritis and other uterine diseases which are poorly detected in postpartum cows? More importantly, the APPs derived locally in the uterus may be key mediators of the innate immune response culminating in clearance of infection and resolution of inflammation, the necessary events for normal uterine involution and re-conception.

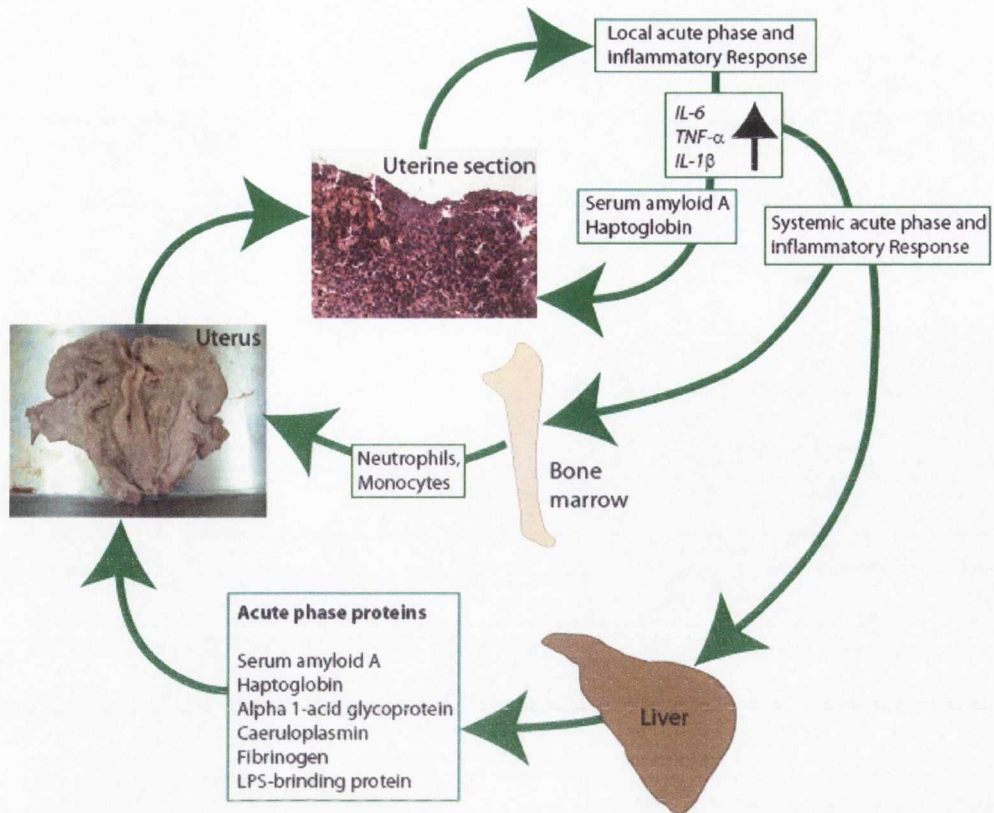


Figure 1.2: Model of uterine APR during uterine involution. Endometrial, stromal or professional cells recognise and phagocytose bacterial pathogens or apoptotic cells. Activation of TLRs such as *TLR4* leads to secretion of chemokines and cytokines, which in turn upregulate effector immune molecule expression. This is followed by local uterine production of APPs which then drive the important inflammatory events of tissue remodelling and reepithelialisation of the endometrial mucosa resulting in a coordinated involutionary response. A systemic loop of the reaction which involves the liver is activated to compliment the local response in pathology.

1.5 Antimicrobial peptides (AMPs)

Amongst the effector molecules induced by the activation of the innate immune system are AMPs. The AMPs are gene-encoded short proteins, generally between 12 and 50 amino acids long, effector molecules of the innate immune system that interact with bacterial membranes through hydrophobic amino acids (Ganz *et al.*, 1985; Sorensen *et al.*, 2008). AMPs are found in a wide range of eukaryotic organisms and are produced by leukocytes and epithelial cells lining the mucosal surface in a variety of tissues (Liang & Kim, 1999). AMPs are constituents of the innate immune system that prevent or limit infections (Zasloff, 2002). The modes of action for AMPs include inhibiting synthesis of specific membrane proteins, arresting DNA synthesis and production of hydrogen peroxide. They have a broad-spectrum activity against Gram-positive and Gram-negative bacteria, yeasts, fungi, protozoa and enveloped viruses by disrupting the membrane integrity resulting in leakage of cell contents. AMPs including defensins, histatins and cathelicidins have the ability to enhance immunity by functioning as immunomodulators (Silva, 2004).

1.5.1 Structure of AMPs

Defensins consist of three classes, namely α -, β - and θ -defensins and are known to participate in immune defence by killing the pathogen and influencing the cellular innate and adaptive immune responses (Lehrer & Ganz, 2002). Defensins exhibit variations in the amino acid sequence. α -Defensins are short amino acid chains (29 – 35 amino acids) containing 3 cysteine bonds in a 1-6, 2-4, 3-6 alignment, whereas a triple-strand β -sheet structure with a β -hairpin loop containing cationic charged molecules is found on β -defensins which are structurally larger than α -defensins (Ganz, 2003). α -Defensins have a C-terminal part (Figure 1.2a) thought to be responsible for the antimicrobial activity (Ganz *et al.*, 1985). In neutrophils, the α -defensins are known to play a role in oxygen-independent killing of phagocytised microorganisms (Lai & Gallo, 2009) and a single metalloproteinase functions to release the active peptide (Ganz, 2003). β -Defensins are larger than α -defensins and are phylogenetically older but have similar tertiary structures due to the presence of three disulphide bridges in a 1-5, 2-4 and 3-6 alignment (Jenssen *et al.*, 2006). The peptides form a triple-stranded β -sheet structure and a β -hairpin loop in which cationic charged molecules are contained as shown in Figure 1.3b.

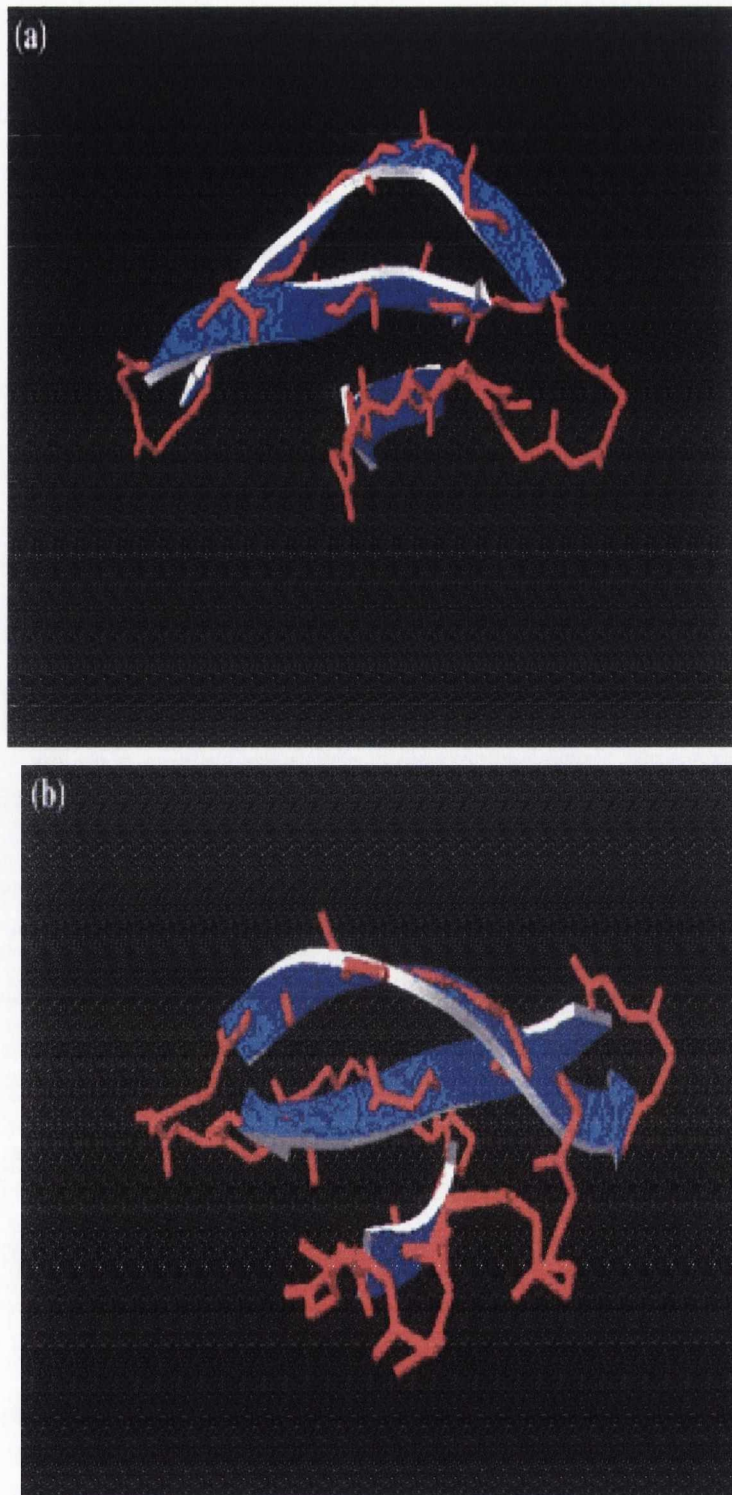


Figure 1.3: Three-dimensional models of the secondary structures of (a) the α -defensin and (b) the β -defensin. The backbone of the peptide and the β -strands are outlined. β -stranded nature is due to the presence of 2 or more disulfide bonds. The backbone of the peptide is outlined in red and β -strands are indicated with blue ribbons.

1.5.2 Role of AMPs

The precise mechanism of action of AMPs is not yet fully described. AMPs target a variety of pathogens, but have been well documented for their potent role against bacteria (Ganz, 2003; Lai & Gallo, 2009; Lehrer & Ganz, 2002). There are suggestions of direct microbial killing via formation of channels and/or destruction of bacterial membranes (Brogden, 2005). Owing to their hydrophobicity, net positive charge, conformational flexibility and secondary structure, AMPs are capable of interacting with, and penetrating bio-membranes to cause disruption of cytoplasmic membranes or intracellular bacterial targets, ultimately leading to killing of the microorganism (Ganz *et al.*, 1985; Lai & Gallo, 2009). Other methods of AMP action proposed include direct inhibition of bacterial intracellular processes such as DNA replication or protein synthesis (Yeaman & Yount, 2003). An important immunomodulation role of α -defensins is increased *TNF- α* and *IL-1* expression in activated monocytes thus driving the proinflammatory response (Ganz, 2003). α -Defensins are also thought to play an important role in protecting the host by attracting T-cells (Chertov, Michiel *et al.* 1996) while β -defensins are known to activate the classical complement pathway (Mayer, Bauer *et al.* 2001). In humans β -defensin 3 (HBD3) binds to the chemokine receptor CCR6, which is expressed by immature dendritic cells and memory T-cells, thereby attracting immature immune cells to the site of injury or inflammation (Duits *et al.*, 2002). In cattle, tracheal antimicrobial peptides (TAP) were characterised and shown to be induced by invading pathogens at the site of inflammation as a protective mechanism (Zaiou & Gallo, 2002). The β -defensins BNBD4 and DEFB5 have been described in the mammary glands of cows with mastitis and perform a direct antibacterial function (Goldammer, Zerbe *et al.* 2004).

1.5.3 Immunomodulation: a key role for AMPs in the local uterine immune response?

In the murine model, in addition to antimicrobial activity, AMPs play an important role in linking the innate and adaptive immune responses (Palffy *et al.*, 2009). Defensins also stimulate proliferation of epithelial and fibroblast cells, and promote wound healing (Selsted & Ouellette, 2005) all of which are necessary steps for successful uterine

involution. Could it be that AMPs perform similar functions in the bovine uterus undergoing involution during the postpartum period?

1.6 Uterine immune system

The uterine mucosal immune system is uniquely adapted to protect against infectious agents. The uterus has evolved into a privileged immune organ contributing to maternal health and foetal survival (Wira *et al.*, 2005b). The dual roles of the uterus include facilitating physiological conditions such as fertilisation, pregnancy and parturition, all of which are necessary for successful reproduction (Horne *et al.*, 2008), and also discriminating between harmless and pathogenic microorganisms they encounter in order to generate protective immune responses (Entrican & Wheelhouse, 2006). The mucosal immune system of the uterus is thought to perform innate immune functions by producing AMPs for limiting bacterial multiplication (King *et al.*, 2003a) and then producing cytokines and chemokines which drive the adaptive immune response (Wira *et al.*, 2005a).

In humans, investigation of female reproductive tract innate and adaptive defence mechanisms has enabled the development of better strategies for preventing sexually transmittable diseases (STDs) or resolving sub-fertility (Wira *et al.*, 2002). The role of TLRs in responding to bacterial or viral pathogens has been described in cervical epithelial cells (Anderson *et al.*, 2006). In the uterus, endometrial cells are known to recognise pathogens, and then present the antigens to autologous T-cells (Wira *et al.*, 2005c). Furthermore, the endometrial cells secrete proinflammatory mediators such as *TNF- α* , *IL-6* and *IL-8* to stimulate and attract other immune cells (Fahey *et al.*, 2005). In the murine model, endometrial epithelial cells are known to present antigens to T-cells under the control of cytokine (Wira & Rossoll, 1995). Together with stromal cells, endometrial cells contribute to the innate immune response via the production of β -defensins such as human defensin 5 (HBD5) antimicrobial peptide known to have antimicrobial activity (Quayle *et al.*, 1998).

The uterine innate immune response during the postpartum period in cows has not yet been fully described. Of particular importance is the local immune response orchestrated during the postpartum period in order to return the postpartum uterus to a state capable of supporting another pregnancy within 6-8 weeks after calving.

1.6.1 Bovine uterine involution

The uterus is a dynamic and complex organ. As shown in Figure 1.4, during pregnancy, the uterus progressively increases in size in order to accommodate the growing foetus such that at term it is 50 times the size of the non-pregnant uterus (VanRees *et al.*, 1981). Classically, all cows experience a postpartum period characterised by infection and drastic reduction in uterine size (Dhaliwal *et al.*, 2001). Postpartum uterine infection is a reproductive risk, therefore the uterus might be expected to possess enhanced mechanisms for combating pathogens during involution (Nilsen-Hamilton *et al.*, 2003). It can be argued that the presence of potential bacterial pathogens and tissue remodelling initiates the uterine inflammatory response after calving. During this period, the involuting uterus undergoes molecular, gross and microscopic changes regulated by signals that are likely to be generated locally within the uterus. Uterine tissue mass and structure change drastically through contraction and physical shrinkage accompanied by necrosis, sloughing off and regeneration of new endometrium (Noakes *et al.*, 1994). There also occurs a drastic induction of APPs which function to limit tissue damage and promote tissue repair (Williams *et al.*, 2005). The normal uterus will lose more than 80% of its pre-calving size during the early postpartum period. The important process of physical reduction in uterine size to less than 50% of the full-term size occurs 10-14 days after calving in cows with an undisturbed puerperum, otherwise the cows are at risk of developing endometritis (Bajcsy *et al.*, 2006). Crucially, any disturbances in physical reduction in size or the inflammatory immune process during this important phase of the reproductive cycle of cows perturb fertility (Ahmadi *et al.*, 2007) thereby contributing to the declining trends in cattle fertility documented in recent times. A fundamental question therefore is, how does the uterus, which is invaded by many potential bacterial pathogens at calving, control the inflammatory response during involution?

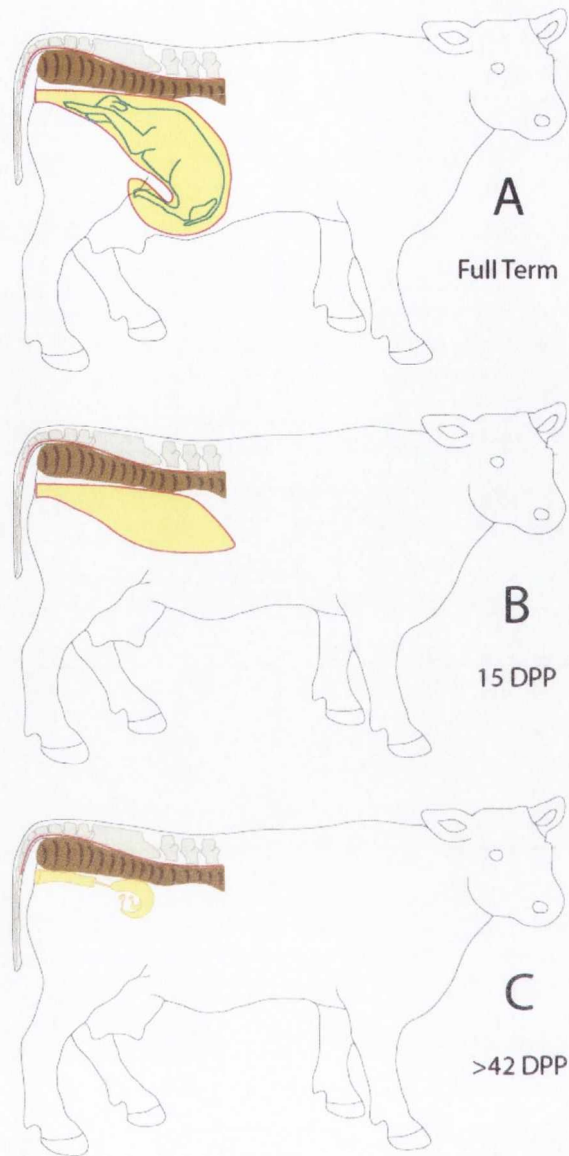


Figure 1.4: Diagrammatic representation illustrating the dramatic variation in the size of the bovine uterus. The full-term pregnancy (A) and 15 days postpartum (B) uterus is shown compared to the non-pregnant 42 days or more after calving (C). As shown, during the postpartum involutionary process, the size of the uterus is reduced by 50 times to the non-pregnant size. In the absence of disturbance, the involution and reduction in size is normally completed within 6 to 8 weeks of calving.

1.6.2 Local uterine innate immunity in postpartum cows

It is clear that dilatation of the cervix during the calving process exposes the uterus to a myriad of potential bacterial pathogens. The types of bacteria colonising the uterus at this stage comprises mixed cultures, although *E. coli* and *Arcanobacterium pyogenes* are commonly isolated from endometritis cases (Foldi *et al.*, 2006). The local uterine innate immune response is activated to eliminate the bacteria, and also to initiate the adaptive immunity arm (Singh *et al.*, 2008). Not surprisingly, an immune response is initiated in the postpartum uterus by TLRs which detect pathogens. Activation of the TLRs then trigger signalling pathways that activate molecules such as NF- κ B (Abrahams *et al.*, 2004; Wietek & O'Neill, 2007). Specific downstream signalling is then achieved through coupling of TLRs with intra-cellular adapter proteins myeloid differentiation primary response gene 88 (MyD88) prior to the activation of NF- κ B (O'Neill, 2006; O'Neill & Bowie, 2007). NF- κ B activation then induces genes encoding *TNF- α* , *IL-1*, *IL-6*, *IL-12* and *IFNG* cytokines and the chemokine *IL-8* for mobilising immune cells and orchestrating the inflammatory response (Hopkins & Sriskandan, 2005). Professional phagocytic cells such as PMN and macrophages recruited to the uterus eliminate the invading bacteria through coordinated adherence, attachment, ingestion and digestion (Kim *et al.*, 2005a). Ultimately hepatically-derived APPs and AMPs involved in resolving infection are induced (Zaiou & Gallo, 2002). Therefore, characterisation of time-sequence events locally in the postpartum uterus, particularly APPs and AMPs production is key to improving fertility in cattle. Could it be that the local acute phase response postpartum is geared towards returning the uterus to a pathogen-free state important for subsequent conception quickly after calving?

1.7 Implications of disturbed uterine involution in cows

1.7.1 Development of bovine uterine disease

There are three possible outcomes of uterine involution in postpartum cows. As shown in Figure 1.5, the ideal scenario is one where cows complete uterine involution within 6-8 weeks of calving and conceive, if mated. Two sub-groups of cows fail to re-

conceive even after repeated mating. Disturbed involution during the postpartum period in these cows results in pathology of the uterus that ultimately results in perturbed fertility (Anaya-Lopez *et al.*, 2006). This thesis explores the local uterine innate involutionary responses in postpartum cows with a view to unlock key immune regulation factors which are crucial for improving cattle fertility.

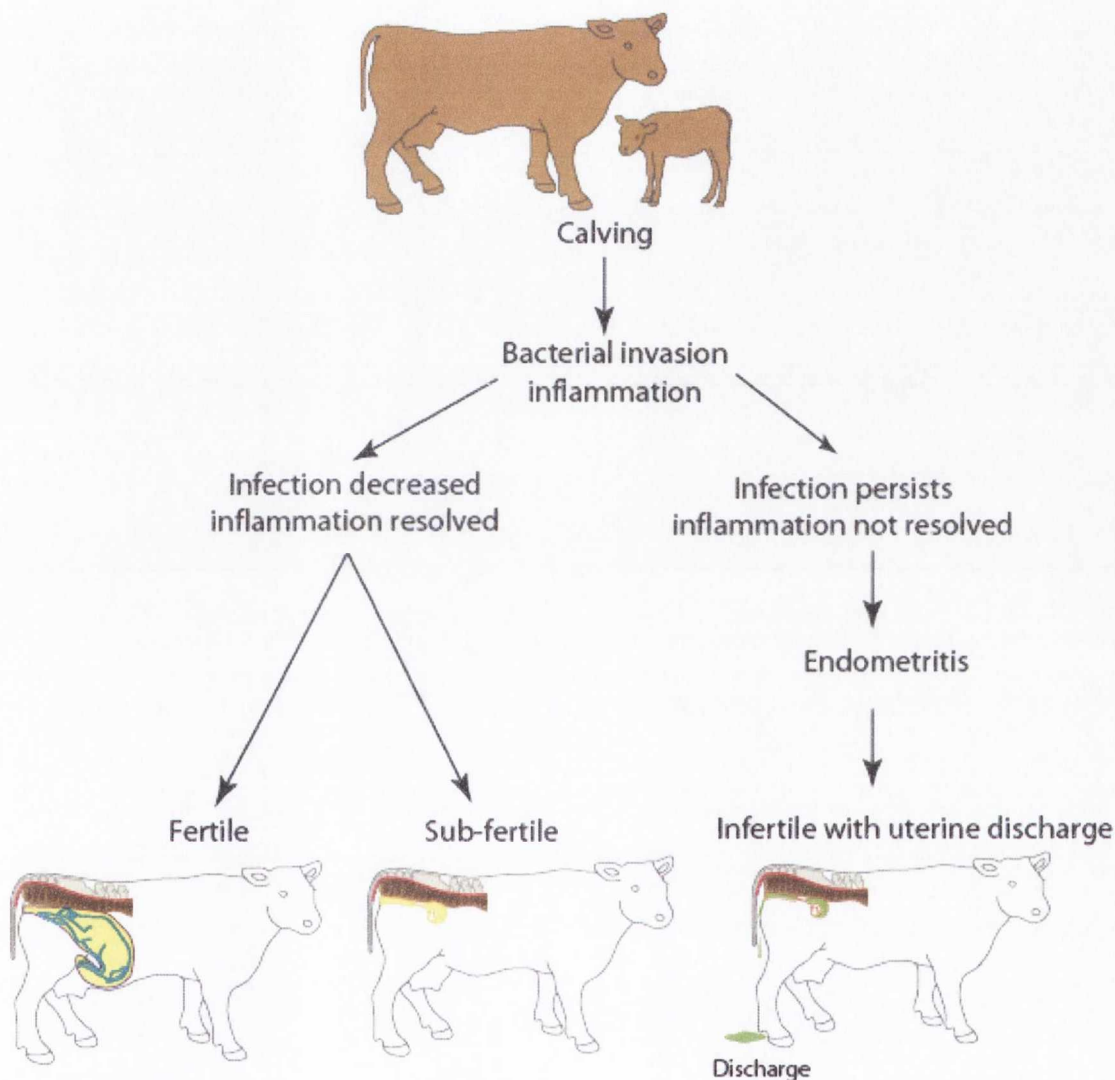


Figure 1.5: Outcome of uterine involution in postpartum cows. The fertile cows complete uterine involution within 6 - 8 weeks of calving exhibit signs of oestrus cyclicity and conceive. The subfertile cows, usually suffering from subclinical uterine disease with no overt signs of clinical illness exhibit signs of oestrous cyclicity even after mating, hence the term ‘repeat breeders’. The third group, the infertile cows do not exhibit signs of clinical ill-health but show signs of oestrus cyclicity. Purulent vulval discharges are commonly detected.

To add further complexity to the problem of decreasing bovine fertility, there are five forms of uterine diseases in postpartum cows as shown in Figure 1.6. Puerperal metritis refers to an abnormally enlarged uterus, fetid discharge and signs of systemic illness up to 10 days postpartum (Herath *et al.*, 2006b). Clinical metritis refers to abnormally enlarged uterus and purulent uterine discharge up to 21 days after calving in apparently normal cows (Foldi *et al.*, 2006). Clinical endometritis refers to cows with a purulent uterine discharge 21 to 30 days after calving (Sheldon *et al.*, 2008). Sub-clinical endometritis refers to uterine discharge with over 18% neutrophils between 30 and 42 days or more after calving; (Sheldon *et al.*, 2006). Pyometra refers to a chronic accumulation of purulent material in the uterus in the presence of a persistent corpus luteum and a closed cervix in cows that calved at least 7 weeks previously (LeBlanc *et al.*, 2002).

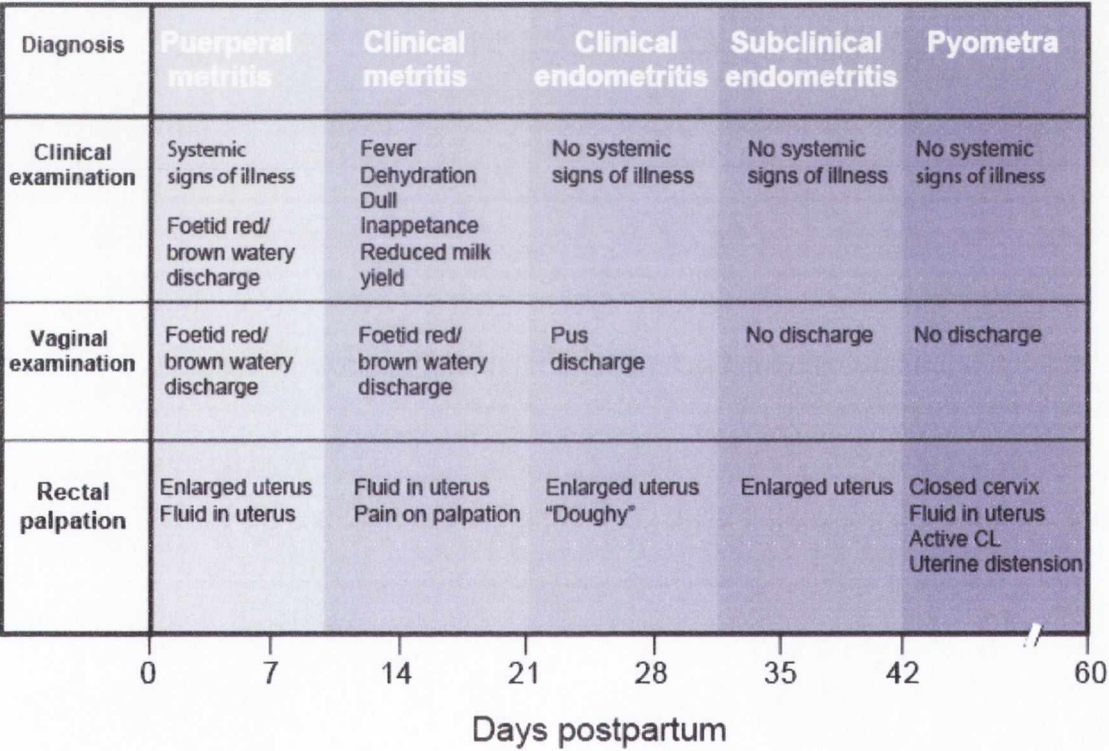


Figure 1.6: Differential diagnosis of uterine diseases in postpartum cows. Metritis develops between 1 and 21 days after calving, endometritis between 22 and 42 days. Pyometra occurs 43 days onwards after calving in cows with an active corpus luteum and closed cervix.

1.7.2 Economic losses

Losses caused by endometritis to dairy producers have been attributed mainly to impaired uterine function, systemic illness, sub-fertility, loss of body condition, increased veterinary bills, the cost of involuntary culling and replacement cows (Stevenson & Lean, 1998). Twenty percent of the cows fail to undergo self-cure, and develop disease, which can be acute, causing severe uterine tissue damage and even death. As a result, financial losses are incurred by dairy farmers and national exchequers totalling nearly €2.5 billion p.a. world-wide (LeBlanc *et al.*, 2002). In Ireland dairy producers incur a financial penalty that ranks among the highest in the world, estimated at nearly €70 million every year, as a result of endometritis (Mc Parland *et al.*, 2007). Furthermore, infertility in the dairy cow was one of the major priorities to emerge from a recent expert opinion (Delphi) study conducted by Animal Health Ireland (www.animalhealthireland.ie/diseases/delphi_study_res.php).

1.7.3 Declining trends in cattle fertility

Over the last five decades, a significant decline in dairy cow fertility has been documented (Royal *et al.*, 2000). The decline is due to a disturbance in the key events of physiological uterine involution and resumption of ovarian cyclicity, particularly in the presence of overwhelming uterine infection. It is currently estimated that only 34 live births occur to first service for every 100 dairy cows in the UK (Dobson *et al.*, 2007). The exact figure for conception rates in beef cows is unknown, but it is thought to be around 45% in Ireland (Mc Parland *et al.*, 2007).

1.8 Evaluation of postpartum cows

A critical component of addressing the worrying decline in cattle fertility involves the accurate identification of postpartum cows with a perturbed uterine involution. After calving, the uterine environment is a dynamic one where tissue involution, and infectious and inflammatory processes occur simultaneously (Chapwanya *et al.*, 2009; Williams *et al.*, 2005). Because most cows experience a bacterial influx postpartum, this can result in excessive inflammation in the absence of adequate regulation in at-risk

cows. A proportion of postpartum cows does not resolve this inflammation, and these cows often develop uterine disease such as endometritis. While clinical assessments frequently occur post calving (Noakes *et al.*, 1994; Sheldon *et al.*, 2009), concurrent laboratory evaluations are rarely performed. Therefore, there is limited information on the mechanisms, particularly at a molecular level, that underlie uterine tissue changes during this period. In this thesis, it is envisaged that an integrated approach to studying innate immunity and histological change on a comprehensive scale in the bovine postpartum uterus will permit identification of, and facilitate ascribing of function to genes that are important for uterine involution during the postpartum period. Identification of novel biomarkers for early and accurate detection of cows at risk of sub-fertility could facilitate the exclusion from breeding programmes of sub-fertile cows.

1.9 Thesis Hypothesis

Local innate immunity is crucial for uterine involution and conception in postpartum cows.

1.9.1 Specific Thesis Aims

- To optimise techniques for investigating bovine uterine clinical, histopathological and immunological status.
- To investigate if the bovine postpartum uterine environment is inflammatory.
- To establish whether local innate immunity mirrors histological change in the postpartum bovine uterus.
- To examine endometrial epithelial specific aspects of inflammation in a monotype cell culture model in response to *E. coli* stimulation.

Chapter 2

Optimisation of techniques for
histopathological and molecular
evaluation of uterine tissue from
postpartum Holstein-Friesian cows

2.1 Introduction

A decline in bovine fertility has become apparent in recent years (Royal *et al.*, 2000) with estimates that only 34 live births occur to first service for every 100 dairy cows in the UK (Dobson *et al.*, 2007). The causes are multifactorial, attributed to nutrition (Caraviello *et al.*, 2006), genetics (Druet *et al.*, 2008; Steine *et al.*, 2008), management (Lawson *et al.*, 2004) and disease (Berends *et al.*, 2008). Almost 20% of cows do not resolve infections that occur during the early postpartum period (Sheldon *et al.*, 2006). This may occur because of unresolved inflammation during involution and warrant study at a molecular level.

Physiological involution is the process of returning the postpartum uterus to a state capable of supporting another pregnancy within 6-8 weeks of calving (Melendez *et al.*, 2004). The events that accompany involution include gross, microscopic and molecular changes, during which the uterus undergoes a series of events including contraction and physical shrinkage, glandular atrophy, necrosis, sloughing and then regeneration of new endometrium (Noakes *et al.*, 1994). The greatest reduction in uterine size in cows with an undisturbed puerperium occurs 10-14 days after calving (Bajcsy *et al.*, 2006).

A common characteristic of involution seems to be bacterial contamination of the uterus (Azawi, 2008; Paisley *et al.*, 1986), that activates the local innate immune response to resolve inflammation and clear infection. Continued inflammation results in the development of uterine disease, which impairs fertility, increases veterinary costs and reduces dairy profitability. Recent studies on uterine health or physiological involution have been based on endocrinological profiling (Etherington *et al.*, 1985) and clinical analysis (Sheldon & Dobson, 2004). Previous studies also reported on the bacteriology and histopathology of postpartum cows (Griffin *et al.*, 1974). Additional objective techniques are needed to accurately identify cows at risk of developing uterine disease from those responding appropriately to infection thus facilitating early breeding after calving (Rodriguez-Sallaberry *et al.*, 2007). The focus of this pilot study was to optimise the techniques for assessing the postpartum uterine tissue, and then combine traditional histopathology and clinical evaluation with molecular gene expression changes at two weeks after calving. The second objective is to build an accurate picture

of the immune status of the postpartum uterus thereby developing a baseline understanding of the immunoregulatory mechanisms that may contribute to resolution of inflammation.

Following infection, an immune response is initiated by TLRs that detect pathogens, and in turn trigger signalling pathways that activate molecules such as NF κ B1 (Janeway *et al.*, 2001a; O'Neill, 2006). NF κ B1 is a crucial transcription factor that controls the induction of a proinflammatory immune response through activation of genes encoding cytokines (Interferon- γ - *IFNG*, Interleukin 1 – *IL-1A*, Interleukin 6 – *IL-6*, Interleukin 12 – *IL-12A*, Tumour necrosis factor – *TNF- α*) and chemokines (Interleukin 8 – *IL-8*) (Bonizzi & Karin, 2004). *IL-8* coordinates recruitment of neutrophils and other innate immune cells to the site of infection. Neutrophils and epithelial cells are primary producers of evolutionary conserved AMPs known to be important in the resolution of infection (Zaiou & Gallo, 2002). A key feature of inflammation in the uterus is the over-production of APPs traditionally thought to be hepatically derived (Sheldon *et al.*, 2001). Combined up-regulation of APP and AMP effector molecules in the uterus may be important for the successful resolution of inflammation and return to a completely involuted state crucial for subsequent conception (Rodriguez-Sallaberry *et al.*, 2007).

To tackle the continuing fertility problems associated with prolonged postpartum uterine infection, understanding the molecular mechanisms controlling the local immune response is crucial. Molecular changes in inflammatory gene expression two weeks postpartum may also identify reliable prognostic indicators of cows that will resolve inflammation and resume cyclicity. Toward this end, in this study, a pilot investigation of a cohort of 13 cows (9 early postpartum, EPP and 4 late postpartum, LPP) was undertaken. Molecular analysis focused on changes in expression of important immune indicator genes including those encoding cell markers, immune sensors TLRs, mediators (chemokines and cytokines) and effector molecules (APPs and AMPs). These genes may hold clues regarding the role of the uterine innate immune response in the resolution of inflammation postpartum.

2.2 Aims

The objectives of the present study were two-fold:

- a. To optimise the techniques for evaluating uterine tissue from postpartum cows;
- b. To investigate whether the postpartum uterine environment is inflammatory.

2.3 Materials and Methods

2.3.1 Cows

Thirteen Holstein-Friesian cows aged 2-8 years from Teagasc Moorepark Research Station (Fermoy, Co. Cork, Ireland) were presented for veterinary examination. Nine had calved 2 weeks previously and were undergoing postpartum uterine involution; these were designated early postpartum (EPP) cows. Four late postpartum (LPP) cows that had calved 9 weeks previously with no clinical signs of uterine disease were selected for reference purposes. Table 2.1 shows the parameters recorded when the individual cows were examined including parity, weight, body condition, time after calving in weeks and calving ease.

2.3.2 Clinical, *per rectum* and ultrasonographic examination

A clinical examination was performed and relevant findings on history, palpation *per rectum*, ultrasonographic imaging and uterine lavage were recorded. The uterus and ovaries were located and cervical diameter estimated *per rectum* (Table 2.1). Manual evaluations were supported by uterine and ovarian imaging using a real-time ultrasound instrument (Aloka SSD-900, UK) with a 5MHz linear array transducer.

2.3.3 Microbial culture

Uterine swabs were obtained using a method previously described (Dohmen *et al.*, 2000; Messier *et al.*, 1984). Briefly, the vulval lips were parted and a guarded sheath advanced into the uterus by cervical manipulation, as if performing AI. A sterile swab

was advanced into the uterus, retracted into a protective sheath and withdrawn. The swab was transferred into sterile tubes containing thioglycolate broth transport medium, stored at room temperature and transported to the laboratory within 24 hours. Swabs were cultured aerobically and anaerobically on Columbia Blood Agar (CBA) base (Oxoid Ltd., Basingstoke, UK; CM0331) with 5% defibrinated sheep blood, CBA with Staph/Strep Selective Supplement (Oxoid SR0070) and MacConkey Agar No. 2 (Oxoid CM0109) and incubated for 72 hours. *Escherichia coli* was identified by colonial morphology and lactose fermentation on MacConkey agar, and confirmed using API 20E (bioMérieux SA).

Table 2.1: Detail for bacteriological culture, clinical, *per rectum*, ultrasonographic, histopathological and cytological examination of each cow analysed.

Cow ID	^a WPP	Parity	^b Wt (kg)	^c BCS	^d T (°C)	^e BC	^j VD	ⁱ CD	^m U/S	^q Histol	^r Concep
1	2	6	728	4.00	38.6	^f <i>E-c</i>	^k Mucop	>7.5cm	ⁿ UF ^o Fo	1	+
2	2	2	612	3.00	38.7	^g nd	Mucoid	<7.5cm	UF, Fo	3	+
3	2	1	546	3.50	38.3	<i>E-c</i>	Bloody	>7.5cm	UF, Fo	2	+
4	2	1	574	3.50	38.9	nd	Mucop	>7.5cm	UF Fo	2	+
5	2	1	540	4.00	38.4	<i>E-c</i>	Mucop	>7.5cm	UF, Fo	2	+
6	2	1	524	3.75	38.3	<i>E-c</i>	Mucop	>7.5cm	UF, Fo	3	+
7	2	1	496	4.25	38.9	nd	Mucop	>7.5cm	UF Fo	1	+
8	2	1	530	4.00	38.7	nd	Mucoid	<7.5cm	UF, Fo	2	+
9	2	4	740	4.25	38.6	nd	Bloody	>7.5cm	UF, Fo	2	+
10	9	2	601	4.00	38.3	^h Mix	None	<5cm	^p CL, Fo	1	-
11	9	4	683	3.25	38.7	ⁱ Nil	None	<5cm	CL, Fo	1	-
12	9	6	742	3.00	38.4	Mix	None	<5cm	CL, Fo	1	-
13	9	6	758	3.25	38.8	Mix	None	<5cm	CL, Fo	1	-

Parity refers to the number of lactations. ^aWeeks postpartum (range 13-15 days for 2 weeks; 63-66 days for 9 weeks). ^bWeight. ^cBody condition score on a scale of 1-5 (Macdonald *et al.*, 2008). ^dTemperature. ^eBacterial culture. ^f*E. coli*. ^gCows not sampled for microbial culture. ^hMixed colonies. ⁱNo colonies. ^jVulval discharge. ^kMucopurulent. ^lCervical diameter. ^mUltrasonography. ⁿUterine fluid. ^oFollicles. ^pCorpus luteum. ^qHistopathology. ^rConception after AI 7-9 weeks postpartum. LPP cows were not bred. Dashed line separates EPP and LPP cows.

2.3.4 Uterine lavage and cytology

Uterine lavage samples were obtained using a previously described method (Brooks, 1993). Briefly, after clinical examination, a clean sleeve was worn and the perineum was wiped with dry paper towel. 10ml phosphate buffered saline (PBS) was drawn into a 50 cm³ syringe and placed on a clean surface. A 30F Foley catheter with an insemination instrument as a stylet was prepared. The Foley catheter was then inserted through the vulva into the vagina. The cervix was identified *per rectum* and the catheter was guided through it into the uterus as if performing AI. The stylet was removed and the syringe containing PBS attached to the catheter. PBS was gently infused into the uterus and negative pressure was then applied to recover fluid. Aspirated fluid was transferred onto labelled glass slides and smears prepared. In the laboratory, smears were fixed with alcohol and stained with Wright-Giemsa. Microscopic evaluation (x400 magnification) of the smears was performed blind of clinical findings. Initially the whole slide was assessed and a representative area was selected to determine the proportion of cells present. Classification was as follows: 0: no evidence of inflammation, mainly endometrial cells (< 5% neutrophils, < 5% lymphocytes, > 30% epithelial cells); 1: mild inflammatory exudates (5 - 20% neutrophils, 5 - 10% lymphocytes, 20 - 30% epithelial cells); 2: moderate inflammatory exudates (20 - 50% neutrophils, 10 - 20% lymphocytes, 10 - 20% epithelial cells); 3: severe suppurative inflammatory exudates, mainly neutrophils (> 50% neutrophils, > 20% lymphocytes, < 10% epithelial cells).

2.3.5 Tissue collection and histopathological assessment

Uterine tissue was obtained from the dairy cows two weeks postpartum using the design shown in Figure 2.1. Uterine biopsies were performed using the Hauptner equine endometrial biopsy instrument using a previously described method (Katagiri & Takahashi, 2004). Briefly, the perineum and external genitalia were cleaned, and the biopsy instrument, covered with a protective sheath, was introduced into the vagina. The instrument was guided into the cervix by manipulation *per rectum*. The instrument alone was introduced into the uterus after rupturing the sheath at the external cervical orifice and guided into the left horn past the uterine bifurcation.

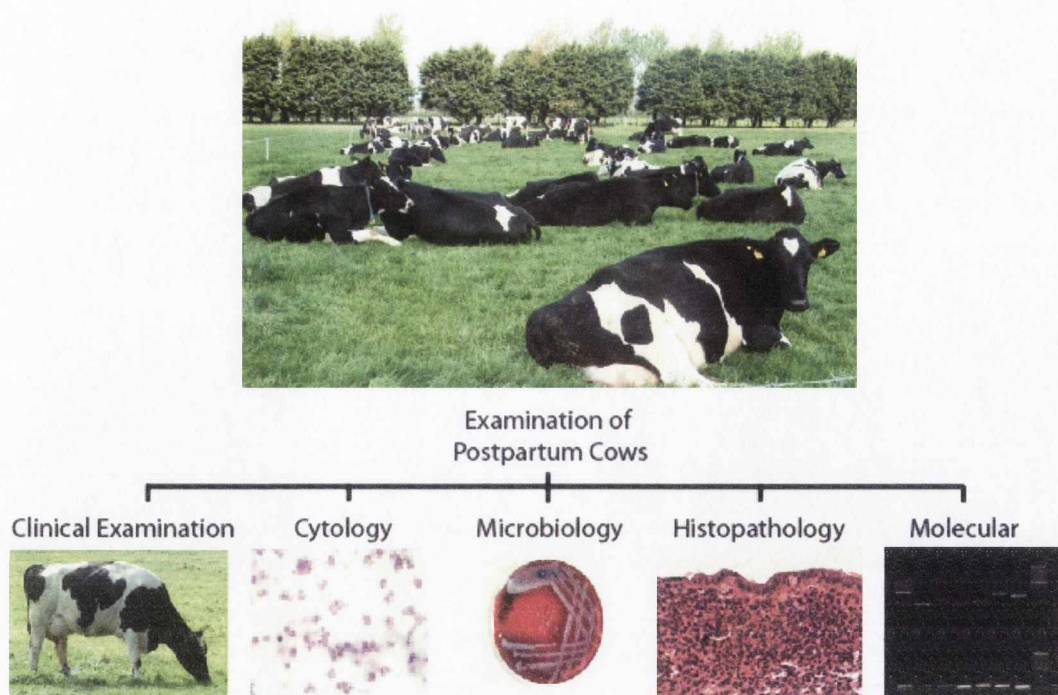


Figure 2.1: Experimental design for sample acquisition from a total of 13 Holstein-Friesian dairy cows at two weeks ($n = 9$) and nine weeks ($n = 4$) after calving. Clinical examination involved physical examination, ultrasonographic imaging and palpation *per rectum*. Cytology smears were prepared from uterine fluid samples obtained using a uterine wash method. Microbial culture was performed using the ‘guarded’ intrauterine swabbing method. The uterine tissues obtained using biopsies were divided into two sections for histopathological and molecular evaluation.

With due care, endometrial tissue measuring 10 x 5 mm was obtained and placed into petri dishes. Tissues were sectioned into 2 pieces and preserved in either liquid nitrogen or 10% formalin saline. Formalin fixed uterine tissues were paraffin-embedded, sectioned at 5-8 μ m thickness and stained with haematoxylin and eosin (H&E). Tissues were graded, independently of clinical findings, based on previously defined criteria (Bonnett *et al.*, 1991a). Histology sections were scored for inflammation as follows: 0: no evidence of inflammation, inert uterine tissue, quiescent; 1: low grade inflammation, infiltration of lymphocytes and plasma cells, few fibrotic areas, cystic glandular degeneration, vascular changes; 2: moderate inflammation showing prominent leukocytes, periglandular or blood vascular fibrosis, few cystic glandular degeneration; 3: severe inflammation, infiltration of polymorphonuclear cells, uterine gland atrophy, cystic degeneration and necrosis, extensive fibrosis of blood vessels, neutrophil and macrophage infiltration of the endometrium, isolated sites of oedema, vascular congestion, haemorrhage, epithelial disruption or loss.

2.3.6 RNA extraction and quality control, cDNA synthesis and quantification

Total RNA was extracted from uterine tissue using the Qiagen RNeasy® method (Qiagen Ltd.) and DNase treated according to the manufacturer's instructions. To ensure integrity and stability of RNA samples, crucial for reliable gene expression analyses, yield and quality were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). The RNA extraction method used produced RNA of high yield and quality (ratios of 18S to 28S ribosomal RNA averaged > 1.75). One μ g of total RNA from each sample was reverse transcribed into cDNA using an OmniScript III® first-strand synthesis kit using oligo (dT) primers according to the manufacturer's recommendations (Invitrogen Ltd, Paisley, UK). cDNA was quantified using a Nanodrop™ spectrophotometer.

2.3.7 Primer design, optimisation and quantitative real time PCR (qRT-PCR)

Twenty-two genes were selected for single gene expression studies by qRT-PCR (Table 2.2). The genes were chosen from the literature and our experience, as key regulators of innate immunity and inflammation. Gene-specific primers for real time qRT-PCR were

designed, intron-spanning where possible, using Primer3 software (Rozen & Skaletsky, 2000) and commercially synthesized (Invitrogen Ltd, Paisley, UK). Primer sequences used in this study are presented in Table A1 and as previously described (Cormican *et al.*, 2008; Eckersall *et al.*, 2006). Each reaction was carried out in a total volume of 25 μ l with 2 μ l of cDNA (40 ng/ μ l), 12.5 μ l 2 $\times$ PCR master mix (Stratagene Corp, La Jolla, CA), and 10.5 μ l primer/H₂O. Optimal primer concentrations were determined by titrating 100, 300 and 900 nM final concentrations and dissociation curves were examined for the presence of a single product. Real time qRT-PCR was performed using an MX3000P® quantitative PCR system (Stratagene Corp, La Jolla, CA) with the following cycling parameters: 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 60°C for 1 min and 72°C for 30 s followed by amplicon dissociation. All reactions were performed in duplicate. A no template control (NTC) was run for each primer used on every 96-well plate.

2.3.8 Selection of Reference (Normaliser) gene: GeNorm

The algorithm programme, GeNorm (Vandesompele *et al.*, 2002) was used to determine the most stable reference genes from a set of tested candidate reference genes in uterine biopsy tissue. GeNorm calculated the gene expression stability measure, M, for a reference gene as the average pair-wise variation, V, for that gene with all other tested reference genes. Stepwise exclusion of the gene with the highest M value allowed ranking of the tested genes according to their expression stability. The most stable reference gene across all uterine samples was selected from a panel of potential normalisers Actin-beta (*ACTB*), Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), Mitochondrial ribosomal protein subunits (*MRPS6*, *MRPS9*, *MRPS15*).

2.3.9 Data analysis and statistics

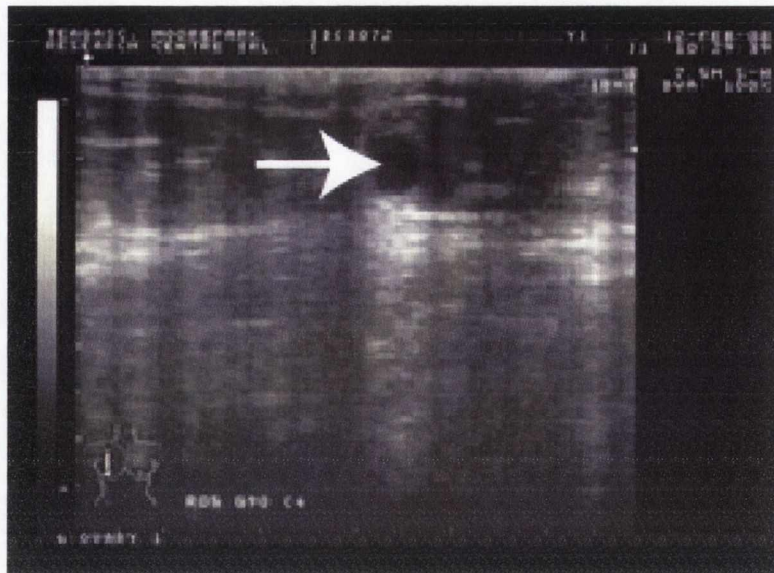
Fold changes for qRT-PCR were calculated using the $2^{-\Delta\Delta C_t}$ method (Schmittgen, 2001; Schmittgen & Livak, 2008) as the expression of the gene of interest in the uterus of each EPP cow, normalized to expression of the normaliser gene (*MRPS15*), and shown relative to the mean of the LPP cow group. Statistical analyses were performed using Mann-Whitney U tests as implemented in version 5.01 of StatView (SAS Institute Inc. Cary, NC, USA) and presented +/- SEM (Standard Error of the Mean).

2.4 Results

2.4.1 Clinical, rectal and ultrasonographic examination

All cows were examined for the presence of vulval discharge, bacteriological culture, intra-uterine fluid, or follicles and findings are summarised in Table 2.1 (on page 51). None of the cows had a history of dystocia or retained foetal membranes. All nine early EPP cows presented with a vulval discharge, five with a mucopurulent, two a bloody and the other two a mucoid discharge. The four LPP reference cows showed no evidence of vulval discharge. Uterine cervical diameter in seven EPP cows measured > 7.5 cm with enlarged and asymmetrical uterine horns. The LPP cows had completely involuted uteri with cervical diameter < 5cm (Table 2.1). No fluid was detected in the uteri of the four LPP cows. Ultrasonographic imaging revealed intrauterine fluid in all nine early EPP cows. There was evidence of follicular development on either one or both ovaries (Figure 2). There were corpora lutea (CL) and follicles on at least one or both ovaries of the LPP cows. All EPP cows were subsequently successfully artificially inseminated 7 - 9 weeks postpartum. The interval from calving to conception for the EPP cows ranged from 56 - 91days.

A



B

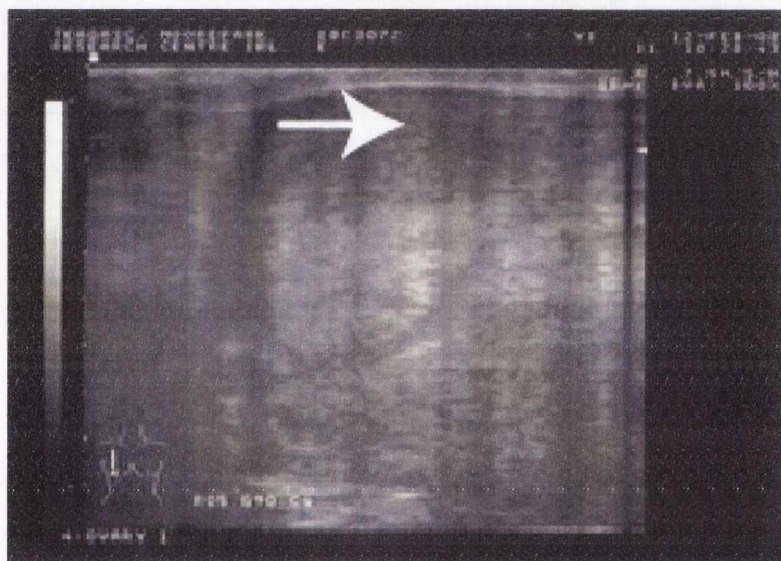


Figure 2.2: Representative ultrasonographic images of an ovary and the uterus obtained from a cow at two weeks postpartum. Developing follicles on the ovary are shown as distinct spherical pockets of fluid-filled cavities (A). Lochia is displayed as echogenic patterns of intrauterine fluid containing cellular particles (B).

2.4.2 Microbial culture

E. coli was the predominant isolate from uteri of the EPP cows (Table 2.1). Other bacterial isolates from the EPP cows included mixed colonies of *Staphylococcus* and *Streptococcus* species. In contrast, no *E. coli* isolates were detected in the LPP cows. Low levels of mixed bacterial colonies were detected from 3 of the 4 LPP cows while the remaining cow had no detectable colonies (Table 2.1).

2.4.3 Histopathology

Histopathological classification of the uterine tissue from each cow (Table 2.1) was based on the inflammatory infiltrate as shown in Figure 2.3. The 4 LPP cows had low grade inflammation in some areas of an otherwise quiescent uterus (Grade 0). This was characterised by an inert uterus devoid of inflammation. Two EPP cows had mild inflammation, characterised by low grade inflammation, little infiltration of lymphocytes and plasma cells with few fibrotic areas, cystic glandular degeneration or vascular changes (Grade 1). Five EPP cows had moderate inflammation, characterized by a diffuse leukocyte infiltration, little periglandular or blood vascular fibrosis with cystic glandular degeneration (Grade 2). Severe inflammation characterised by high infiltration of polymorphonuclear cells, uterine gland atrophy, cystic degeneration and necrosis was seen in 2 EPP cows. There was also extensive fibrosis of blood vessels, neutrophil and macrophage infiltration of the endometrium, isolated sites of oedema, vascular congestion, haemorrhage and areas with epithelial disruption or loss (Grade 3).

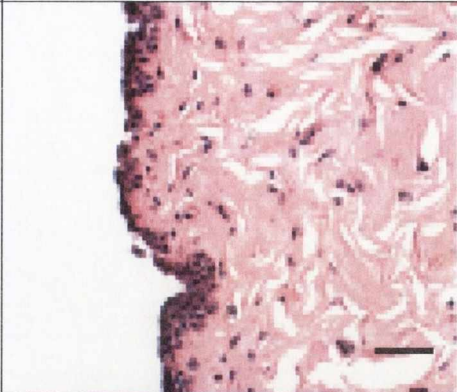
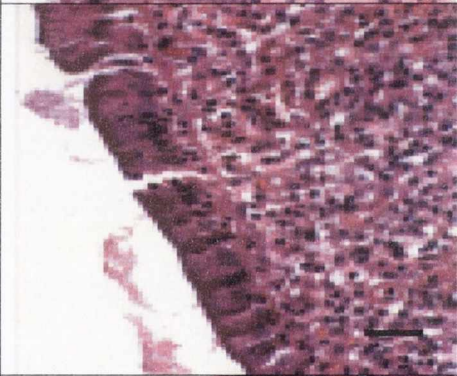
Grade	Histopathology	Characteristics	^a Cows
0		Histology: Inert or quiescent uterus with no inflammation.	4
1		Histology: Minimal inflammation characterised by low-grade lymphocytic infiltration.	2
2		Histology: Moderate inflammation characterised by a prominent lymphocytic-rich infiltration.	5
3		Histology: Severe inflammation characterised by monocyte and neutrophil migration, oedema, vascular congestion and haemorrhage.	2

Figure 2.3: Histopathological analysis of endometrial biopsy sections stain with H&E (magnification x400, Bar = 250 µm) obtained from postpartum cows showing varying degrees of uterine inflammation. Grade 0 is an inert uterus from a cow nine weeks postpartum. Inflammation varied from mild (Grade 1) to moderate (Grade 2) and severe (Grade 3). ^aNumber of cows per grade for both histopathology.

2.4.4 Cytology

Cytological classification of the uterine smears was based on neutrophil, lymphocyte and epithelial cell counts (Figure 2.4). Smears from the 4 LPP cows showed mainly proteinacious material, occasional endometrial epithelial cells, neutrophil count of < 5% and > 30% epithelial cells (Grade 0). Smears from EPP cows revealed pink proteinacious material, smeared nuclear material and lysed cells that lacked cytoplasmic borders. Intact nucleated cells consisted mainly of neutrophils and medium-sized clusters of atypical epithelial cells. Cytological classification was carried out based on both neutrophils and epithelial cell counts. Smears from 2 EPP cows had 5 - 20% neutrophils and 20 - 30% epithelial cells (Grade 1) and considered mild. Three EPP cows had a moderate response consisting 20 - 50% neutrophils and 10 - 20% epithelial cells (Grade 2) while the other 4 had a severe response with > 50% neutrophils but < 10% epithelial cells (Grade 3).

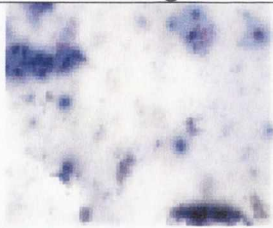
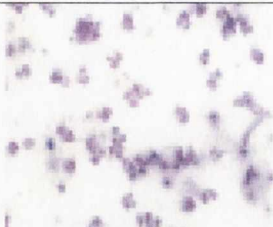
Grade	Image	Features	Cows (n)
0	 A	Proteinacious material, > 30% epithelial cells and < 5% neutrophils	4
1	 B	Mild exudates, 20 - 30% epithelial cells and 5 - 20% neutrophils	2
2	 C	Moderate exudates, 10 - 20% epithelial cells and 20-50% neutrophils	3
3	 D	Severe suppurative exudates. Mainly neutrophils (> 50% neutrophils) and < 10% epithelial cells.	4

Figure 2.4: Cytology analysis of smears (uterine wash) obtained from postpartum cows. Initially the whole slide was assessed and a representative area was selected to determine the cellular content. ^aNumber of cows per grade for cytology.

2.4.5 Normaliser gene selection

Gene expression patterns for the potential normaliser genes are shown. *MRPS15* and *GAPDH* were the two most stable genes (Figure 2.5). *MRPS15* was selected on the basis of primer efficiency. All gene amplifications were then normalised to *MRPS15* for qRT-PCR analysis.

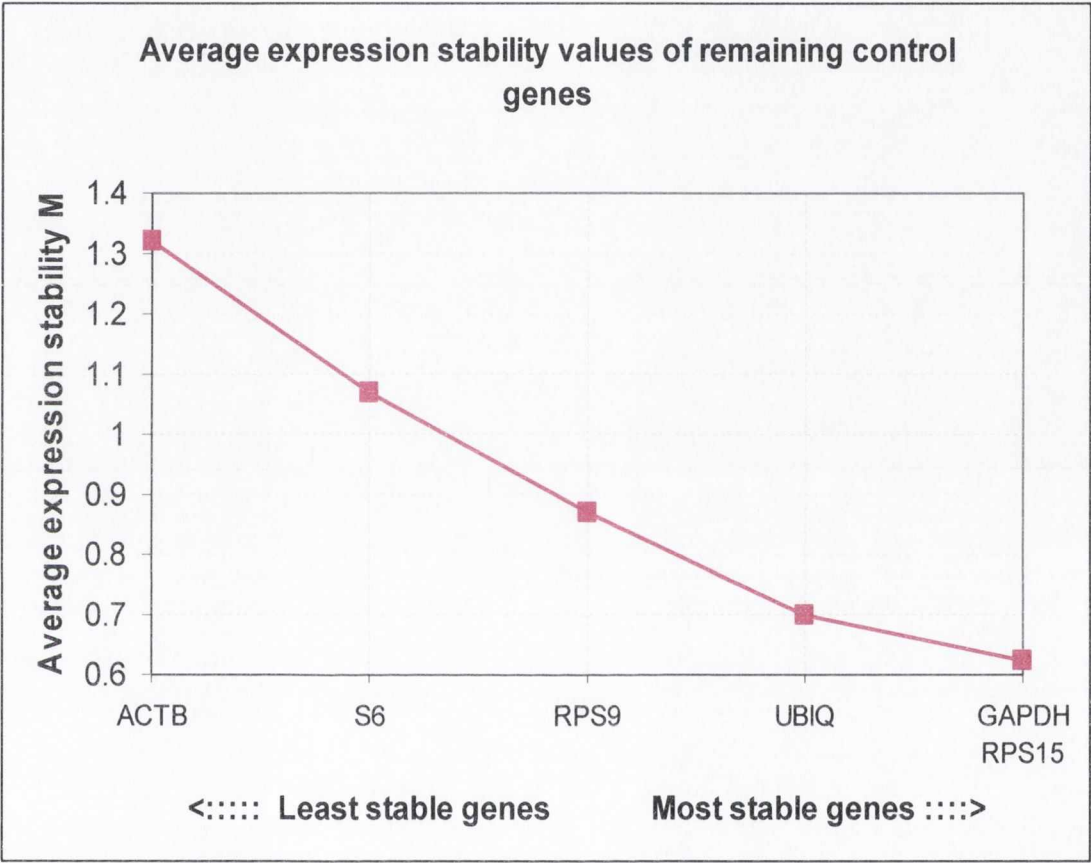


Figure 2.5: GeNorm calculation showing *MRPS15* and *GAPDH* as the two most stable normaliser genes in uterine tissues. Gene expression stability measure for the reference gene is determined as the average pair-wise variation, for that gene with all other tested reference genes. *MRPS15* was selected on the basis of superior PCR efficiency.

Table 2.2: Innate immune gene expression profiles in the uterus of early postpartum (EPP) cows shown relative to late postpartum (LPP) cows

[§]Base pairs. [¥]Genes not significantly differentially expressed.

Gene Class	Gene Symbol	Amplicon size ([§] bp)	Mean fold change	P value	Change in expression
Cell markers	<i>CD45</i>	845	5	0.01	Increase
	<i>CD14</i>	600	2	0.01	Increase
Sensors	<i>TLR2</i>	292	-0.1	0.83	[¥] n/s
	<i>TLR4</i>	452	6	0.01	Increase
	<i>TLR5</i>	417	3	0.01	Increase
	<i>TLR6</i>	214	3	0.01	Increase
	<i>TLR10</i>	378	4	0.01	Increase
	<i>NFKB1</i>	131	6	0.03	Increase
Mediators	<i>IL-1A</i>	120	3	0.04	Increase
	<i>IL-2</i>	120	0.7	0.82	n/s
	<i>IL-6</i>	120	3	0.01	Increase
	<i>IL-8</i>	120	4	0.01	Increase
	<i>IL-12A</i>	120	10	0.01	Increase
	<i>IFNG</i>	125	2	0.03	Increase
	<i>TNF</i>	120	-55	0.01	Decrease
	<i>CRP</i>	827	1.8	0.40	n/s
Effectors	<i>HP</i>	1374	3	0.01	Increase
	<i>SAA3</i>	601	16	0.01	Increase
	<i>BNBD4</i>	278	-4.5	0.83	n/s
	<i>DEFB5</i>	143	53	0.01	Increase
	<i>LAP</i>	273	9	0.40	n/s
	<i>TAP</i>	134	97	0.01	Increase

2.4.6 Analysis of genes encoding cell surface markers using qRT-PCR

Expression of *CD45*, also known as protein tyrosine phosphatase receptor type C and a common marker for hematopoietic cells was increased significantly in EPP cows by 5 fold ($P < 0.05$; Table 2.2). Similarly, expression of *CD14*, a marker of myeloid cells was also significantly increased (2 fold) in EPP cows compared to LPP cows ($P < 0.05$).

2.4.7 Novel immune gene expression indicative of a proinflammatory postpartum uterine environment

Of the 5 TLR genes examined by qRT-PCR, 4 were significantly increased in expression in EPP cows relative to reference LPP cows - *TLR4* (6 fold), *TLR5* (3 fold), *TLR6* (3 fold) and *TLR10* (4 fold) ($P < 0.05$; Table 2.2). *TLR2* was not significantly differentially expressed.

NFkB1 gene expression was significantly increased by 6 fold in EPP cows ($P < 0.05$). The gene encoding the chemokine *IL-8* was also differentially expressed, showing a significant increase in the EPP cows (4 fold; $P < 0.05$). The expression of genes encoding Interleukin 1, a major proinflammatory cytokine, Interleukin 6, which drives the production of APPs (Andus *et al.*, 1988), and Interleukin 12, which modulates the expression of Interferon- γ (Collins *et al.*, 1999), were also examined. Gene expression of these cytokines was significantly increased – *IL-1A* (3 fold), *IL-6* (3 fold) and *IL-12A* (10 fold) in EPP cows ($P < 0.05$, Table 2.2). Also significantly differentially expressed was the *IFNG* gene, showing a 2 fold increase.

In contrast, expression of the cytokine - *TNF* gene was significantly reduced in all EPP cows by 55 fold ($P < 0.05$) relative to the LPP group. *IL-2* gene expression was not differentially expressed.

2.4.8 Analysis of APP and AMP gene expression during the early postpartum period

Four AMP genes *LAP*, *TAP*; *BNBD4* and *DEFB5* and 3 APP genes *HP*, *SAA3* and *CRP* were examined for changes in expression in EPP cows relative to LPP cows. Expression

of 2 of the 3 APP genes - *SAA3* and *HP* were significantly increased 16 and 3 fold in the EPP cows respectively ($P < 0.05$; Table 2.2). Expression of the *CRP* gene was not significantly changed. *TAP* and *DEFB5* genes were highly significantly differentially expressed between the two groups, with increase in expression of 97 and 53 fold in the EPP cows respectively ($P < 0.05$; Table 2.2). *LAP* and *BNBD4* gene expression were not significantly different.

2.4.9 Gene expression and severity of uterine inflammation

It was an important aim of this study to examine the relationship between histopathological classifications with changes detected at a molecular level in uterine tissue. Each animal had been blindly classified into one of three histology grades from 1 (mild inflammation) to 3 (severe inflammation). Of the 22 genes examined by qRT-PCR, increased expression for 16 genes was detected in cows with uterine inflammation (Table 2.2). A 10 fold increase in *CD45* gene expression indicated an increase in leukocytes in inflamed uteri of EPP cows during the early postpartum period (Figure 2.6a). Gene expression of the TLRs - *TLR4*, *TLR6* and *TLR10* - mirrored severity of uterine inflammation grading based on histopathology. All 3 increased consistently from grade 1 to grade 3 in EPP cows. *TLR4* and *TLR10* gene expression showed the most pronounced change, with a 4 and 5 times increase, respectively in grade 3 over grade 1 EPP cows (Figure 2.6a).

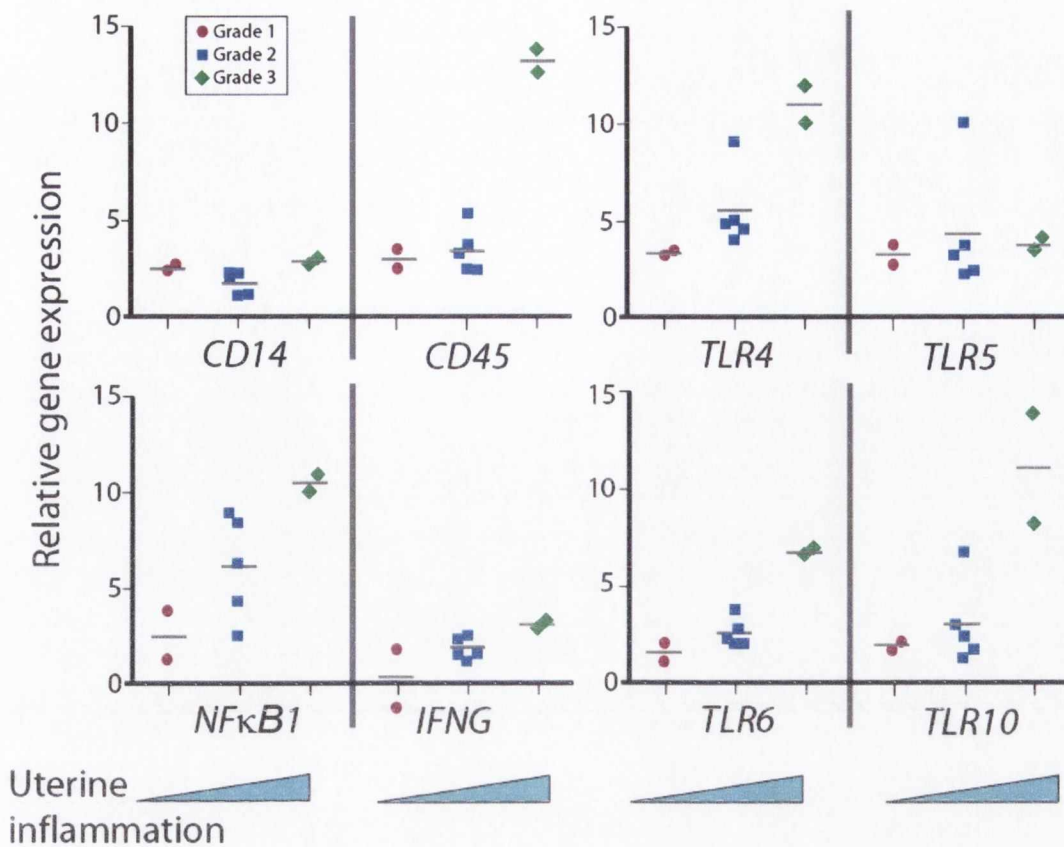


Figure 2.6A: Uterine innate immune gene expression in early postpartum (EPP) relative to late postpartum (LPP) cows. Results are shown for genes encoding cell surface marker and sensor, mediator and molecules of innate immunity.

In relation to proinflammatory immune genes, a close relationship between the level of gene expression and severity of uterine inflammation was also evident. Increased expression of the transcription factor *NFkB1*, cytokines *IFNG*, *IL-6*, *IL-8* and *IL-12A* was observed across grades of inflammation (Figure 2.6A & 2.6C). *IL-12A* displayed the most significant increase in expression with a 25 fold increase in grade 3 EPP cows over grade 1 ($P < 0.05$). In contrast, a significant decrease in *TNF- α* expression by 150 fold in grade 3 EPP cows, showed an inverse relationship between gene expression and severity of uterine pathology (Figure 2.6B).

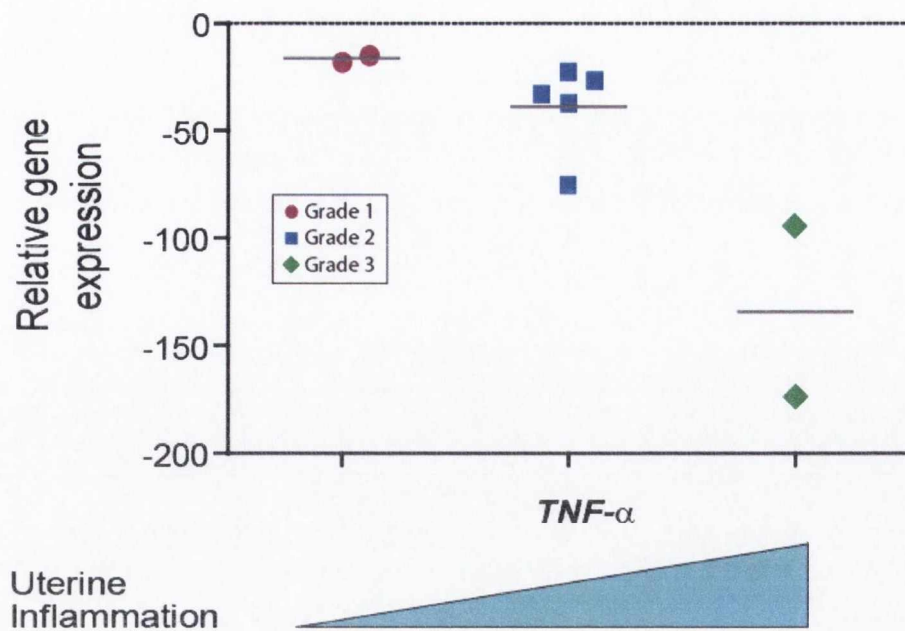


Figure 2.6B: The expression of $TNF-\alpha$ was inversely proportional to the severity of uterine inflammation in early postpartum (EPP) relative to late postpartum (LPP) cows.

Expression of genes encoding the APPs - *SAA3* and *HP* - also mirrored the severity of uterine inflammation (Figure 2.6C). Although the fold change in gene expression for *HP* was low, *SAA3* expression increased to 38 fold in grade 3 EPP cows. The expression of both AMPs - *TAP* and *DEFB5* - was significantly induced at high levels in EPP cows with higher grade uterine inflammation. *TAP* gene expression was 3 times higher in grade 3 compared to grade 1 EPP cows. Expression of *DEFB5* gene was similarly 3 times higher in grade 3 versus grade 2 EPP cows, showing a direct link with severity of pathology (Figure 2.6C).

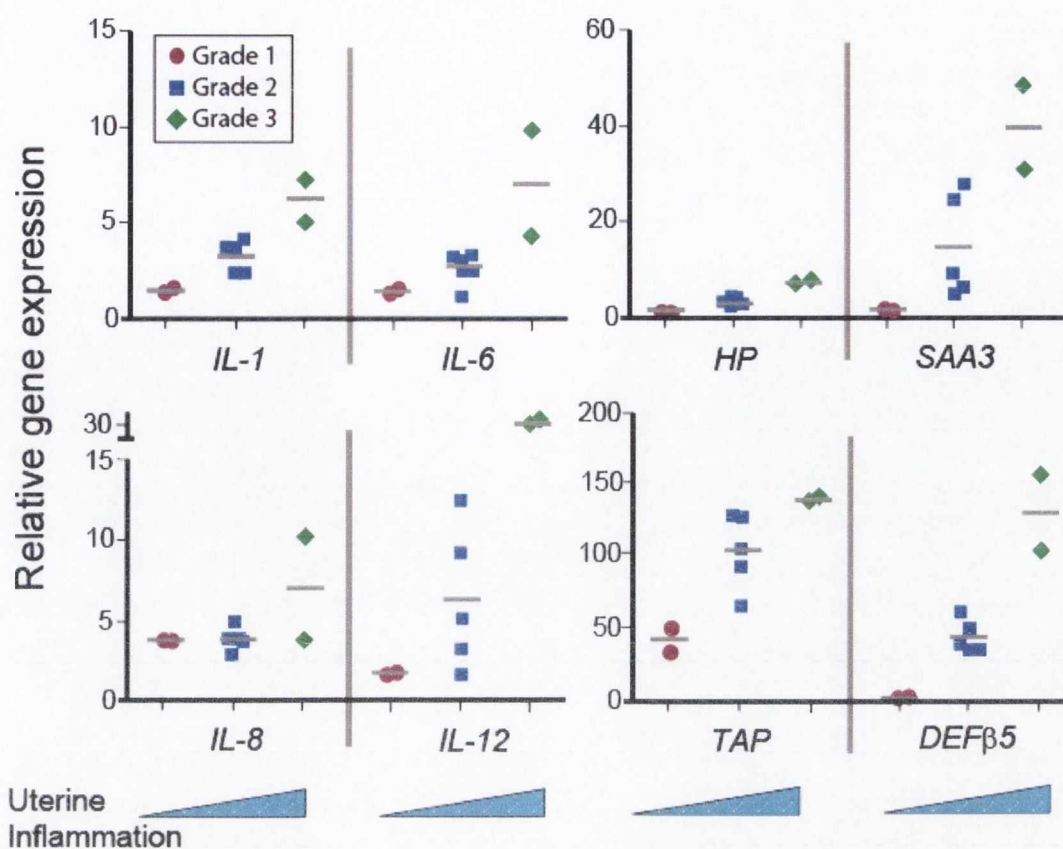


Figure 2.6C: Uterine innate immune gene expression in early postpartum (EPP) relative to late postpartum (LPP) cows varies with severity of inflammation within each of the 3 histopathology grades. Results are shown for genes encoding mediator and effector molecules of innate immunity.

2.5 Discussion

In fertile cows, uterine involution is usually completed within 6-8 weeks of calving thus facilitating conception. Although the specific mechanisms controlling uterine involution remain unclear, endocrinological and physical events in the early postpartum period have been documented (Etherington *et al.*, 1985; Llewellyn *et al.*, 2008) with major changes occurring around 2 weeks postpartum (Noakes *et al.*, 1994). Clinical tools are routinely used for diagnosis in the field, without appreciation of the molecular mechanisms underlying changes at a cellular or indeed systemic level. The objective of this study was to characterise the local innate immune response of the uterus during physiological involution, and to examine the relationship between histopathology and molecular markers of inflammation. To tackle the continuing fertility problems associated with prolonged postpartum uterine inflammation, understanding the molecular mechanisms regulating the local immune response is crucial.

The capacity of the uterus to resolve an infection depends on its ability to detect and respond to microbial ligands. *E. coli* and *Arcanobacterium pyogenes* were predominant pathogens previously isolated from clinical cases of uterine disease (Williams *et al.*, 2005) and are thought to be the primary causes of infertility (Foldi *et al.*, 2006). Similarly, reports have suggested that cows with *E. coli* infection 10-21 days after calving run the risk of developing uterine disease (Foldi *et al.*, 2006). In this study, *E. coli* was identified as the dominant isolate from 4 EPP cows with confirmed uterine pathology.

Histopathological examination of biopsy sections provides accurate and reliable characterisation of the uterine immune environment (Moraitis *et al.*, 2004), and as such, was used to classify uterine biopsy samples from each cow. The blind classification identified three classes of early postpartum (EPP) cows, graded on the basis of the severity of uterine inflammation. Two cows were Grade 1 (mild), 5 cows Grade 2 (moderate) and 2 cows Grade 3 (severe inflammation). Relative gene expression analysis was then performed for a range of common innate immune markers between EPP and LPP cows. In the present study, the histopathological and molecular characterization of the early postpartum uterus reveals evidence of an inflammatory uterine environment early postpartum.

Importantly, histopathological changes are always preceded and underpinned by changes at a molecular level (Greenfield *et al.*, 2000). Increased relative expression of genes encoding cell-surface receptor markers *CD45* and *CD14* indicates cell recruitment to the postpartum uterus of EPP cows during involution. The increased expression mirrored severity of uterine inflammation. *CD45* is primarily expressed by neutrophils, and increased *CD45* gene expression in the EPP cows indicates an influx of leukocytes, a necessary step to combat the bacterial infection at calving. Coupled with cytological findings in these cows, these data suggests that neutrophils are the primary innate immune cell responding to infection early postpartum. The expression of *CD14* was not significantly different between histopathology grades, indicating that increased monocyte influx is not a characteristic of uterine inflammation in these animals.

TLR expression in uterine cells has been previously demonstrated *in vitro*, where stimulated bovine endometrial and stromal cells expressed *TLR4* necessary for recognition of *E. coli* or LPS ligand (Herath *et al.*, 2006a). In this study, increased expression of *NF-κB1*, and inflammatory cytokines, suggests that the predominant immune response in early postpartum cows is proinflammatory, and that this response is TLR-NFκB mediated. IFN-γ is a main driving cytokine of a proinflammatory immune response. It activates macrophage and T-lymphocyte proliferation and antigen-presenting cell stimulation (Karpuzoglu-Sahin *et al.*, 2001), all of which function to eliminate pathogens. The expression of *IL-6*, another proinflammatory cytokine, which regulates the synthesis of APPs crucial for the acute phase response to infection (Ishikawa *et al.*, 2004) was enhanced locally in the uterus two weeks after calving. Expression of *IL-1A* and *IL-12A* genes was also increased with severity of uterine pathology, suggesting that these cytokines play a key role in inflammation during uterine involution. *IL-12A* controls T-lymphocyte differentiation, a step necessary for efficient elimination of pathogens. Gene expression of these mediators increased significantly with severity of inflammation.

Interestingly, levels of *TNF-α*, another important inflammatory cytokine, were significantly decreased in cows with uterine pathology. Classically, in humans, *TNF-α* is increased in a range of inflammatory conditions where it is an immunotherapeutic target (Danese *et al.*, 2006). So its decreased expression in postpartum cows warrants further investigation. Diminished *TNF-α* gene expression was also detected in the early

stages of inflammation in bovine mammary gland (Yang *et al.*, 2008). Increased expression of *TNF- α* has been described in endometrial and stromal cells stimulated *in vitro* with the *TLR4* ligand LPS (Herath *et al.*, 2006b), indicating important differences between *in vivo* and *in vitro* studies of uterine cellular responses. It is likely that the downregulation of *TNF- α* may facilitate rapid resolution of inflammation in postpartum cows.

Even though *TNF- α* gene expression was decreased in the uterus, there was considerable expression of APP genes *HP* and *SAA3* in uterine tissue. APPs and AMPs are effector molecules involved in clearing pathogenic infection in a range of disease conditions. Although expression of APPs in the uterus has been previously analyzed (Sheldon *et al.*, 2001), this study presents the first report of expression of AMPs in the local uterine immune response in the early postpartum period. Increased expression of the *TAP* and *DEFB5* genes varied directly with severity of uterine inflammation. Because of direct antimicrobial activity and immune regulatory potential, AMPs are thought to be important for control and clearance of infection (Fjell *et al.*, 2008; Jenssen *et al.*, 2006). Increased expression of *DEFB5* (Goldammer *et al.*, 2004) and *LAP* (Swanson *et al.*, 2004) has been described in mammary tissue during mastitis. Abundant *TAP* message in the present study points towards a specific and inducible expression during involution. Increased expression of specific genes encoding AMPs in the uterus suggests that antimicrobial peptides are integral components of local innate immunity (Roosen *et al.*, 2004) and may be considered candidate genes for bovine uterine disease resistance.

Given that the cost of uterine disease continues to increase while fertility declines, understanding molecular changes that underpin inflammatory responses and their resolution during uterine involution is critical to unlocking the local uterine innate immune mechanisms. The elevated expression of genes encoding toll-like receptors (*TLR4*), inflammatory mediators (*NF- κ B1*, *IL-8*, *IL-1A*, *IL-6*, *IL-12A*) and the effector molecules (APPs, AMPs) during the postpartum period is indicative of local uterine inflammation response. There is a need, therefore, to study the temporal innate immunity and associated histological changes in recently calved cows to identify involutionary responses beneficial for conception.

Chapter 3

Investigation of local innate immunity
and histological change during uterine
involution in postpartum cows:
relevance to fertility?

3.1 Introduction

Infectious bovine endometritis is the most common reproductive disease of postpartum cows. Endometritis is particularly important in cows not only because of financial penalty, but also because of detrimental effects on herd reproductive performance (Foldi *et al.*, 2006). The endometrium undergoes marked morphological and functional changes regulated by the hormones progesterone, oestradiol, and oxytocin (Spencer *et al.*, 2004) during involution. Clearly these events are underpinned by changes at a molecular level (Greenfield *et al.*, 2000). Cows, also characteristically, experience an influx of pathogens in the immediate post-calving period. Among the many genes differentially expressed during this period are immune genes important for clearance of infection and resolution of inflammation.

To study the important postpartum events such as clearance of infection, resolution of inflammation or resumption of oestrus cyclicity at the mRNA level, the endometrial transcriptome was interrogated using a combination of histology and qRT-PCR analysis. Histological assessment was performed to identify and differentiate the cellular composition of the tissue under microscopy while qRT-PCR was used to identify temporal differential expression changes of innate immune genes in the same animal.

In humans, there are reports of studies comparing gene expression in the endometrium during the menstrual cycle (Horcajadas *et al.*, 2007; Tanaka & Umesaki, 2003). Similarly, in bovine, the endometrium transcriptome was interrogated during the oestrous cycle and also during pregnancy (Bauersachs *et al.*, 2005). However, there are no comparable studies so far in cows during the postpartum period focussing on the local uterine innate immune response. Such information may allow the development of better strategies for combating uterine infection and resolving inflammation, the important steps for reversing the declining trends in bovine fertility. In addition, novel traits for targeting during genetic selection of cattle of superior fertility for breeding may be identified.

3.1.1 Integrated approach to study of innate immunity and histological change in uterine tissues from postpartum cows

The techniques currently applied in clinical veterinary practice for the diagnosis of uterine disease in postpartum cows are clinical examination, microbiological culture and cytological assessment. In order to reverse the decline in cattle fertility, there is a requirement for other technologies to assess cows after calving. Understanding uterine innate immunity and associated histological change would facilitate accurate diagnosis and treatment of metritis and endometritis, as well as develop biomarkers for use in cattle breeding programmes. The experimental work in this chapter focuses on an integrated approach to the study of postpartum uterine temporal events in cows (Figure 3.1), with particular emphasis on histological assessment and qRT-PCR analysis techniques, which are described in detail here.

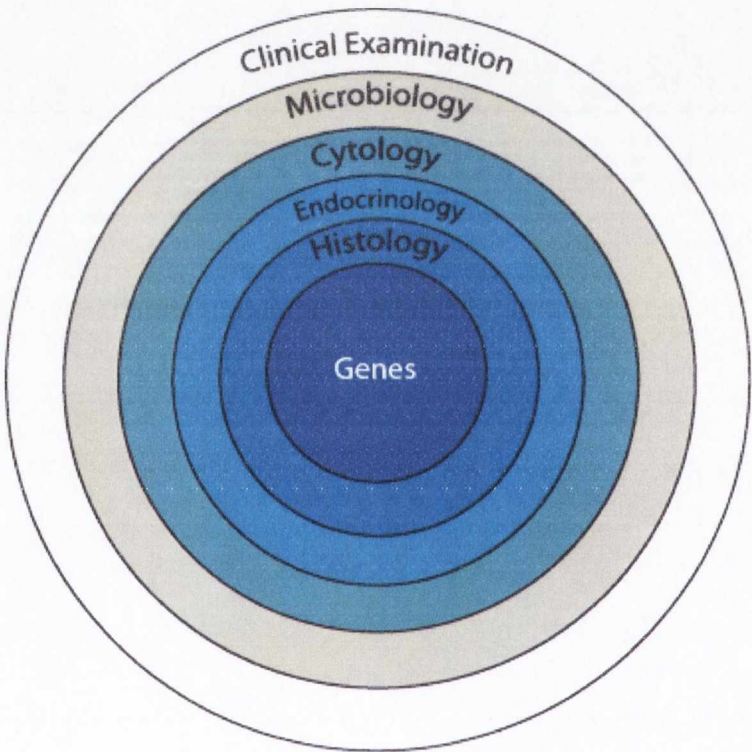


Figure 3.1: Integrated approach to studying innate immunity and histological change in the bovine postpartum uterus employed in the present study. Histological assessment and qRT-PCR analysis were performed in the same tissue acquired by uterine biopsy and divided into two sections from each animal at 15, 30 and 60 days postpartum (DPP).

3.1.2 Histology

Histology is the study of the microscopic appearance of cells and tissues of animals. It is routinely performed by examining cells or thin sections of tissue under a light microscope, although electron microscopes are now available. The visualisation or differential identification of the microscopic structures of tissues or cells, is aided by the use of a number of histological stains to provide contrast (Lynch *et al.*, 1969).

In order to perform a histological assessment, chemical fixatives are used to preserve tissue from degradation, and to maintain the structure of the cells. The most commonly used fixative for light microscopy is 10% neutral buffered formalin. In addition, tissues are commonly rapidly cryo-preserved in compounds such as liquid nitrogen, where the frozen tissue is later sectioned using a microtome.

3.1.2.1 Processing of tissue for histology

3.1.2.1.1 Dehydration and embedding

In order to obtain sufficiently thin sections (4 – 8 μm) for microscopic assessment, tissues are supported in a hard matrix. For light microscopy, paraffin wax is the matrix of choice used. For dehydration, tissues are transferred through baths of progressively more concentrated ethanol to remove the water, followed by a clearing agent, usually xylene to remove the alcohol.

After the tissues have been dehydrated, they are embedded in paraffin wax. Formalin-fixed, paraffin-embedded (FFPE) tissues may be stored indefinitely at room temperature, and DNA can be extracted, it is often of a poor quality, depending on the length of time the samples remain in formalin prior to embedding (Baloglu *et al.*, 2008; van Maldegem *et al.*, 2008). The amount of RNA extracted from the FFPE tissues is usually small due to time-dependent fragmentation caused by length of stay in formalin (Ciotti *et al.*, 2009). FFPE tissues are sectioned at 4 - 8 μm for light microscopy evaluation using microtomes.

3.1.2.1.2 Staining

Staining is employed to provide both a background for contrast to the tissue, as well as highlighting particular features of interest. The most commonly used stain for light microscopy is haematoxylin and eosin (H&E). The oxidation product of haematoxylin, haematin, is the active ingredient in the staining solution. Haematin exhibits indicator-like properties, being blue and less soluble in aqueous alkaline conditions, and red and more soluble in alcoholic acidic conditions (Lynch *et al.*, 1969). The haematin demonstrates cell nuclei. Full cellular detail is obtained by counterstaining with the eosin solution. Figure 3.2 shows a histological section of uterine tissue stained with H&E.

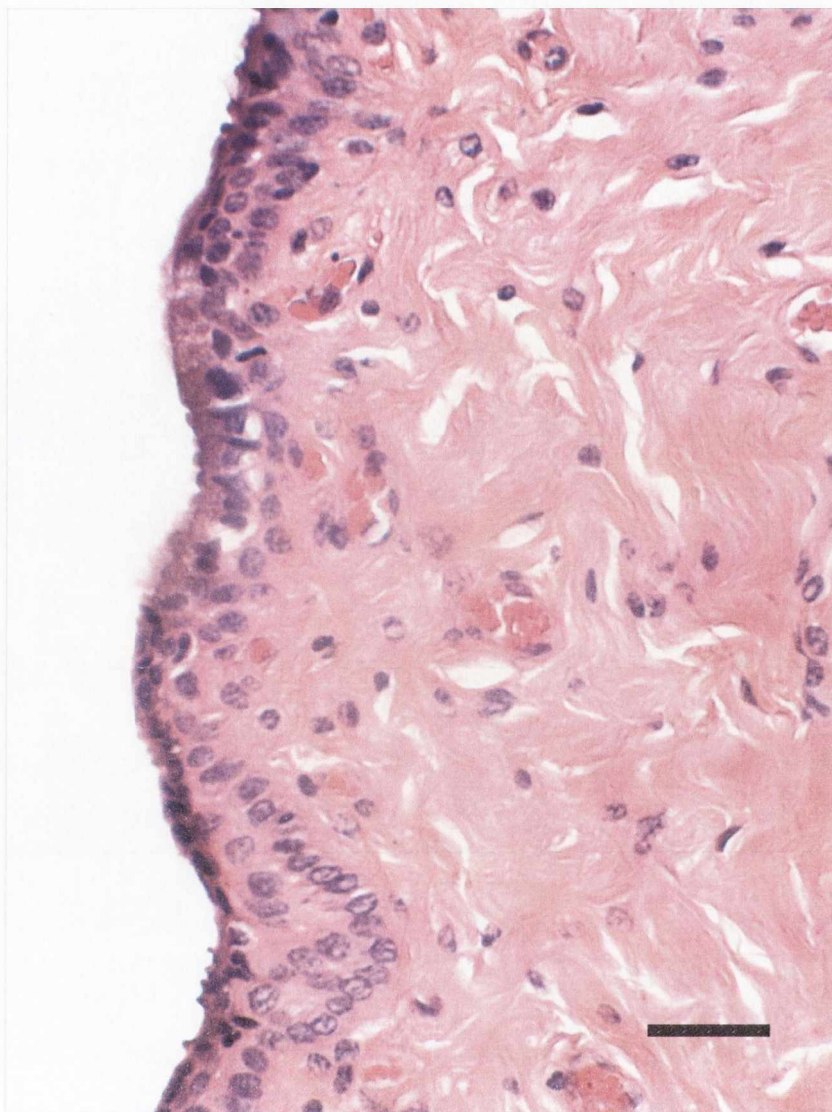


Figure 3.2: Histological section of uterine tissue obtained from a cow 60 DPP and stained by H&E (magnification x400, Bar = 250 μ m). Endometrial epithelial cells are clearly visible in a quiescent uterine tissue devoid of inflammation.

3.1.3 Quantitative real time polymerase chain reaction PCR (qRT-PCR)

qRT-PCR is a state-of-the-art technique first described in 1996 which can be employed to investigate specific molecular changes in gene expression (Gibson *et al.*, 1996). Although similar to conventional PCR in principle, the fundamental difference is that the end-product in conventional PCR is usually fractionated on an agarose gel to visualise the DNA and confirm amplification in a non-quantitative manner (Saiki *et al.*, 1985). Unlike conventional PCR, qRT-PCR allows monitoring of the PCR amplicons in real-time during the reaction and can be used to estimate the mRNA transcript abundance for a particular gene. In addition, quantification is an absolute estimation of the actual original copy number of any gene transcripts (Bustin, 2000). Comparisons of mRNA between two or more samples are possible (Bustin *et al.*, 2005).

PCR amplification is monitored by recording fluorescence of non-specific DNA intercalating dyes such as SYBR green or target reporter *Taqman*® probes which identify individual amplified sequences (Vet & Marras, 2005). To carry out a qRT-PCR study, RNA from the experimental samples is reverse-transcribed to produce complementary DNA (cDNA). Then a fluorescent dye, such as SYBR green, which binds specifically to double-stranded DNA, is used. The quantity of fluorescence is proportional to DNA abundance as the amplicon copy number increases with each round of PCR amplification completed (Wilhelm & Pingoud, 2003).

If using the Light Cycler PCR system from Roche Molecular Biochemicals, the melting curves can be analysed and characterised with respect to the melting temperature (T_m), which is a function of the product length and nucleotide composition. The T_m of the amplified DNA can also be used to detect non-specific DNA contamination which can introduce error in relative gene expression comparisons (Bustin, 2000).

Relative gene expression comparisons require a sensitive and precise measurement of specific mRNA sequences using a reference or 'normaliser' gene, which is a constitutive gene transcribed at a relatively constant level in all the experimental samples (Dheda *et al.*, 2004; Radonic *et al.*, 2004). The normaliser is then used as a calibrator for the expression of genes of interest (Livak & Schmittgen, 2001). For the analysis, relative gene expression is calculated by comparing Ct ratio for each group,

where the Ct values are estimates of cycle number at which amplification reaction reaches the log phase of the PCR reaction. An example of the plot of amplification from two uterine tissues is shown in Figure 3.3. A dissociating or melting curve (Figure 3.4) is also analysed at the end of the assay to ensure that only a single PCR is formed. The dissociation curve is also used to ascertain that the PCR product formed melts continuously within the same temperature range for all experimental samples.

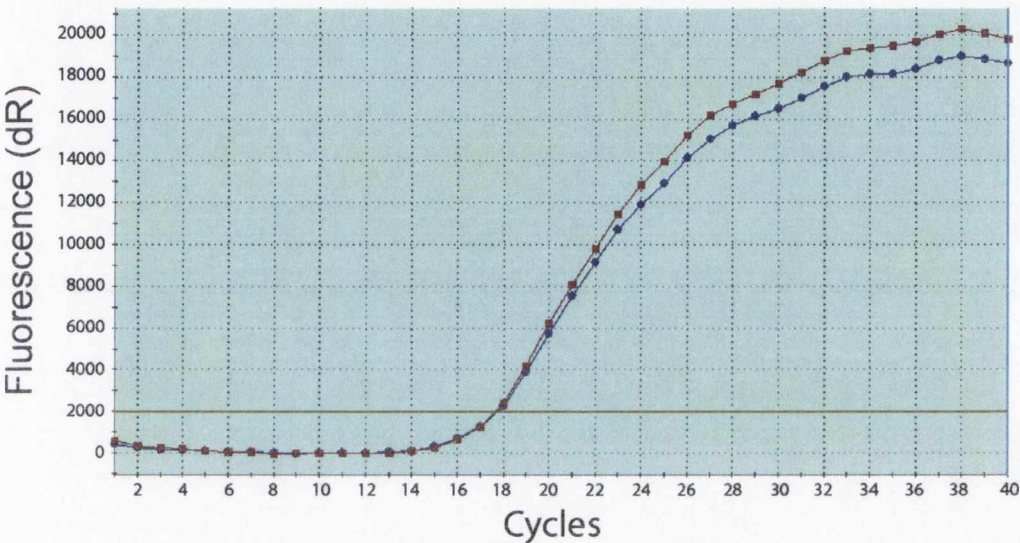


Figure 3.3: Gene amplification plots in real-time for duplicate samples using qRT-PCR machine. The comparison values (Ct) are recorded during the log phase of the amplification cycle when the amount of gene transcripts exceeds the set cycle threshold.

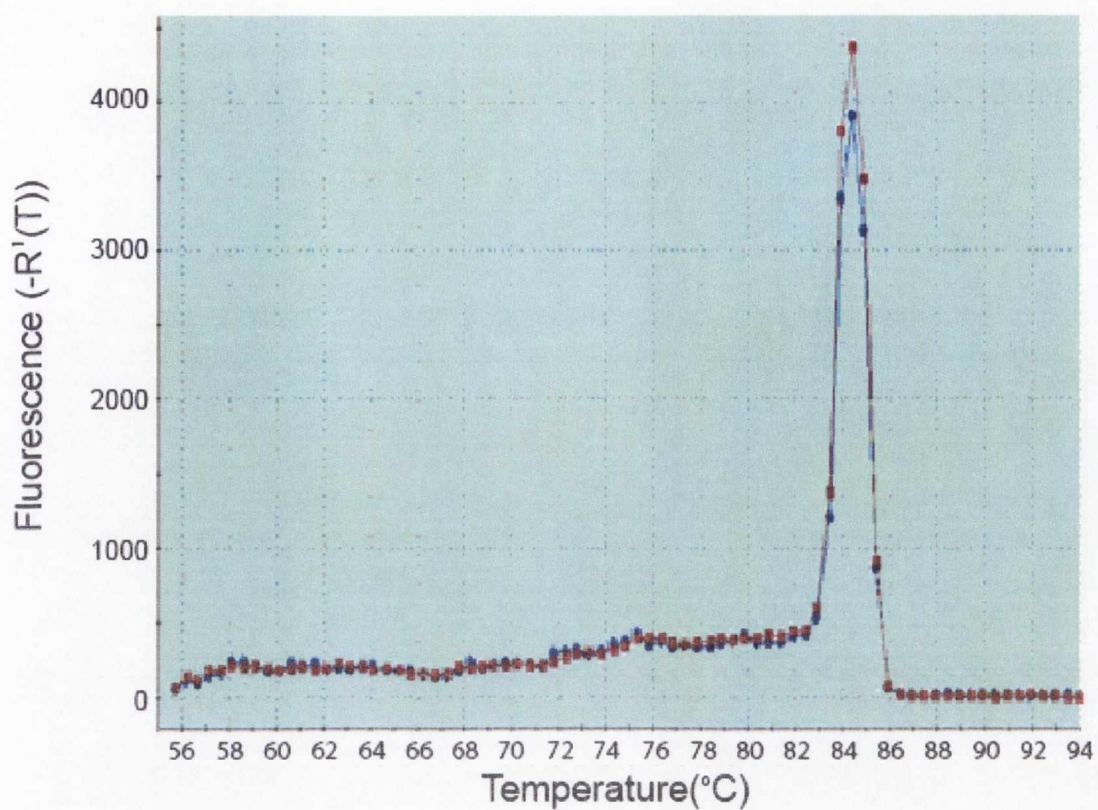


Figure 3.4: Dissociation curve showing the melting of gene amplicons in uterine tissue. The single amplification peak indicates the formation of a single PCR product in the duplicate wells ruling out non-specific amplification errors.

In the present study, histology and qRT-PCR approaches were used to analyse microscopic change and differential gene expression in endometrial tissue obtained from postpartum cows, respectively. The tissues were acquired at three important time points after calving – 15 DPP (clearance of infection), 30 DPP (resolution of inflammation and resumption of oestrus cyclicity) and 60 DPP (completion of microscopic uterine involution when the uterus is prepared for another pregnancy).

3.2 Aims

The objectives of this study were three-fold:

- a.** To characterise the endometrial transcriptome during the postpartum period using histology and qRT-PCR
- b.** To examine differential expression of novel and known innate immune uterine-specific genes (e.g AMP, APP) from postpartum cows
- c.** To determine if histological change mirrors local innate immunity in the postpartum bovine uterus

3.3 Methods and Materials

3.3.1 Cows

A total of 13 first parity mixed-bred cows that had been synchronised for artificial insemination 9 months previously were presented for veterinary examination 15, 30 and 60 days postpartum. For the in-depth study, the 13 cows were selected based on the fact that they had not been subjected to nutritional restriction at any point. The cows included Charolais (n = 9), Simmental (n = 2), Belgian Blue (n = 1) and Holstein-Friesian (n = 1) cross-breds from a beef herd at Lyons Research Farm, University College Dublin. The average age of the cows was 31 months (range 27 - 32 months) with a mean body condition score of 3 on a scale of 1 – 5 (Macdonald *et al.*, 2005) suckling one calf each. No calving assistance was required. All calves were viable following normal calving and no cows had retained foetal membranes. No cow received intrauterine antibiotic therapy during this study, which was approved by the ethics committee of University College Dublin.

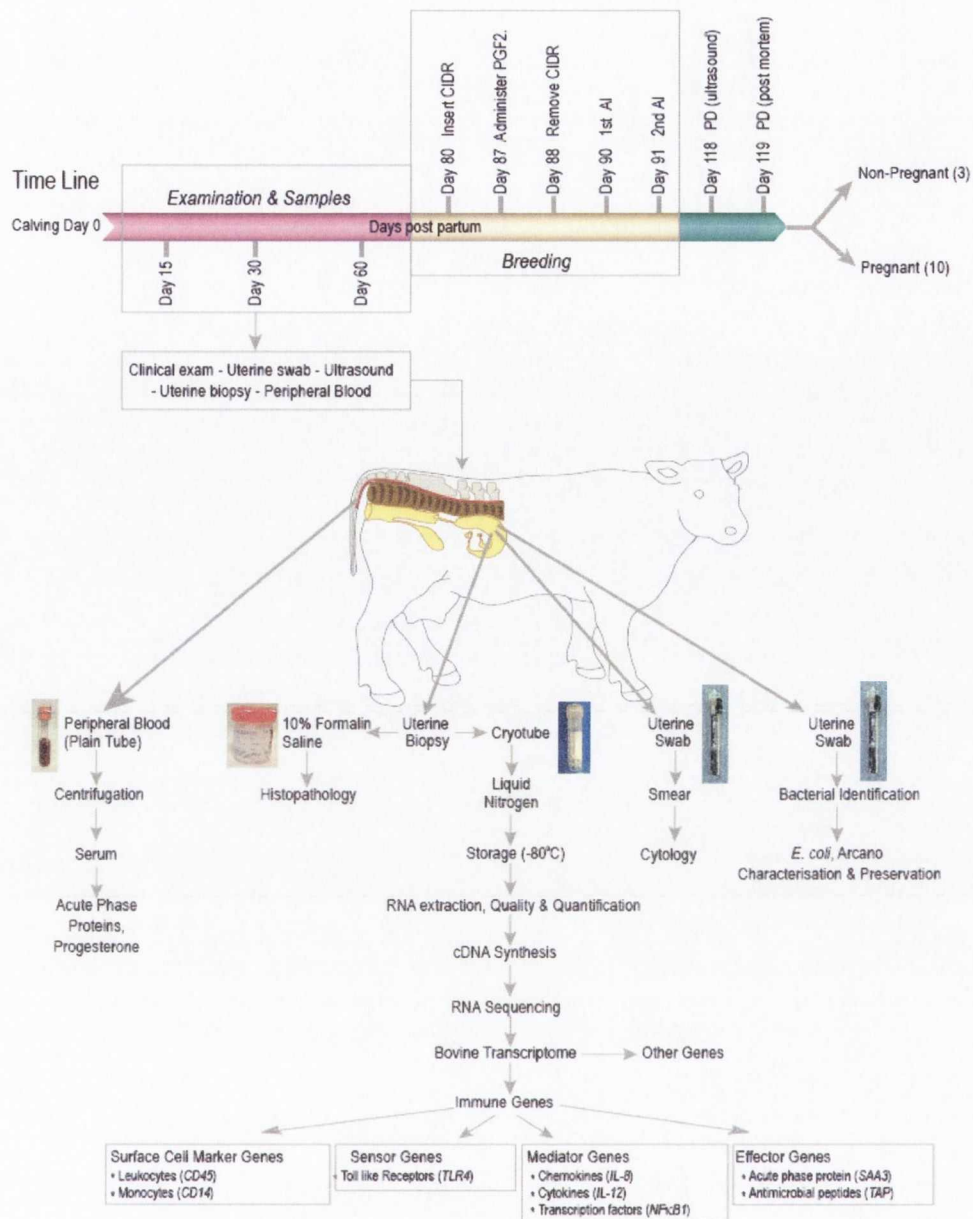


Figure 3.5: Experimental protocol for collection, preservation, processing and analysis of biological samples obtained from every cow at each time point (n = 13). Clinical examination involved physical examination, ultrasonographic imaging and palpation *per rectum* and blood sampling. Microbial culture was performed using the ‘guarded’ intrauterine swabbing method. A second swab was obtained to prepare cytology smears. The uterine tissue obtained from each cow using biopsy was divided into two sections for histopathological and molecular evaluation. Oestrus cyclicity was then synchronised, AI performed, pregnancy assessed and the cows slaughtered.

3.3.2 Clinical, *per rectum* and ultrasonographic examination

A clinical examination was performed and relevant findings on history, palpation *per rectum*, ultrasonographic imaging and uterine lavage were recorded. The uterus and ovaries were located and cervical diameter estimated *per rectum*. Manual evaluations were supported by uterine and ovarian imaging using a real-time ultrasound instrument (Aloka SSD-900, UK) with a 5MHz linear array transducer.

3.3.3 Tissue acquisition

Peripheral blood, uterine swabs and biopsies were obtained from the cows 15, 30 and 60 days after calving. Figure 3.5 shows the experimental protocol for obtaining the tissue samples from the 27 cows. At each time point, uterine swabs, peripheral blood and uterine biopsies were obtained from every cow. All cows were clinically examined after each biopsy.

3.3.3.1 Peripheral blood sampling, progesterone assay and haptoglobin measurement

Peripheral blood was collected by coccygeal venipuncture into plain and lithium heparin coated vacutainer test tubes at each sample acquisition time point. After four hours, serum was separated from the plain tubes by centrifugation at 800 x g for 10 min and stored frozen at -20°C until required. Serum progesterone concentration was measured by using Coat-A Count RIA kits (Diagnostic Products Corporation, USA) according to the procedure previously described (Rodbard & Lewald, 1970). The assay had a sensitivity of 0.1 ng/ml and intra- and inter-assay coefficients of variation of 10.2% and 6.5% respectively. Haptoglobin was measured by determining haemoglobin binding capacity of serum (Chan *et al.*, 2004; Eckersall *et al.*, 2006) according to the manufacturer's instruction using the PhaseTM Range Haptoglobin assay kit (Tridelta Development Ltd, www.trideltaltd.com). Calibration curves were created using 50 µl of purified bHp at dilutions from 0 - 10 µg/ml in duplicate. 50 µl of the antiserum (dilution 1/50,000) was then added and incubated for 2 h at room temperature. After 3

washes, 100 µL of the second antibody conjugated to peroxidase (1/20,000 dilution) was added and incubated for 30 min, and then washed. Each well was then filled with 150 µl of freshly prepared substrate solution containing 0.05 M citric acid, 0.055 M Na₂HPO₄, 0.05% urea hydrogen peroxide, 2% ProClin 150, and 2% of a tetramethylbenzidine solution (12.5 mg/ml dimethylsulfoxide). After 30 min, the reaction was stopped using 50 µl of 1 M oxalic acid. Optical density (OD) was then determined at 450 nm with a microtiter plate reader (ELX800, Bio-Tec Instruments, Inc., Winooski, VT) and the Hp concentrations in unknown samples were calculated from the calibration curve. The concentrations of serum amyloid were determined using an enzyme linked immunosorbent assay kit (ELISA, Tridelta Development PLC, Dublin, Ireland) according to the manufacturer's instructions. The limits of detection of the haptoglobin and serum amyloid A assays were 20 µg/ml and 0.33 µg/ml, respectively.

3.3.3.2 Uterine microbiological culture

Uterine swabs were obtained using a method previously described (Dohmen *et al.*, 2000; Messier *et al.*, 1984). Briefly, the vulval lips were parted and a guarded sheath advanced into the uterus by cervical manipulation, as if performing AI. A sterile swab was advanced into the uterus, retracted into a protective sheath and withdrawn. The swab was transferred into sterile tubes containing thioglycolate broth transport medium, stored at room temperature and transported to the laboratory within 2 hours. All culture swabs were submitted to a specialist diagnostic laboratory (Irish Equine Centre, Johnstown, Co. Kildare, Ireland). At the laboratory, the swabs were cultured aerobically and anaerobically on Columbia Blood Agar (CBA) base (Oxoid Ltd., Basingstoke, UK; CM0331) with 5% defibrinated sheep blood, CBA with Staph/Strep Selective Supplement (Oxoid SR0070) and MacConkey Agar No. 2 (Oxoid CM0109) and incubated for 24, 48 and 72 hours. *Escherichia coli* was identified by colonial morphology and lactose fermentation on MacConkey agar, and confirmed using API 20E (bioMérieux SA).

3.3.3.3 Uterine cytology

Uterine swabs were obtained using a previously described method (Brooks, 1993). The method was modified to exclude infusion of PBS into the uterus. Briefly, after clinical

examination, a clean sleeve was worn and the perineum was wiped with dry paper towel. A guarded swab was inserted through the cervix into the uterus as if performing AI. The swab was rolled on labelled glass slides and smears prepared. In the laboratory, smears were fixed with alcohol and stained with Wright-Giemsa. Microscopic evaluation (x400 magnification) of the smears was performed blind of clinical findings. Classification was as follows: 0: no evidence of inflammation, mainly endometrial cells (< 5% neutrophils, < 5% lymphocytes, > 30% epithelial cells); 1: mild inflammatory exudate (5 - 20% neutrophils, 5 - 10% lymphocytes, 20 - 30% epithelial cells); 2: moderate inflammatory exudate (20 - 50% neutrophils, 10 - 20% lymphocytes, 10 - 20% epithelial cells); 3: severe suppurative inflammatory exudate, mainly neutrophils (> 50% neutrophils, > 20% lymphocytes, < 10% epithelial cells).

3.3.3.4 Endometrial biopsy

Endometrial biopsies were obtained with the Hauptner equine endometrial biopsy instrument using a previously described method (Chapwanya *et al.*, 2009; Katagiri & Takahashi, 2004). Samples were obtained on day 15 after calving and repeat biopsies were taken on days 30 and 60 postpartum. Briefly, after cleaning the perineum and external genitalia, the biopsy instrument in a protective sheath was introduced into the vagina. The instrument was guided into the cervix by manipulation *per rectum*. Then the instrument alone was introduced into the uterus after rupturing the sheath at the external cervical orifice, and guided into the previously gravid horn approximately 3 cm past the uterine bifurcation as shown in Figure 3.6. To ensure reproducible tissue procurement, with due care, the tip of the biopsy instrument inside the uterus was identified using the non-operating hand *per rectum*. Then the biopsy jaws were opened and, with the help of the hand in the rectum, the medial uterine wall was gently pressed into the instrument jaws. Endometrial tissue measuring 10 x 5 mm was clipped off by closing the jaws and withdrawing the instrument. All biopsies were performed by the same operator. The tissue was then placed on petri dishes, orientated to visualise the mucosal surface, sectioned into 2 pieces and placed in an eppendorf tube for preservation in liquid nitrogen or in a sample bottle in 10% formalin saline. For histological assessment, formalin-fixed tissues were paraffin-embedded, sectioned at 5-8 µm thickness and stained with haematoxylin and eosin (H&E). Tissues were classified based on previously defined criteria (Bonnett *et al.*, 1991a). For molecular analysis, tissue was cryo-preserved in liquid nitrogen and stored at -80°C for later processing.

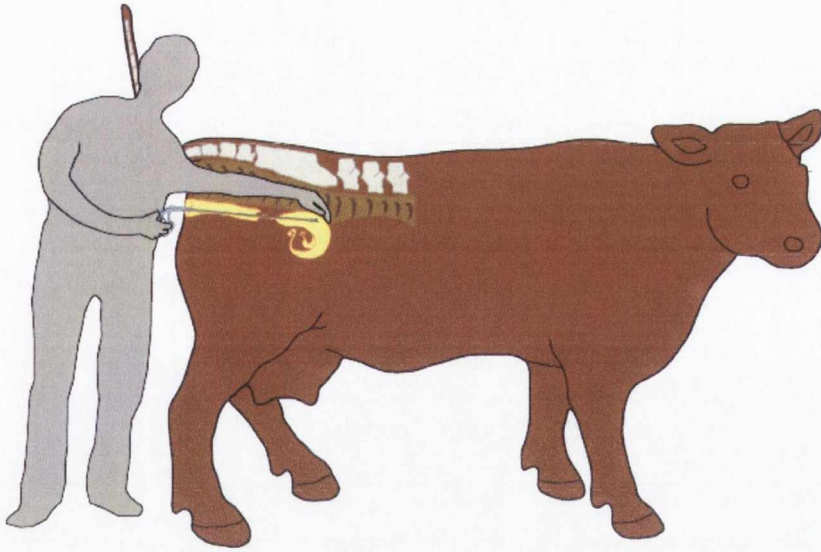


Figure 3.6: To perform a biopsy, the uterus is located *per rectum* and the previously gravid horn identified. The non-operating hand manipulates the uterus to identify the tip of the instrument thereby safeguarding the uterine wall prior to acquiring biopsy tissue.

3.3.3.5 Post-biopsy evaluation

All cows were clinically examined by visual appraisal for any signs of abnormal vulval discharge or discomfort at 1, 6, 24 and 48 hours following endometrial biopsy. Vital parameters of temperature, pulse rate, respiration rate and colour of the mucosae were also recorded for each cow at each time point.

3.3.3.6 Synchronisation of oestrus cyclicity and AI

Oestrous cycles were synchronised at least 80 days postpartum with progesterone-releasing intravaginal inserts (CIDR[®]; Pfizer Animal Health, Chesham, UK) for 8 days as shown in Figure 3.5. Seven days after the PRID insertion, heifers were injected with a prostaglandin F2 α analogue (Prosolvin[®], 15 mg dinoprost tromethamine per injection, Intervet, Loughrea, Co. Galway, Ireland). Starting 36 hours after CIDR removal, visual oestrous detection was conducted every 4 hours. The cows were artificially inseminated 8 and 16 hours after oestrus using sex-sorted semen from a single sire (LIM IRB5 - Jack, AI Station, Enfield, Co. Meath, Ireland).

3.3.3.7 Pregnancy diagnosis

Assessment for pregnancy was performed 27 days after AI using *per rectum* palpation and ultrasonographic imaging. To visualise the conceptus, a systematic lengthwise and crosswise examination was performed on both uterine horns. On the 28th day of gestation all the cows were slaughtered, and the embryos were recovered.

3.3.3.8 Histology

For histological assessment, formalin-fixed tissues were paraffin-embedded, sectioned at 5-8 μ m thickness and stained with haematoxylin and eosin (H&E). Tissues were classified independently of clinical findings, based on previously defined criteria (Bonnett *et al.*, 1991a) with modifications. Histology sections were scored for inflammation, gland morphology and presence of endometrial epithelium. The

inflammatory reaction was scored based on classical inflammatory cell infiltration. The cells were counted per high power field (HPF, 400x magnification) and the sections graded as follows:

0: normal uterine tissue, 0 – 25 cells per hpf, no evidence of inflammation, quiescent tissue, normal gland morphology and no fibrosis, intact endometrial epithelium;

1: low grade inflammation, 26 – 50 cells per hpf, infiltration of lymphocytes, neutrophils or plasma cells, fibrotic areas, cystic glandular degeneration, vascular changes, endometrial epithelium intact;

2: moderate inflammation showing prominent leukocytes, 51 – 75 cells per hpf, periglandular or blood vascular congestion, cystic glandular degeneration, endometrial epithelium disruption;

3: severe inflammation, infiltration of polymorphonuclear cells, > 75 cells per hpf, uterine gland atrophy, cystic degeneration and necrosis, extensive fibrosis of blood vessels, focal aggregates of neutrophil and macrophage, isolated sites of oedema, vascular congestion, haemorrhage, endometrial epithelial disruption or loss.

3.3.3.9 RNA extraction, quantification, cDNA synthesis

Thirty-nine uterine tissue sections preserved in liquid nitrogen and stored at -80°C were processed for molecular analysis. Total RNA was extracted using the Qiagen RNeasy® method (Qiagen Ltd; www.qiagen.com) and DNase treated according to the manufacturer's instructions. Tissue sections were removed from the -80°C freezer and placed in 600 µl RTL lysis buffer, disrupted using a homogeniser and 200 µl chloroform was added and the mixture shaken for 15 sec. After the 5 min incubation at room temperature, tubes were centrifuged at 800 x g for 1 min at 4°C to separate the phases. The upper aqueous phase was transferred to labeled 1.5 ml eppendorf tubes. 600 µl 70% ethanol was added and then passed through a column to isolate RNA. The columns were washed with 350 µl RNase free water and then 5 µl DNase added and incubated for 15 min at 37°C to digest any residual DNA. A further wash with RNase free water was performed followed by 2 washes with 500 µl buffer. The RNA was eluted twice, in 30 and 20 µl of nuclease free water, and stored at -80°C. To ensure integrity and stability of RNA samples, yield and quality were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). One µg of total RNA from each sample was reverse transcribed into cDNA using an OmniScript III® first-strand synthesis kit using oligo (dT) primers according to the manufacturer's recommendations (Invitrogen Ltd,

Paisley, UK). RNA was combined with 10 mM oligo (dT)₁₂₋₁₈ primer and DNase/RNase-free water in a 10 µl volume. Master mixes containing 2 µl of reaction buffer (supplied by the manufacturer; final reagent concentration of 50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl₂ and 10 mM DDT), 200 U M-MuLV reverse transcriptase and a final concentration of 0.5 mM each dNTP were added to obtain a final reaction volume of 20 µl. Reverse transcription reaction was allowed to proceed for 1 h at 37°C, then the samples were heated to 95°C for 5 min before being cooled to 4°C for 5 min. cDNA was quantified using a Nanodrop™ spectrophotometer and the samples diluted to a final working concentration of 40 ng/µl and stored at -20°C until required for qRT-PCR.

Gene-specific oligonucleotide primer pairs were designed using Primer Express® version 2.0 software (Applied Biosystems: www.appliedbiosystems.com) and synthesised commercially (Invitrogen Corp: www.invitrogen.com). The primer pairs used in this study are presented in Appendix A1.

3.3.3.10 qRT-PCR

All qRT-PCR reactions were performed in a total volume of 25 μ l with 2 μ l of cDNA (80 ng/ μ l), 12.5 μ l 2 $\times$ PCR DNA polymerase master mix (Taq polymerase, anti-Taq antibody, MgCl₂, nucleotides and optimised reaction buffer components; Stratagene Corp, La Jolla, CA), 2.5 μ l each of the sense and antisense primers, 1/60 000 dilutions of SBYR® Green I dye (Invitrogen: www.invitrogen.com) and 80 ng of cDNA template. Optimal primer concentrations were determined by titrating 100, 600 and 900 nM final concentrations and dissociation curves were examined for the presence of a single product. The optimal concentrations are presented in Appendix A1 for each gene. qRT-PCR was performed using an MX3000P® quantitative PCR system (Stratagene Corp, La Jolla, CA) with the following cycling parameters: 95°C for 1 min followed by 40 cycles of 95°C for 30 s, 60°C for 1 min and 72°C for 30 s followed by amplicon dissociation. All reactions were performed in duplicate and oligonucleotide primers were used to amplify *H3F3A* mRNA transcript as the reference for normalising the data. An interplate calibrator (IPC) and no template control (NTC) were run for each primer used on every 96-well plate in a predetermined plan.

3.3.3.10.1 Selection of Reference (Normaliser) gene: geNorm

The algorithm programme, geNorm (Vandesompele *et al.*, 2002) was used to determine the most stable reference (normaliser) genes from a set of tested candidate reference genes in uterine biopsy tissue. GeNorm calculates the gene expression stability measure, M, for a reference gene as the average pair-wise variation, V, for that gene with all other tested reference genes. Stepwise exclusion of the gene with the highest M value allowed ranking of the tested genes according to their expression stability. The most stable reference gene across all uterine samples was selected from a panel of potential normalisers - Actin-beta (*ACTB*), Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and Mitochondrial ribosomal protein subunit genes (*MRPS6*, *MRPS9*, *MRPS15*). From this, a gene expression normalisation factor was calculated for each sample based on the geometric mean of each reference gene.

3.3.3.10.2 Interplate calibrator (IPC)

A plan of assaying was devised where the normaliser gene *H3F3A* was amplified in all uterine cDNA at all time points on the first plate with an interplate calibrator (IPC) included on each plate. Then expression of one gene of interest (GOI) was measured in the 13 cows at 3 time points (15, 30 and 60 DPP), in duplicates. This gave a total of 78 samples. The 78 cDNA samples were analysed per run together with IPC and NTC as shown in Figure 3.7. For interplate correction, an identical sample (IPC) was included in all runs, and analysed for all genes. In the present study, the IPC gene was *H3F3A* amplified in bovine endometrial epithelial cDNA. Setting the Ct threshold to the same value for each plate facilitated valid interplate comparisons.

PLATE 1	1 15DPP	2 15DPP	3 30DPP	4 30DPP	5 60DPP	6 60DPP	7 15DPP	8 15DPP	9 30DPP	10 30DPP	11 60DPP	12 60DPP
Gene												
A	Cow 1	Cow 1	Cow 1	Cow 1	Cow 1	Cow 1	Cow 9	Cow 9	Cow 9	Cow 9	Cow 9	Cow 9
B	Cow 2	Cow 2	Cow 2	Cow 2	Cow 2	Cow 2	Cow 10	Cow 10	Cow 10	Cow 10	Cow 10	Cow 10
C	Cow 3	Cow 3	Cow 3	Cow 3	Cow 3	Cow 3	Cow 11	Cow 11	Cow 11	Cow 11	Cow 11	Cow 11
D	Cow 4	Cow 4	Cow 4	Cow 4	Cow 4	Cow 4	Cow 12	Cow 12	Cow 12	Cow 12	Cow 12	Cow 12
E	Cow 5	Cow 5	Cow 5	Cow 5	Cow 5	Cow 5	Cow 13	Cow 13	Cow 13	Cow 13	Cow 13	Cow 13
F	Cow 6	Cow 6	Cow 6	Cow 6	Cow 6	Cow 6						
G	Cow 7	Cow 7	Cow 7	Cow 7	Cow 7	Cow 7					IPC	IPC
H	Cow 8	Cow 8	Cow 8	Cow 8	Cow 8	Cow 8					NTC	NTC

Figure 3.7: qRT-PCR plate layout for analysis of innate immune gene expression in endometrial tissues. A single gene of interest was assayed on each plate together with the IPC and NTC. IPC – Interplate calibrator. NTC – Non-template control.

3.3.3.10.3 qRT-PCR data analysis

qRT-PCR data were analysed using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001). Relative expression of mRNA in each sample was determined and a ratio of each gene of interest relative to the reference gene (H3F3A) was calculated (to correct for differences in efficiency of cDNA production). The relative abundance of mRNAs was calculated by dividing 15 and 30 DPP by 60 DPP values in the same animal after reference gene correction to give a fold-change value ($\Delta\Delta C_t$). (For $\Delta\Delta C_t$ values < 1 , $-1/\Delta\Delta C_t = \text{fold change}$). The average of the duplicate values was used to determine a more accurate indication of the difference. Values where the duplicates differed by more than one cycle C_t were re-run. Values from the three time points (15 and 30 DPP vs 60DPP) were compared using the Mann-Whitney test on logged data to account for non-normal distribution in the variance. For presentation of the data, 15 and 30DPP values are expressed relative to 60DPP values in the same animal, presented as standard error of the median.

3.4 Results

3.4.1 Clinical findings

All cows selected for endometrial biopsy in the present study were in good health and no cow showed any signs of uterine or systemic illness. There was no history of dystocia at calving or retained foetal membranes post-calving. No cow required antimicrobial treatment during the study. All the cows were examined and BCS, temperature, respiration rate and colour of mucosae recorded. Table 3.1 shows the findings at each of the time points (15, 30, 60 DPP).

At 15 DPP, the cows were at an average BCS of 3.5 (range 3 – 3.75). Rectal temperature ($38 - 38.8^\circ\text{C}$), heart rate (56 - 72 beats per minute), respiration rate (14 - 18 breaths per minute) and ocular mucosal colour (pink, moist) were all normal.

At 30 DPP, as shown in Table 3.1, the BCS improved to an average of 3.75 (3.25 – 4). Temperature, respiration and heart rate were within normal ranges, similar to findings at 15 DPP.

At 60 DPP, BCS improved to an average of 4, and all other parameters were within normal ranges.

Table 3.1: Clinical findings of the 13 cows at each examination time point (15, 30 and 60 DPP).

Cow	15 DPP					30 DPP					60 DPP				
	BCS	T (°C)	RR	HR	MM	BCS	T (°C)	RR	HR	MM	BCS	T (°C)	RR	HR	MM
1	3	38.4	16	56	Pink	3.75	38	16	64	Pink	3.75	38.8	16	86	Pink
2	3.75	38.6	14	60	Pink	4	38.7	22	76	Pink	3.75	37.4	20	78	Pink
3	3.5	38.9	15	68	Pink	3.25	38.8	18	64	Pink	4	38.2	20	76	Pink
4	3.25	38.3	18	72	Pink	3.75	38.9	22	72	Pink	3.75	38.6	18	80	Pink
5	3.5	38	16	68	Pink	3.25	38.9	16	62	Pink	4	38.3	22	76	Pink
6	3.5	38.2	16	58	Pink	3.75	38.7	20	76	Pink	4	38.5	18	70	Pink
7	3.5	38.7	16	60	Pink	3.75	38.1	18	76	Pink	3.75	38	18	76	Pink
8	3.25	38.4	14	60	Pink	3.5	38	18	72	Pink	3.75	36.7	18	72	Pink
9	3.5	38.4	14	68	Pink	4	38.6	20	72	Pink	3.75	38.1	16	72	Pink
10	3.25	38.7	16	72	Pink	3.75	38.4	22	76	Pink	4	38.2	16	68	Pink
11	3.25	38.4	18	64	Pink	4	38.8	18	72	Pink	4	38.3	16	72	Pink
12	3.75	38	16	72	Pink	3.75	38.5	14	68	Pink	4	37.8	24	76	Pink
13	3.5	38.8	16	60	Pink	3.75	39.3	22	76	Pink	4	38.1	22	80	Pink

BCS- Body condition score, scale 1-5 (Macdonald *et al.*, 2005), T – Body temperature (°C), RR – Respiration rate (breaths per minute), HR – Heart rate (beats per minute), MM – Mucosal membranes.

3.4.2 Progesterone and haptoglobin measurement

Progesterone concentration profiles did not differ among the cows at the three time points. As shown in Table 3.2, at 15 DPP, all the 13 cows were in the postpartum anoestrous physiological status as indicated by serum progesterone levels of less than 1ng/ml of blood. The concentration values ranged between 0.01 and 0.80 ng/ml at 15 DPP.

At 30 DPP, the majority of the cows had ovulated, and the progesterone concentration levels were between 0.2 and 5.2 ng/ml of blood. With this frequency of sampling for progesterone, levels could have already increased and subsequently decreased by 30 DPP because the first cycle postpartum is often short. At this stage, 9 cows had serum progesterone values which had increased to 1 ng/ml of blood or more, the critical value which indicates the presence of an active *corpus luteum* (CL). Only 4 cows had progesterone values below 1 ng/ml of blood, although one had a value of 0.99ng/ml of blood.

At 60 DPP, 11 cows had recently ovulated and had peripheral progesterone levels equal to or more than 1 ng/ml of blood (Table 3.2). Of the two cows with progesterone levels below 1 ng/ml of blood, one cow had a value of 5 ng/ml of blood at 30 DPP. Only one cow had progesterone levels less than 1ng/ml at the three time points. Figure 3.8 shows the temporal increases in the levels of serum progesterone in the individual cows. In each cow, the progesterone concentration at 30 and 60 DPP is higher than at 15 DPP.

Table 3.2: Serum progesterone levels (ng/ml of blood) in postpartum cows at 15, 30 and 60 days after calving. A value of 1 ng/ml of blood or higher signifies functional luteal tissue following ovulation.

Cow	15 DPP (ng/ml)	30 DPP (ng/ml)	60 DPP (ng/ml)
1	0.2	5.0	0.3
2	0.1	2.8	3.5
3	0.2	0.8	1.4
4	0.8	1.9	10.1
5	0	1	3.1
6	0	1.4	9.8
7	0	0.2	0.2
8	0	0.5	3.4
9	0.4	4.8	4.2
10	0.1	2.1	7.4
11	0	5.2	3.1
12	0.2	3.9	1.2
13	0.5	1.3	6.8

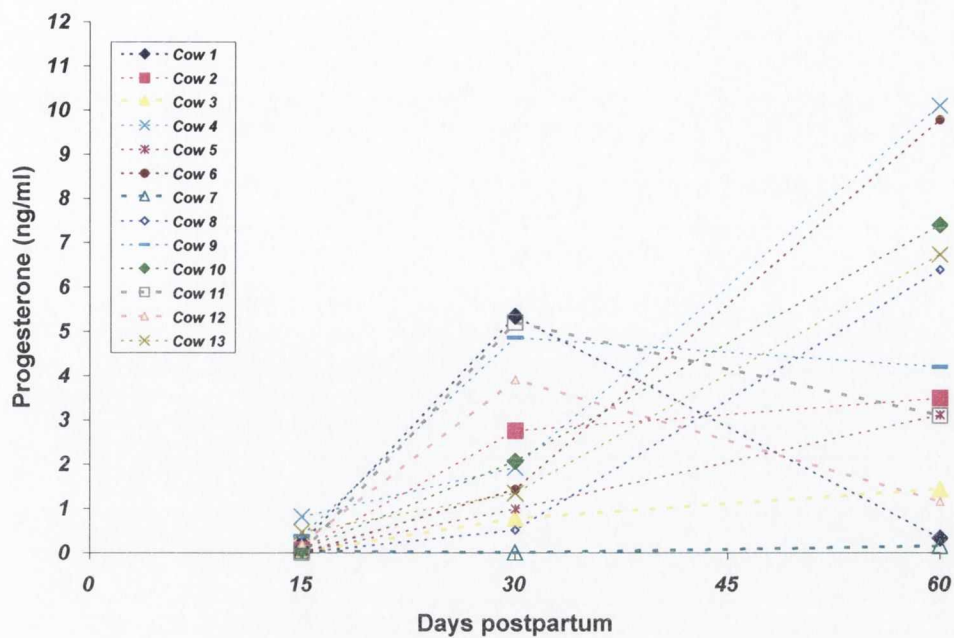


Figure 3.8: Temporal fluctuation in the progesterone levels of the individual cows at 15, 30 and 60 DPP. For every cow, the level at 30 and 60 DPP is higher than the level at 15 DPP, indicating that all the cows resumed oestrous cyclicity by 30 or 60 days after calving.

As shown in Table 3.3, at 15 DPP the majority of the cows had average peripheral HP levels of 126 g/l (range 42 – 212). At 30 DPP the HP levels decreased to 72 g/l (range 39 – 202) as shown in Table 3.8. Interestingly, at 60 DPP, three cows had elevated HP levels over 250 g/l. The HP levels at 15, 30 and 60 DPP in all the cows is shown in Figure 3.9.

Table 3.3: Mean values for HP concentrations in serum samples from postpartum cows.

Animal	HP concentration g/L		
	15 DPP	30 DPP	60 DPP
Cow 1	42	43	39
Cow 2	204	56	49
Cow 3	115	62	45
Cow 4	196	41	40
Cow 5	70	47	40
Cow 6	171	39	62
Cow 7	209	202	39
Cow 8	192	63	44
Cow 9	52	55	41
Cow 10	171	49	61
Cow 11	209	77	273
Cow 12	59	50	289
Cow 13	212	80	313

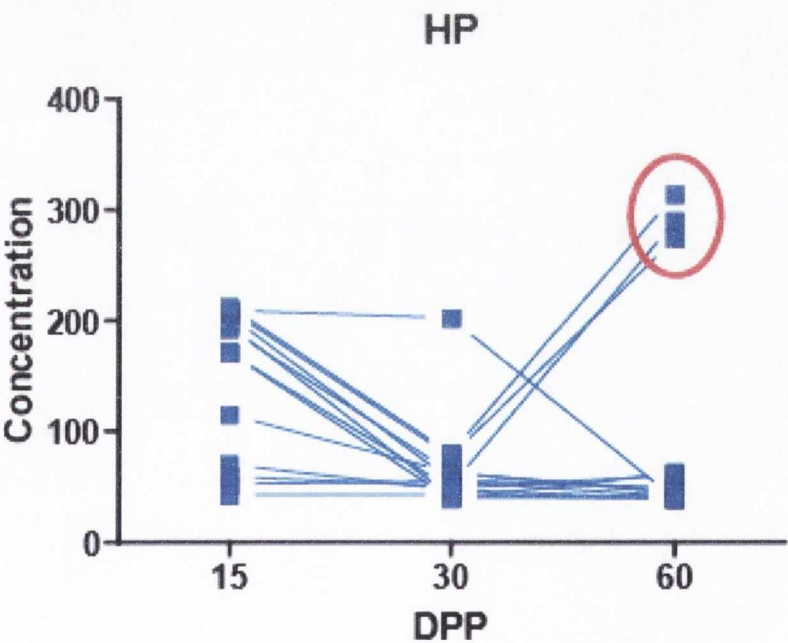


Figure 3.9: Haptoglobin (HP) protein expression profiles from serum of the cows at 15, 30 and 60 DPP. Haptoglobin concentration is in g/l. Each point on the graph represents expression in the individual cows.

3.4.3 Microbial culture

In total, seven different bacterial species were isolated from the uteri of the 13 cows in the present study. In many cases, more than one bacterial species were detected in the uterus of the cows at each time point. The variety and prevalence of the bacteria species identified are listed in Table 3.4. The table shows the number and percentage of cows inhabited by the bacteria at each time point.

At 15 DPP, all the cows had positive culture. The most frequently isolated bacteria, as shown in Table 3.5, were α – haemolytic *Streptococcus spp* (13 cows, 100%) followed by *Staphylococcus aureus* (8 cows, 62 %). *E. coli* and *Arcanobacterium pyogenes*, the bacterial species commonly implicated in clinical incidences of uterine disease, were identified with a frequency of 38 % (5 cows) and 15 % (2 cows), respectively. Also identified were *Bacillus spp.* (15 %, 2 cows), and *Corynebacterium spp.* (7.69%, 1 cow). *Micrococcus spp* were not present in the uterus of any cow.

At 30 DPP, no bacteria were isolated from the uteri of 7 cows (54 %). Similar to 15 DPP, the predominant bacteria species detected were α – haemolytic *Streptococcus spp* (46 %, 6 cows). Two cows (15 %) had positive culture for *Staphylococcus aureus*. *E. coli* and *A. pyogenes* both accounted for 8 % (1 cow) at 30 DPP. The other bacterial species isolated were *Corynebacterium spp* (4 cows, 31 %), *Micrococcus spp* (1 cow, 7.69%) and *Bacillus spp* (1 cow, 8 %).

At 60 DPP, no positive bacterial cultures were detected in 4 cows (31 %). Unlike 30 DPP, the number of cows showing positive growths of α – haemolytic *Streptococcus spp* at 60 DPP increased to 9 (69 %). Three cows (23 %) had *Staphylococcus aureus* present in the uteri. *E. coli* were identified in one cow (8 %). There were no *A. pyogenes* present in any of the cows. As shown in Table 3.5 and Figure 3.10, 5 cows (38 %) had positive cultures of both *Baccillus spp* and *Corynebacterium spp*. No *Micrococcus spp* were detected.

A trend where the prevalence of *E. coli* and *A. pyogenes* decreases with time after calving and the number of cows with no bacterial growth starts at 0 at 15 DPP and

peaks at 7 (54 %) at 30 DPP and then decreasing to 4 (31 %) at 60 DPP is illustrated in Figure 3.10.

Table 3.4: Bacterial species isolated from individual cows at 15, 30 and 60 DPP. ‘Primary’ refers to known uterine pathogens while ‘Other’ refers to potential pathogens or commensal bacteria.

Cow	15 DPP		30 DPP		60 DPP	
	Bacteria		Bacteria		Bacteria	
	Primary	Other	Primary	Other	Primary	Other
1	0	sa, stah	0	0	0	0
2	0	sa, stah	0	stah, cor, bac	0	stah, cor, bac
3	EC, AP	stah	AP	sa, cor	0	stah
4	0	stah	0	0	EC	sa, stah, bac
5	0	sa, stah, bac	0	stah, mic	0	0
6	EC	sa, stah	0	sa, stah	0	0
7	0	sa, stah, cor	0	0	0	stah, cor
8	0	sa, stah	0	0	0	sa, stah, bac
9	0	bac, stah	0	0	0	stah, cor, bac
10	0	stah	0	0	0	0
11	EC	stah	0	stah, cor	0	stah, cor, bac
12	EC	sa, stah	0	0	0	sa, stah
13	EC, AP	sa, stah	EC	stah	0	stah, cor

EC- *Escherichia coli*
AP- *Arcanobacterium pyogenes*
sa- *Staphylococcus aureus*
stah- *α-haemolytic Streptococcus spp*

bac- *Bacillus spp*
cor- *Corynebacterium spp*

Table 3.5: Prevalence of bacteria isolated from the uteri of postpartum cows at 15, 30 and 60 DPP. Overall, 7 different bacterial species were isolated from the 13 cows during the postpartum period. All the cows had bacteria present in the uterus 15 DPP.

Bacteria species	Bacterial prevalence					
	15 DPP		30 DPP		60 DPP	
	N	%	N	%	N	%
<i>E. coli</i>	5	38	1	8	1	8
<i>A. pyogenes</i>	2	15	1	8	0	0
<i>Streptococcus spp</i>	13	100	6	46	9	69
<i>Staphylococcus aureus</i>	8	62	2	15	3	23
<i>Bacillus spp</i>	2	15	1	8	5	38
<i>Micrococcus spp</i>	0	0	1	8	0	0
<i>Corynebacterium spp</i>	1	8	4	31	5	38
No isolate	0	0	7	54	4	31

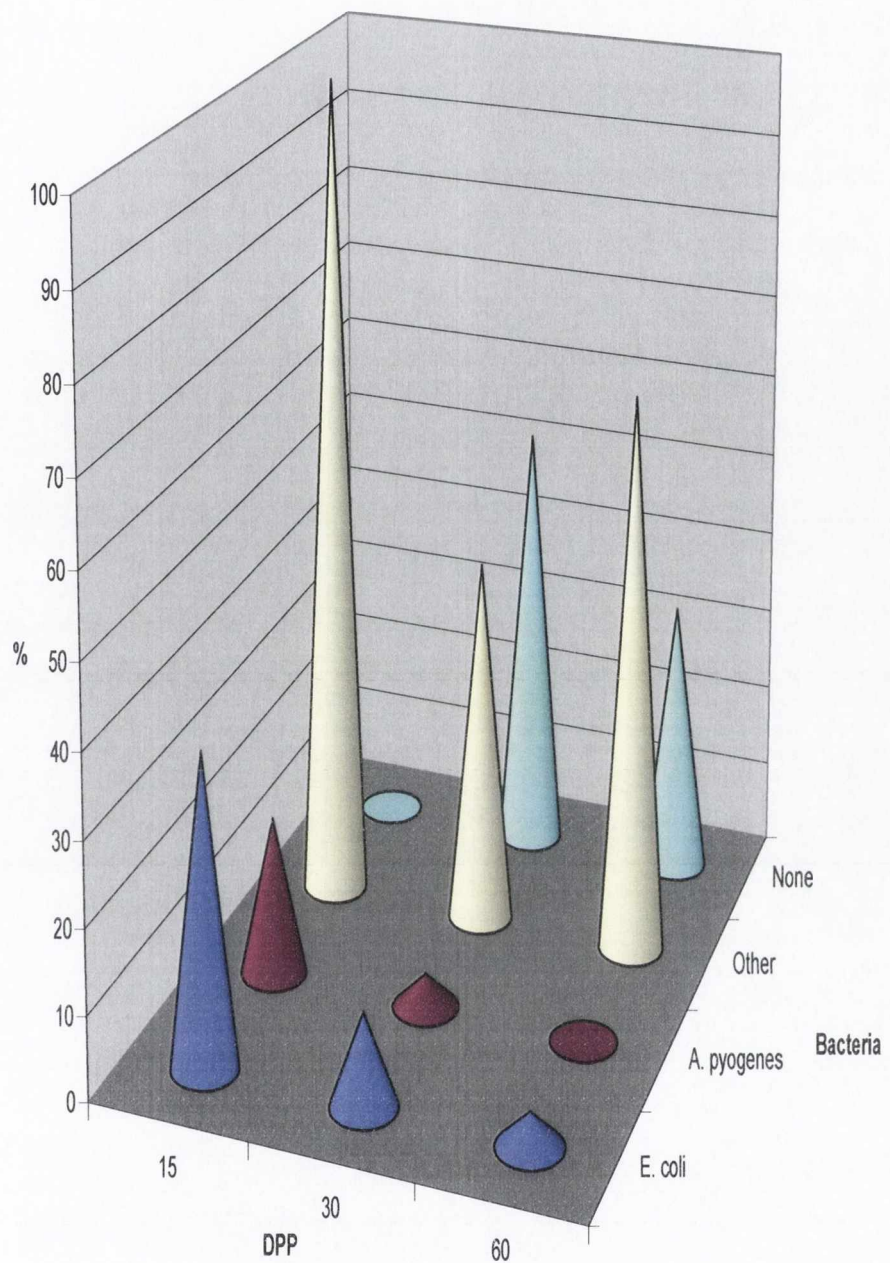


Figure 3.10: Prevalence of bacterial species isolated from the uterus of cows during the postpartum period. The pathogens *E. coli* and *A. pyogenes* isolated are shown individually 15, 30 and 60 days postpartum. There is a decrease in the number of both *E. coli* and *A. pyogenes* with increase in number of days postpartum. Other and None the proportions of cows with other bacterial or no bacterial growth at the 3 time points respectively.

3.4.4 Post-biopsy evaluation

Clinical examination by visual appraisal for any signs of abnormal vulval discharge or discomfort at 1, 6, 24 and 48 hours following endometrial biopsy detected no signs of ill-health in any cow. Vital parameters of temperature, pulse rate, respiration rate and colour of the mucosae recorded for each cow at each time point were within normal ranges.

3.4.5 Synchronisation of oestrus cyclicity

Oestrus cyclicity was successfully synchronised in all cows. Some or all of the following behavioural signs indicative of oestrus activity (Diskin *et al.*, 2003; Noakes *et al.*, 1994) were observed and recorded:

- a. Sniffing of vulva or urine of other cows
- b. Resting chin on other cows
- c. Soliciting
- d. Licking other cows
- e. Scuffed tail heads, dirty flanks and sweating
- f. Bunting
- g. Bellowing and restlessness
- h. Frequent urination
- i. Mounting other cows head to head
- j. Standing to be mounted.

3.4.6 Pregnancy diagnosis

A re-conception rate of 77% (10/13) after oestrus cyclicity synchronisation and AI was detected by ultrasonography at 27 days after AI, and confirmed by embryo recovery at slaughter at 28 days gestation. Systematic imaging of the entire length of both uterine horns revealed the presence of fluid within the uterine lumen (Figure 3.11). The embryo, measuring approximately 1 cm in length, either straight or C-shaped and usually located close to the uterine wall, is visible in the gravid horn.

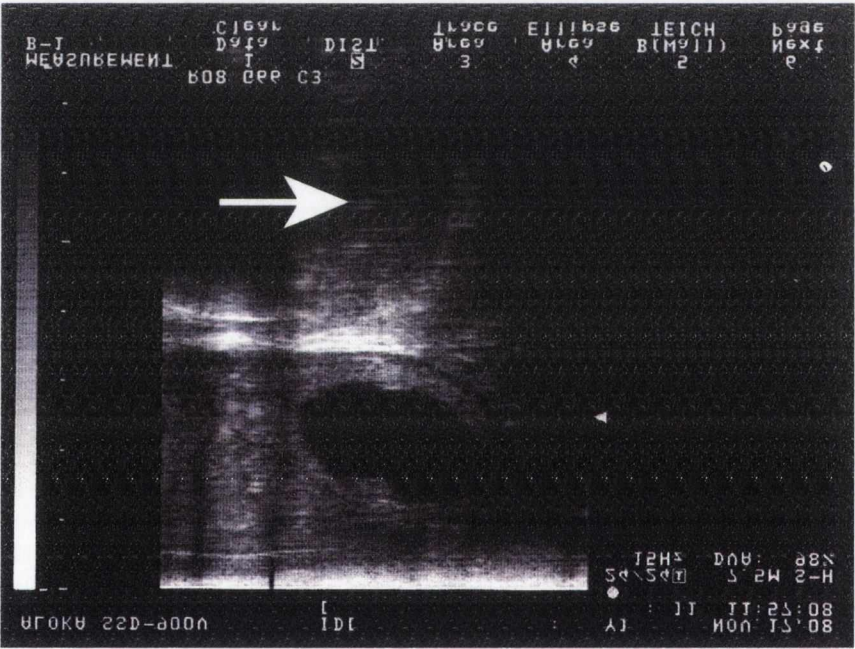
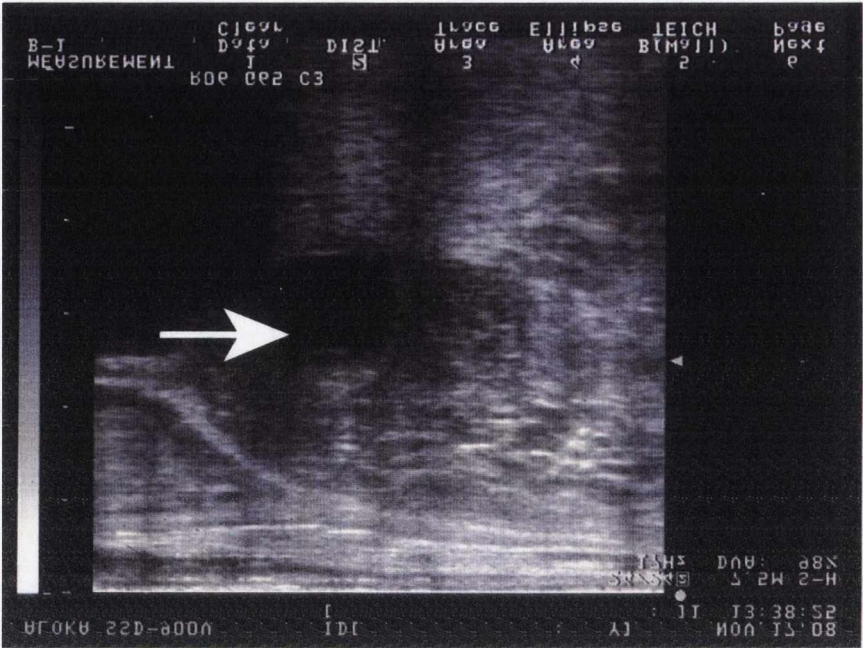


Figure 3.11: Ultrasonographic images showing 27 day-old embryos. Endometrial biopsy was performed 15, 30 and 60 days postpartum prior to synchronisation and AI of the cows. Pregnancy is confirmed by the presence of uterine intra-lumen fluid (arrow).

3.4.7 Histology

Conventional microscopic examination of the tissue sections allowed visualisation of the mucosae, and distinguished sequential involutionary uterine change. Histological classification of the uterine tissue from each cow at each time point was based on the inflammatory infiltrate and number of endometrial glands per high power field (hpf, x 400 magnification). The histological classification of the individual uterine tissue sections at 15, 30 and 60 DPP is shown in Table 3.6.

Table 3.6: Inflammation score in uterine tissues obtained from cows at 15, 30 and 60 days postpartum. Grade 0 is quiescent uterine tissues devoid of inflammation. Inflammation scores were mild (Grade 1), moderate (Grade 2) or severe (Grade 3).

Cow	15 DPP	30 DPP	60 DPP
1	3	2	1
2	3	2	0
3	3	3	1
4	3	1	0
5	3	3	1
6	3	2	0
7	3	1	0
8	3	1	0
9	3	1	0
10	3	3	0
11	3	1	1
12	3	2	0
13	3	2	1

3.4.7.1 Histology at 15 DPP

At 15 days postpartum there is evidence of severe inflammation accompanied by a prominent infiltration of leukocytes including polymorphonuclear cells and lymphocytes, numbering greater than 75 cells per hpf (magnification x400) in each tissue section. All the uterine sections were in histological category 3 (Figure 3.12). The leucocyte cell counts ranged between 89 - 213 cells per hpf. The cell counts and histology sections of the individual sections at 15 DPP are shown in Figure A3 in the Appendices section. Also detected was endometrial gland atrophy. The number of endometrial glands detected per hpf ranged in number between 1 and 4 (Figure A3). Other pathological features detected at this stage included cystic degeneration and necrosis, extensive fibrosis of blood vessels, as well as focal aggregates of neutrophils and macrophages. In addition, other types of pathology present were isolated sites of oedema, vascular congestion, haemorrhage and endometrial epithelial disruption or loss. The epithelial cell type detected at 15 DPP was predominantly columnar which was sloughed off in some sections.

Category	15 DPP		
	Histology	Features	Cows (n)
3		Severe inflammation, diffuse leukocyte infiltration. Leukocyte cells >75 cells per hpf, Columnar epithelium with areas of disruption or loss. Endometrial oedema. Vascular congestion. Focal aggregates of neutrophils and macrophages are detected in some areas of the section. Bar = 250 μm	13
2			0
1			0
0			0

Figure 3.12: Histological classification of the 13 uterine tissue sections at 15 DPP (H&E stain, magnification x400). All the sections were in category 3, characterised by prominent leukocyte infiltration and vascular congestion. The majority of leukocyte cells detected were neutrophils and lymphocytes.

3.4.7.2 Histology at 30 DPP

At 30 DPP, the uterine sections were classified into categories 1, 2 and 3 (Table 3.6). Microscopic assessment revealed endometrial mucosal surfaces lined by columnar or cuboidal epithelial cells. The endometrial sections in category 3 ($n = 2$) had a severe inflammatory infiltrate (greater than 75 cells per hpf). Although there was no evidence of endometrial gland atrophy in the two sections, one had only a single uterine gland per hpf, and the other had five per hpf (Figure 3.13). Four uterine sections classified as category 2 had prominent glandular and myometrial tissue and/or a surface lined by columnar, cuboidal or pseudo-stratified epithelium on a mature collagen-rich non-glandular and relatively non-inflamed stroma. A low-grade mononuclear and neutrophil infiltration with a leucocyte count of 51 to 75 cells per hpf was detected. Moderate periglandular congestion or blood vascular congestion was seen with minimal endometrial epithelial disruption. The majority of the endometrial sections ($n = 7$) were grouped in histological category 1 characterised by a low grade inflammation, 26 – 50 leukocyte cells per hpf, mild infiltration of lymphocytes, neutrophils or plasma cells, few fibrotic areas, little cystic glandular degeneration, vascular changes and intact endometrial epithelia (Figure 3.13). Uterine glands were also prominent in the uterine sections at 30 DPP.

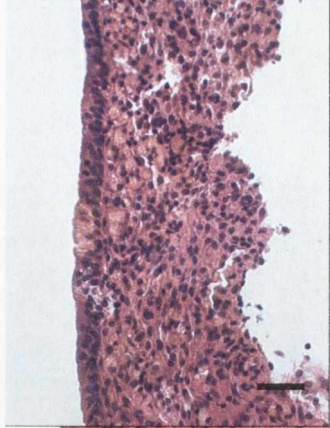
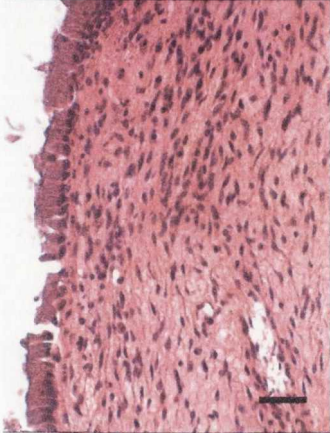
Category	30 DPP		
	Histology	Features	Cows (n)
3		Columnar epithelium with a mononuclear cell infiltration and neutrophils. 99 cells/hpf Bar = 250 μ m	2
2		Columnar epithelium, low grade lymphocyte (and occasional neutrophil) infiltration. 106 cells/hpf Bar = 250 μ m	4
1		Columnar epithelium with a low-grade mononuclear cell infiltration and occasional neutrophils. 47 cells/hpf Bar = 250 μ m	7
0			0

Figure 3.13: Histological classification of the 13 endometrial tissue sections at 30 DPP (H&E stain, magnification x400). Two sections were in category 3, four in category 2 and seven in category 1. Endometrial epithelial cells were present.

3.4.7.3 Histology at 60 DPP

At 60 DPP, glandular-rich mucosae with small numbers of inflammatory cells in a collagen-rich environment were the main features in the majority of the tissue sections. As shown in Figure 3.14, five uterine tissue sections given a score of 1 were characterised by a low grade inflammation, with 26 – 50 leukocyte cells per hpf, consisting of lymphocytes, neutrophils and plasma cells. Also present were some fibrotic areas, with intact uterine glands and endometrial epithelia. Eight endometrial sections deemed to be category 0 had predominantly normal uterine tissue and 0 – 25 leukocyte cells per hpf. There was no evidence of inflammation or fibrosis in the quiescent endometrial tissues. Intact endometrial epithelia were detected (Figure 3.14).

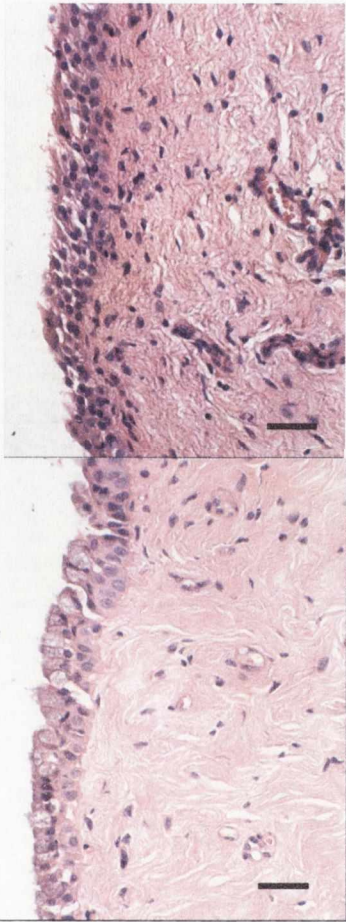
60 DPP			
Category	Histology	Features	Cows (n)
3			0
2			0
1		Pseudostratified ciliated epithelia on a mature collagen-rich non-inflamed stroma.	5
0		Pseudostratified to columnar ciliated epithelium.	8

Figure 3.14: Histological classification of the 13 endometrial sections at 60 DPP (H&E stain, magnification x400). Five tissue sections were in category 1, and eight in category 0.

3.4.8 Molecular assessment of innate immune gene expression in uterine tissues from postpartum cows

3.4.8.1 RNA quality and quantity

The extraction method used yielded RNA of high quality (RNA integrity number RIN average 7.6; range 6.3 – 9.6) suitable for reverse transcription to cDNA and analysis using qRT-PCR. Quality of the RNA, which is essential for qRT-PCR analysis, was assessed using an Agilent Bioanalyzer. The bioanalyzer separates the RNA (1 µl) components in microfabricated chips according to molecular weight and automatically calculates the ratio of ribosomal bands in total RNA samples. The RNA is labeled with a dye and then detected via laser induced fluorescence. The result is visualised as an electrogram (Figure 3.15b) and a digital format RIN value is calculated. RIN values range from 1 (low quality degraded RNA) to 10 (high quality intact RNA). Calculation and publication of an RIN facilitated reliable comparison of tissue RNA samples as well as accurate repeatability of experiments. Figure 3.15a illustrates the high RNA quality isolated from the endometrial biopsy tissue from each animal at each time point (Ratios of 18S to 28S ribosomal RNA averaged > 1.7). Table 3.7 shows the corresponding RIN values and sample concentrations. Any RNA degradation was detected by a shift in the RNA size distribution towards smaller fragments and a decrease in fluorescence signal of ribosomal peaks as shown in Figure 3.15b. Any degraded RNA samples were discarded, and isolation was repeated.

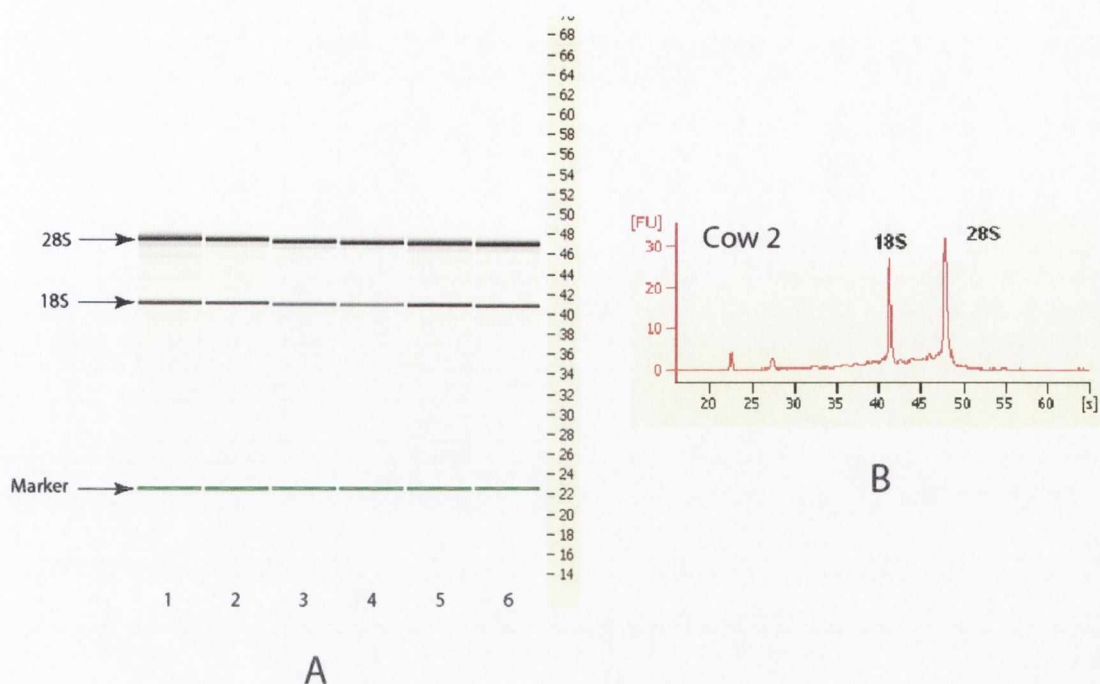


Figure 3.15: A. Agilent bioanalyzer profiles of total RNA isolated from endometrial biopsy tissue. The RNA bands 18S and 28S are shown, with little or no degraded bands of different sizes indicating intact good quality RNA yield. B. Electrophoresis trace (electrogram) showing the 18S and 28S bands of high quality intact RNA.

Table 3.7: Integrity and concentration of the RNA isolated from endometrial biopsy tissue at each of the three time points. RNA Integrity Number (RIN) is a classification system of eukaryotic RNA based on a score between 1 and 10. 1 represents most degraded and 10 most intact RNA and is calculated using the Agilent Bioanalyser™ (Agilent Technologies). Conc. is the concentration of the RNA per µl of sample and is calculated using the Nanodrop™ spectrophotometer (Thermo Scientific).

Cow	RNA Integrity and Concentration					
	15 DPP		30DPP		60DPP	
	RIN	Conc. (ng/µl)	RIN	Conc. (ng/µl)	RIN	Conc. (ng/µl)
1	8.2	364	7.0	412	9.4	422
2	7.4	528	7.2	456	8.9	406
3	9.6	488	6.6	330	8.9	386
4	8.0	378	7.6	386	8.7	390
5	7.1	390	6.9	302	8.2	328
6	6.9	286	6.3	288	9.3	406
7	7.3	306	7.8	346	8.9	400
8	7.8	356	7.0	352	8.8	382
9	6.7	312	7.3	364	7.7	384
10	7.6	402	7.7	396	6.9	296
11	6.9	284	8.9	398	8.0	338
12	7.3	334	7.9	384	7.3	376
13	8.1	396	8.6	420	8.5	382

3.4.8.2 Normaliser gene selection: GeNorm

Stepwise exclusion of the gene with the highest M value allowed ranking of the tested genes according to their expression stability in the uterine tissue. As shown in Figure 3.16, *H3F3A* and *GAPDH* were the two most stable normaliser genes. *H3F3A* was selected on the basis of standard efficiency curves generated using serial dilutions (1: 2 to 1:100) of cDNA samples.

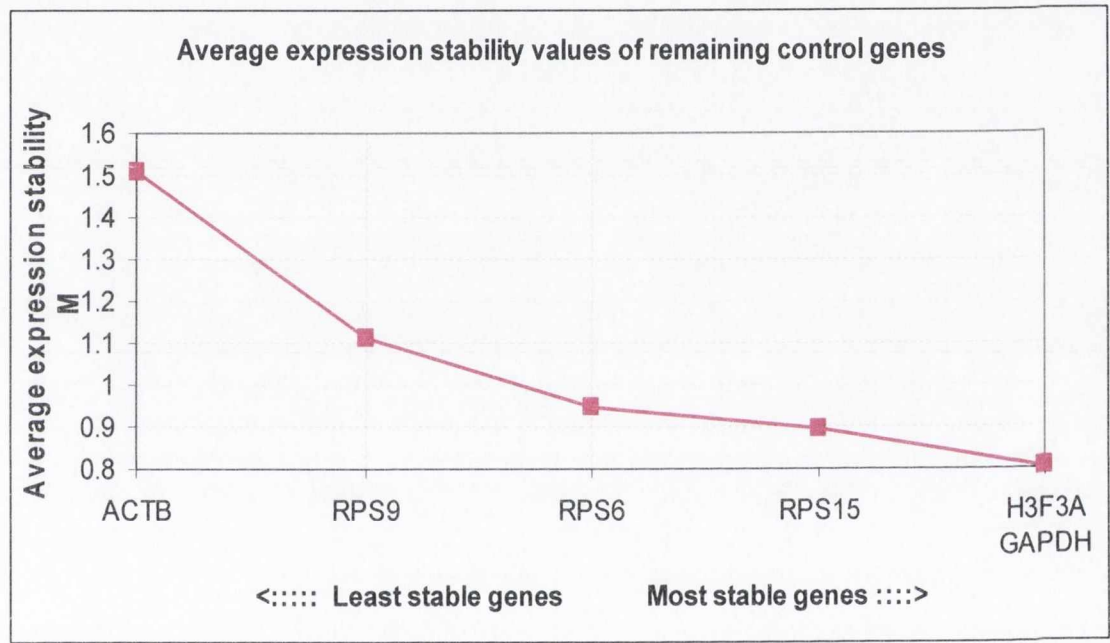


Figure 3.16: GeNorm calculation showing *H3F3A* and *GAPDH* as the two most stable normaliser genes in endometrial tissues. Gene expression stability measurement for the reference gene is determined as the average pair-wise variation, for that gene with all other tested reference genes. *H3F3A* was selected on the basis of superior PCR efficiency.

3.4.8.3 Analysis of expression of innate immune genes and pathways that constitute uterine inflammation in postpartum cows

Each PCR measurement generated a cycle threshold (CT) value, which is the number of amplification cycles required to reach a threshold signal level. The CT values, which are inversely proportional to the logarithm of the initial number of target copies present in each sample, were converted to relative expression fold changes in proinflammatory and innate immune genes. The relative gene expression levels were determined at 15 and 30 DPP compared to expression at 60 DPP in the same animal. It was an important aim of this study to investigate whether postpartum uterine involutionary responses are inflammatory in cows after calving. The levels of mRNA expression for the innate immune genes analysed in the present study for the individual animals are presented as appendix figures in this thesis. The innate immune genes were grouped into four categories based on their immune role based on previous reports in the literature. The classes of genes were markers, sensors, chemokines, cytokines, transcription factor, AMPs and APPs. The genes, mean expression values and significance levels are summarised in Table 3.8. The normaliser gene used in the present study was *H3F3A*.

Table 3.8: Mean mRNA fold changes for innate immune gene expression in uterine tissue from cows at 15 and 30 DPP. *H3F3A* was the normaliser for gene expression relative to 60 DPP.

Classs	Gene Symbol	Gene Name	15 DPP	<i>P</i> value	30 DPP	<i>P</i> value
Marker	<i>CD45</i>	Protein tyrosine phosphatase receptor type C	104	0.01	13	0.05
	<i>CD14</i>	Myeloid marker	8	0.01	3	0.05
Sensor	<i>TLR2</i>	Toll-like receptor 2	11	0.03	3	0.05
	<i>TLR3</i>	Toll-like receptor 3	5	0.05	1	0.05
	<i>TLR4</i>	Toll-like receptor 4	16	0.02	3	0.04
Chemokine	<i>I-L8</i>	Interleukin 8	117	0.01	27	0.03
Cytokine	<i>TNF-α</i>	Tumour necrosis factor	42	0.01	7	0.05
	<i>IL-1β</i>	Interleukin 1, beta	31	0.01	4	0.05
	<i>IL-6</i>	Interleukin 6	18	0.02	4	0.03
	<i>IFNG</i>	Interferon, gamma	68	0.01	10	0.05
Transcription factor	<i>NFκB1</i>	Nuclear factor KappaB1	35	0.04	8	0.05
AMPs	<i>BNBD4</i>	Bovine neutrophil beta-defensin 4	23	0.01	2	0.04
	<i>DEFB5</i>	Defensin beta 5	58	0.01	17	0.02
	<i>TAP</i>	Tracheal antimicrobial peptide	110	0.01	10	0.05
	<i>LAP</i>	Lingual antimicrobial peptide	11	0.03	11	0.03
APPs	<i>SAA3</i>	Serum amyloid A 3	37	0.01	18	0.04
	<i>HP</i>	Haptoglobin	36	0.04	4	0.05
	<i>SAA4</i>	Serum amyloid A 4	-5	#ns	2	#ns

#Genes not significantly differentially expressed.

3.4.8.3.1 Analysis of genes encoding cell surface markers in uterine tissue during the postpartum period

To assess the infiltration of professional immune cells into the uterus after calving, the mRNA encoding the pan-leukocyte marker protein tyrosine phosphatase receptor type C, *CD45* was analysed. At 15 DPP, compared to 60 DPP, the relative *CD45* gene expression was upregulated significantly by 104 fold (Table 3.8, $P < 0.05$). At 30 DPP there was a 13 fold mean increase in *CD45* gene expression. Figure 3.16 shows the individual animal gene expression at 15 and 30 DPP, showing a decrease in the professional immune cell population from 15 to 30 DPP. Similarly, expression of *CD14*, a marker of myeloid cells was also significantly increased (8 fold at 15 DPP and 3 fold at 30 DPP) in all the cows compared to expression at 60 DPP ($P < 0.05$). The level of gene transcript expression for *CD14* decreases with days after calving from 15 to 30 DPP, as shown in Figure 3.17.

3.4.8.3.2 Analysis of expression of genes encoding TLRs in uterine tissue during the postpartum period

Gene transcripts for the three mammalian *TLRs* were detected in bovine endometrium (Figure 3.18) using qRT-PCR. The level of expression did not differ significantly between the individual cows at each time point. All the animals expressed mRNA for *TLR2*, *TLR3*, and *TLR4* in endometrial biopsies at a higher level at 15 and 30 DPP compared to 60 DPP ($P < 0.05$; Table 3.8). Gene expression profiles for *TLR4* revealed significant upregulation by 16 and 2 mean fold at 15 and 30 DPP, respectively. Similarly, the levels of *TLR2* and *TLR3* gene expression were significantly increased at 15 and 30 DPP in all the endometrial tissues. Although significantly upregulated compared to expression at 60 DPP, the relative fold change for *TLR3* gene expression was small (5 at 15 DPP and 1 at 30 DPP). As shown in Figure 3.18, there was a similar pattern of decreasing expression from 15 to 30 DPP for the three TLR genes.

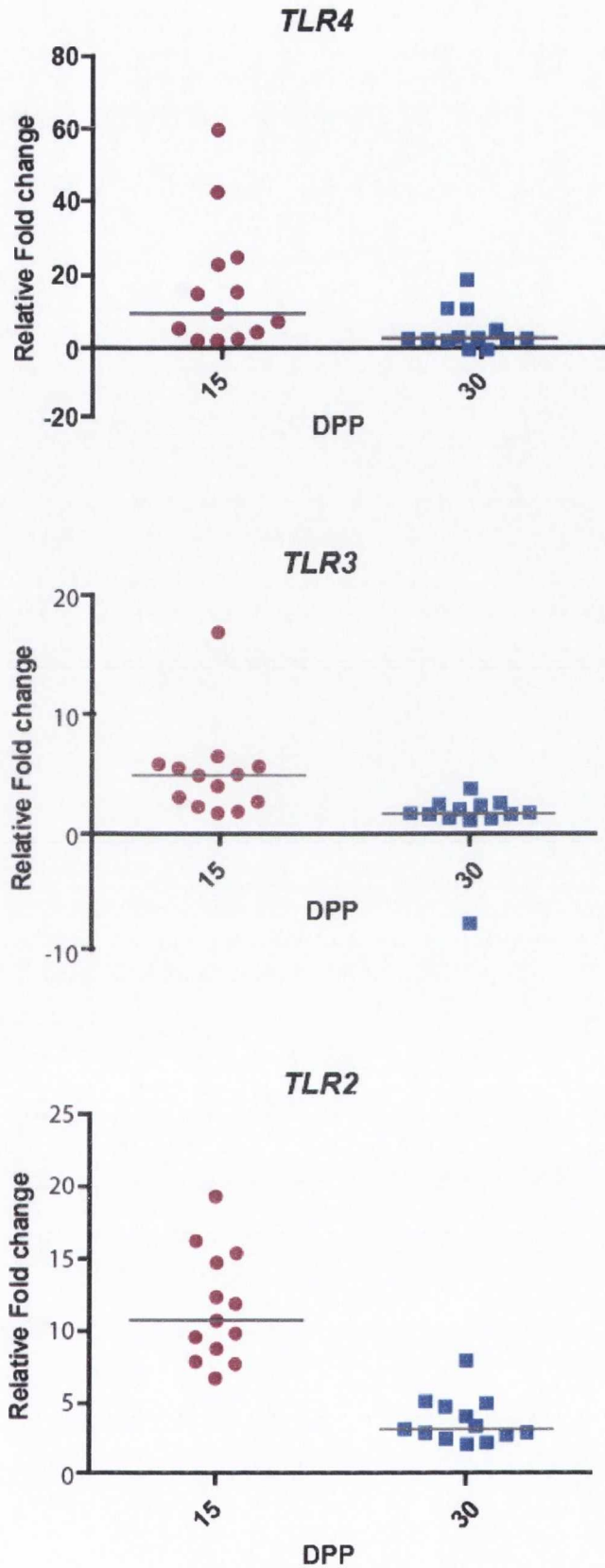


Figure 3.18: mRNA for TLRs (*TLR4*, *TLR3* and *TLR2*) quantified in the endometrial tissue sections from the individual cows at 15 and 30 DPP. The expression levels of the genes were calculated as mean fold increase compared to the expression level in the same animal tissue at 60 DPP ($P < 0.05$). The median level of gene expression is indicated by the horizontal line.

3.4.8.3.3 Analysis of expression of genes encoding transcription factor NFκB1 in uterine tissue during the postpartum period

To determine whether downstream signalling in the uterus of cows after calving involves the nuclear transcription factor *NFκB1*, relative gene expression was analysed at 15 and 30 DPP using qRT-PCR. As shown in Figure 3.19, mRNA levels for *NFκB1* were significantly increased by mean fold of 35 and 8 fold at 15 and 30 DPP, respectively ($P < 0.05$). The level of the mRNA in the uterine tissues decreased in relative quantity from 15 to 30 DPP.

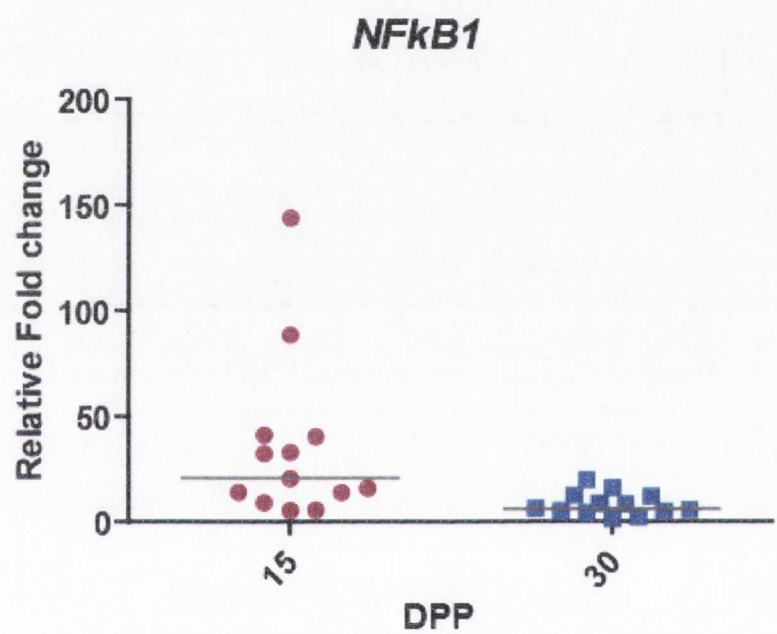


Figure 3.19: mRNA for the transcription factor *NFκB1* quantified in the endometrial tissue sections from the individual cows at 15 and 30 DPP. The expression levels of the genes were calculated as mean fold increase compared to the expression level in the same animal tissue at 60 DPP ($P < 0.05$). The median level of gene expression is indicated by the horizontal line.

3.4.8.3.4 Analysis of expression of genes encoding chemokine IL-8 in uterine tissue during the postpartum period

The mRNA expression of the chemokine *IL8* analysed by qRT-PCR was upregulated in all the animals at 15 and 30 DPP. As shown in Figure 3.19, although significantly induced after calving, *IL-8* gene expression decreased from mean fold of 117 fold at 15 DPP to 27 at 30 DPP, relative to 60 DPP in all the cows ($P < 0.05$).

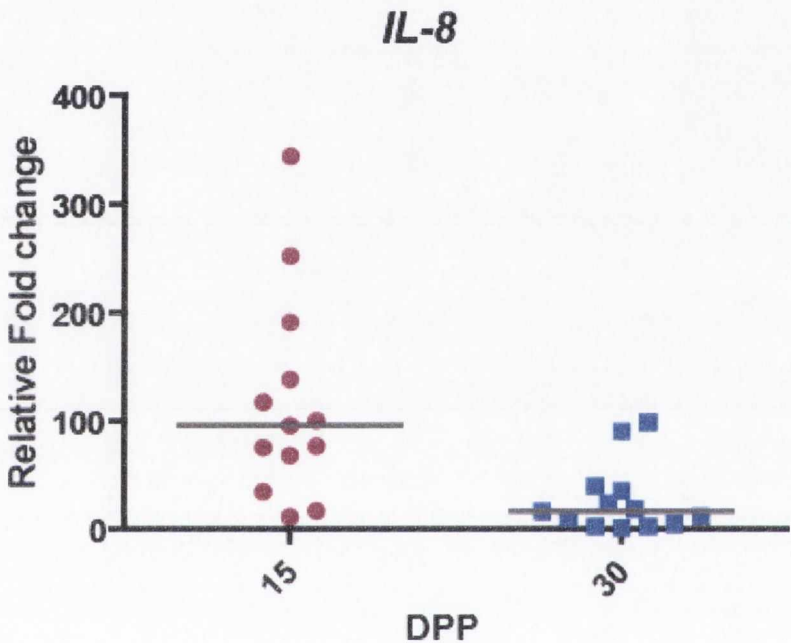


Figure 3.20: mRNA for the chemokine *IL-8* quantified in the endometrial tissue sections from the individual cows at 15 and 30 DPP. The expression levels of the genes were calculated as mean fold change compared to the expression level in the same animal at 60 DPP ($P < 0.05$). The median level of gene expression is indicated by the horizontal line.

3.4.8.3.5 Analysis of expression of genes encoding cytokines *IL-1 β* and *IL-6* in uterine tissue during the postpartum period

The expression of genes encoding the proinflammatory cytokines *IL-1 β* and *IL-6*, which drive inflammatory responses as well as the production of APPs (Andus *et al.*, 1988), were also examined by qRT-PCR. Gene expression of these cytokines was significantly increased at 15 DPP by 31 and 18 fold, respectively ($P < 0.05$, Table 3.8). Interestingly, both cytokines were similarly upregulated by a mean fold change of 4 at 30 DPP (Figure 3.21). The significant upregulation of both cytokines at 15 DPP in all animals suggests a proinflammatory uterine environment. In one cow with a high *IL-6* expression at 30 DPP, severe inflammation was detected at histological assessment.

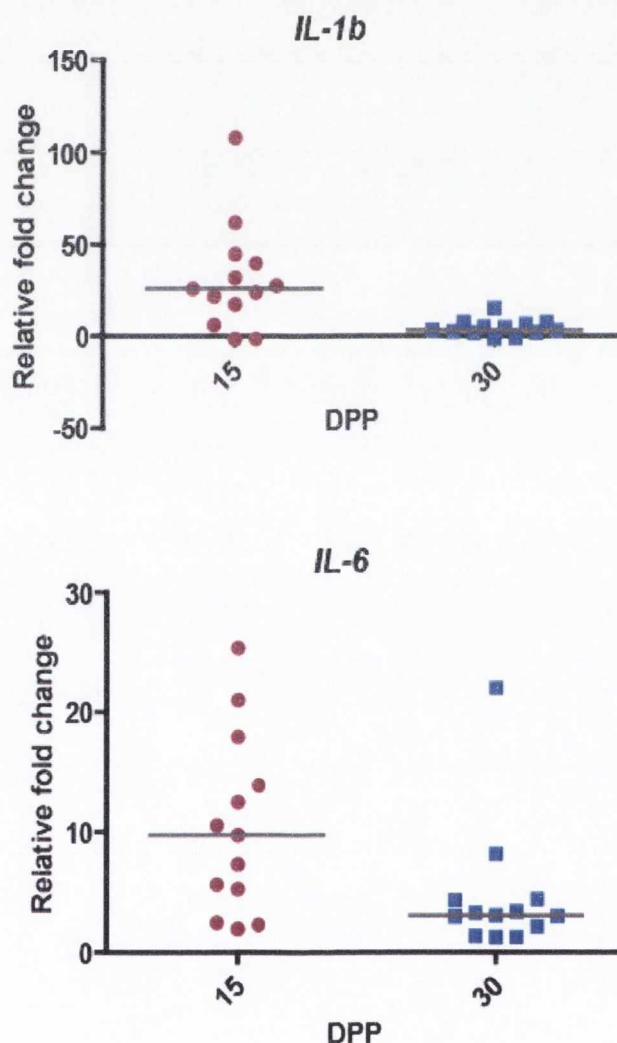


Figure 3.21: mRNA for cytokines quantified in the endometrial tissue sections from the individual cows at 15 and 30 DPP. The expression levels of the genes were calculated as mean fold change compared to the expression level in the same animal tissue at 60

DPP ($P < 0.05$). A similar pattern of expression is shown for *IL-1 β* and *IL-6*. The median level of gene expression is indicated by the horizontal line.

3.4.8.3.6 Analysis of expression of genes encoding cytokines *TNF- α* and *IFNG* in uterine tissue during the postpartum period

Relative *TNF* and *IFNG* gene expression was significantly upregulated, showing mean fold increases of 42 and 68 at 15 DPP, respectively ($P < 0.05$, Table 3.8). At 30 DPP, expression of the two cytokines was significantly increased by mean fold changes of 7 and 10 ($P < 0.05$) relative to expression at 60 DPP in each animal tissue. Similar to the trend of expression in other cytokines, *IFNG* and *TNF* relative gene expression was lower at 30 DPP compared to 15 DPP (Figure 3.22).

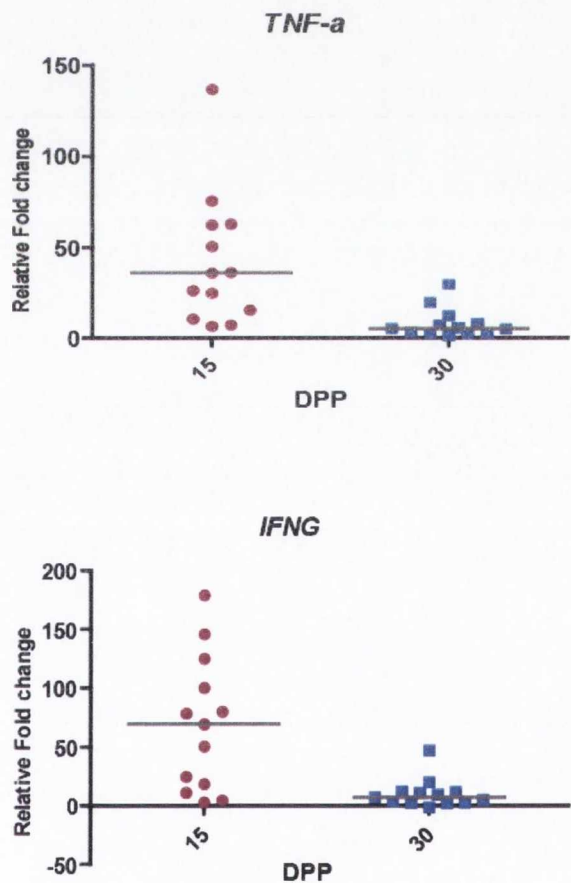


Figure 3.22: mRNA for proinflammatory cytokines (*TNF- α* and *IFNG*) quantified in the endometrial tissue sections from the individual cows at 15 and 30 DPP. The expression levels of the genes were calculated as fold induction compared to the expression level in the same animal tissue at 60 DPP ($P < 0.05$). The median level of gene expression is indicated by the horizontal line.

3.4.8.3.7 Analysis of expression of genes encoding APPs in uterine tissue during the postpartum period

Three APP genes *HP*, *SAA3* and *SAA4* were examined for changes in expression at 15 and 30 DPP in all the endometrial tissues relative to 60 DPP using qRT-PCR. A novel significant upregulation in mRNA levels in two of the three APP genes - *SAA3* and *HP* by mean fold values of 37 and 36 fold in the endometrial tissues, respectively, relative to 60 DPP was detected ($P < 0.05$; Table 3.7). *SAA3* is highly induced during the inflammatory response of the APR, and was significantly upregulated by a mean fold value of 18 at 30 DPP (Figure 2.23). All the endometrial tissues displayed a significant, although low *HP* gene expression at 30 DPP (mean fold increase of 3, Figure 2.23). The expression of *HP* was confirmed by calorimetric assay of haptoglobin in serum samples obtained at the same time as the endometrial biopsy. Expression of the *SAA4* gene was not significantly induced; some endometrial tissues showed upregulation while others showed downregulation at 15 and 30 DPP ($P > 0.05$; Table 3.8).

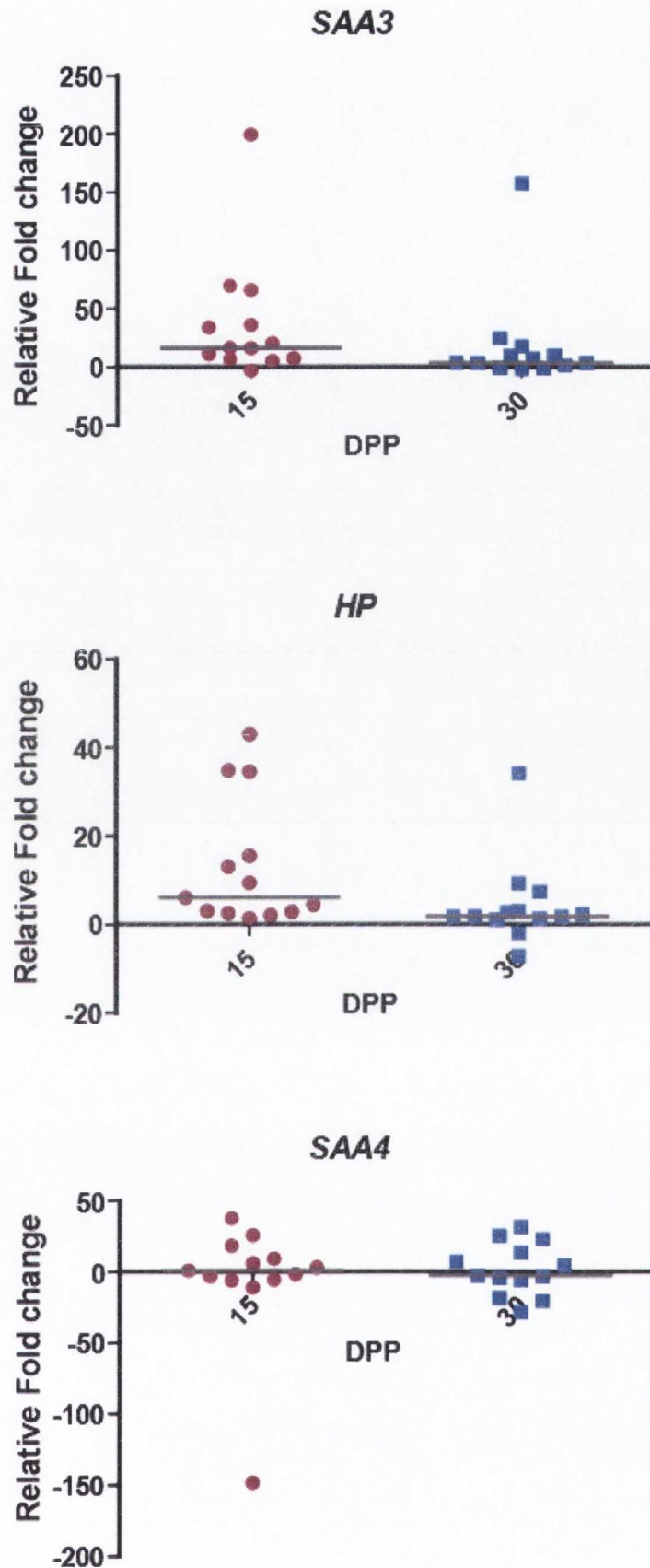


Figure 3.23: Expression levels of mRNA for APPs (*SAA3*, *HP* and *SAA4*) quantified in the endometrial tissue sections from the individual cows at 15 and 30 DPP. The expression levels of the genes were calculated as mean fold change compared to the

expression level in the same animal tissue at 60 DPP ($P < 0.05$). The median level of gene expression is indicated by the horizontal line.

3.4.8.3.8 Analysis of AMP gene expression during the postpartum period

Four AMP genes *LAP*, *TAP*, *BNBD4*, and *DEFB5* were examined for changes in expression in all the cows at 15 and 30 DPP relative to 60 DPP in the same animal, and the findings are presented in Table 3.8. All 4 genes showed a similar pattern of expression at 15 and 30 DPP in all the animals (Figure 3.24 and 3.25). *TAP* and *DEFB5* were the two genes which were highly significantly upregulated, with increase in mean fold expression of 110 and 58, respectively, at 15 DPP ($P < 0.05$; Table 3.7). At 30 DPP, expression for the two AMPs was 10 and 17 mean fold increase, respectively, which was consistent with moderate inflammation detected in the uterine sections on histological assessment. An interesting observation was *LAP*, which was similarly expressed in all the cows, showing a fold increase of 11 at both time points, despite differences in histological inflammation scores at 15 and 30 DPP. *BNBD4* gene expression was significantly increased by 23 mean fold at 15 DPP and 2 mean fold at 30 DPP.

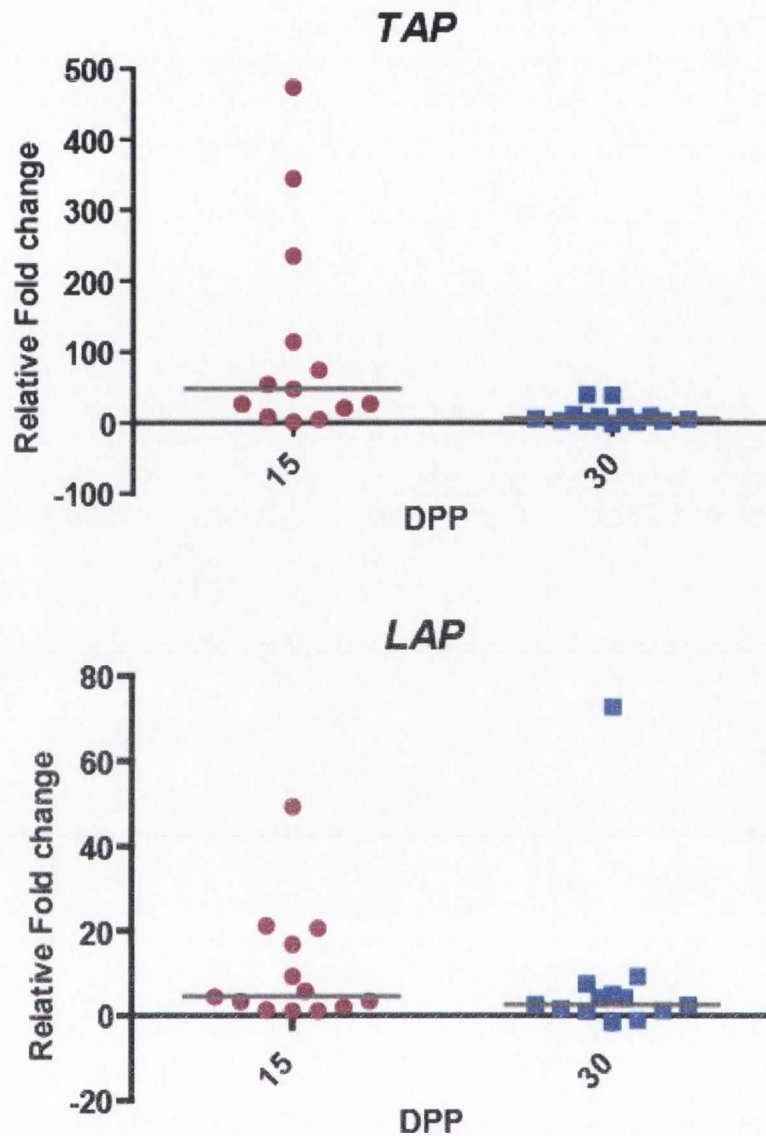


Figure 3.24: Expression of mRNA for AMPs *TAP* and *LAP* quantified in the endometrial tissue sections from the individual cows at 15 and 30 DPP. The expression levels of the genes were calculated as mean fold increase compared to the expression level in the same animal tissue at 60 DPP ($P < 0.05$). The median level of gene expression is indicated by the horizontal line.

3.4.9 Unique bovine innate immune gene expression mirrors histological change in uterine tissues during the postpartum period

It was an important aim of this study to examine whether innate immune gene expression mirrors histological change detected in uterine tissues during the postpartum period. In the present study, the detected histological changes depict temporal appearance of normal involution in cows. The gene expression results imply that immunomodulatory mechanisms underline effective involutionary reaction to restore a healthy uterine environment suitable for the next pregnancy.

The uterine inflammation of each tissue at each time point (15, 30, 60 DPP) was characterised and blindly classified into four histology grades from 0 (no inflammation) to 3 (severe inflammation). Of the 18 genes examined by qRT-PCR, increased expression for 16 genes was detected in the cows with uterine inflammation at 15 DPP and 30 DPP ($P < 0.05$, Table 3.8). The upregulated genes included markers (*CD45*) sensors (*TLR4*), mediators (*IFN*, *IL-1 β* , *TNF- α*) and effector immune proteins (*SAA3*, *HP*, *TAP*, *DEFB5*), and the results are shown in Figure 3.26. A mean fold increase of 104 in *CD45* gene expression at 15 DPP indicated an influx of leukocytes (severe inflammation) in the uteri of all the cows. At 30 DPP, *CD45* relative gene expression was only mean fold increase of 13, suggesting a drastic reduction in immune cell influx with resolution of inflammation detected histologically. Gene expression of the *TLR4* mirrored resolution of inflammation from a severe score (histological category 3) at 15 DPP to a mild score (category 1 or 2). Similarly the 3 cytokines- *IFNG*, *IL-1 β* , *TNF- α* – were increased in expression at 15 DPP in all the cows, consistent with severe endometrial inflammation (category 3). *NFkB1* gene expression showed a pronounced change, with a 35 and 8 mean fold increase, respectively, at 15 and 30 DPP. In relation to *SAA3* and *HP* genes, a close relationship between the level of gene expression and severity of uterine inflammation was evident, as shown in Figure 3.26. Similarly, increased expression of the AMPs *TAP* and *DEFB5* was observed at 15 DPP when the endometrial tissues were severely inflamed. Overall, the expression pattern of the immune genes showed a direct link with histological changes observed.

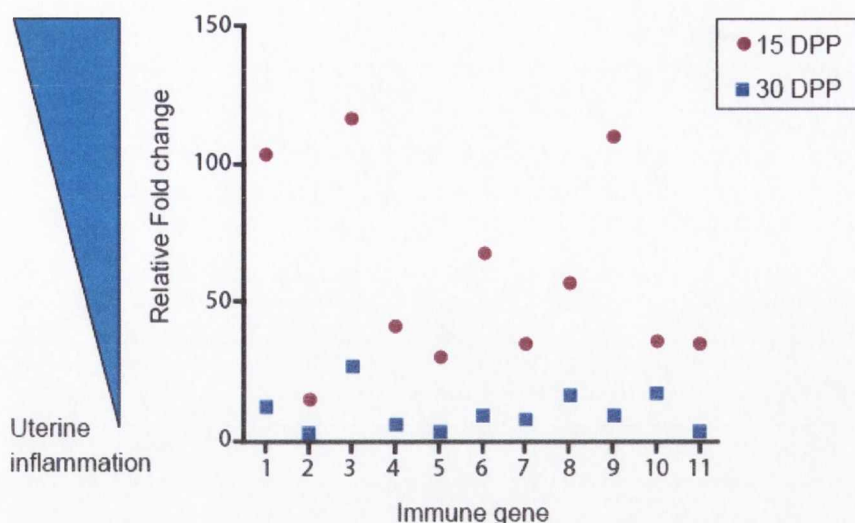


Figure 3.26: Uterine innate immune gene expression mirrored inflammation at 15 (circles) and 30 (squares) DPP in the uterine tissue sections relative to expression in the same cow at 60 DPP. The blue triangle represents the severity of uterine inflammation (histology score 0 – 3). mRNA expression of the genes was reduced in abundance with resolution of inflammation. Results are shown for mean fold increases for genes encoding markers, sensors, mediator and effector molecules of innate immunity listed below. As shown, there was increased expression for 16 innate immune genes in the cows with uterine inflammation at 15 DPP and 30 DPP ($P < 0.05$,

- | | |
|----------------------------------|-----------------------------------|
| 1- <i>CD45</i> | 2- <i>TLR4</i> |
| 3- <i>IL-8</i> | 4- <i>TNF-α</i> |
| 5- <i>IL-1β</i> | 6- <i>IFNG</i> |
| 7- <i>NFkB1</i> | 8- <i>DEFB5</i> |
| 9- <i>TAP</i> | 10- <i>SAA3</i> |
| 11- <i>HP</i> | |

3.5 Discussion

Significant progress has been made in recent times on the understanding of postpartum uterine immunology (Chapwanya *et al.*, 2009; Dhaliwal *et al.*, 2001; Hussain, 1989; Kim *et al.*, 2005a; Llewellyn *et al.*, 2008; Sheldon *et al.*, 2009). To keep economic losses as low as possible, it is important to have an early and sensitive diagnosis of cows at risk of poor fertility after calving. During the postpartum period, characteristic changes occur in the bovine endometrium regarding its composition and differentiation status. Scientists, veterinarians and cattle producers are increasingly interested in finding out how these changes affect fertility. The dilemma is that there has been intense selection pressure for production traits such that every cow is expected to re-conceive within a short period after calving to ensure economic viability. The present study investigated postpartum uterine immunity and related histological changes observed in uterine tissue after calving. Characterisation of cellular and molecular events governing inflammation has greatly enhanced our knowledge of mammalian immune responses, and continues to do so. In this chapter therefore, we have addressed two important aspects of bovine uterine immunity - the innate immune gene expression pattern and histological change during the postpartum period. These elements of uterine involution warrant particular attention because they have wide ranging implications in resolution of inflammation and successful conception. The bovine postpartum uterine system is a complex network of cellular and intracellular molecules that effect responses to a diverse range of stimuli. For this reason we employed an integrated approach to investigating involutionary inflammatory responses in the bovine uterus after calving.

Early diagnosis and treatment of any uterine disease are the key components of successful fertility outcome in postpartum cows. In our study we evaluated the importance of systematic clinical examinations on 15, 30 and 60 DPP in recently calved cows. While clinical examinations are frequently performed in postpartum cows, and are highly diagnostic for overt signs of ill-health (Noakes *et al.*, 1994; Sheldon *et al.*, 2009), they yield limited information on the mechanisms that underlie the tissue changes at a molecular level during this period. Thus, there is a need for further techniques for evaluating cows after calving.

Based on clinical evaluations, all the cows in this study had clinical parameters within normal ranges, similar to findings in other studies (Drillich *et al.*, 2000). In that study, Drillich and co-workers did not find any significant differences in reproductive performance in cows systematically examined by rectal palpation between Day 20 and Day 26 after calving. Clinical examination becomes valuable if any cows have uterine disease particularly subclinical endometritis (Azawi *et al.*, 2008b; Drillich *et al.*, 2003; LeBlanc *et al.*, 2002). However, clinical examinations alone do not shed light on the cows at risk of infertility, hence the need for additional techniques to assess postpartum cows.

Here, extensive ultrasonography, endocrinology and microbial culture revealed that all the cows had similar profiles and that none of the cows developed clinical uterine disease. The use of transrectal ultrasonography to evaluate reproduction in cows has enhanced our understanding of the ovarian and uterine processes during the bovine oestrous cycle and pregnancy (Buczinski *et al.*, 2009; Thiengtham *et al.*, 2008). Ultrasonographic methods have enhanced our ability to manipulate pregnancy and oestrous cyclicity in order to improve cattle fertility. In the present study, ultrasound provided a noninvasive view of the uterine environment in all the cows. There were no detectable differences in the ultrasonographic images of the cows at 15, 30 and 60 DPP. Although the technique does not reveal the physiological processes in the involuting uterus, imaging must be performed during postpartum to provide realtime images, which could not be attainable on palpation or post-mortem examination (Fricke & Lamb, 2005; Rajamahendran *et al.*, 1994). For pregnancy diagnosis, ultrasonographic imaging accurately identified the embryo as a distinct C-shaped echogenic structure within the nonechogenic, fluid-filled vesicle in the uteri of 10 fertile cows. This was a distinct advantage because no direct physical manipulation of the gravid reproductive tract was necessary and the embryos were detected earlier than would have been possible by palpation *per rectum* alone.

In previous studies, the uteri of postpartum cows have been shown to experience an influx of pathogens, particularly bacteria (Foldi *et al.*, 2006; LeBlanc *et al.*, 2002; Williams *et al.*, 2007). The capacity of the postpartum uterus to resolve infection depends on the ability to recognise a wide range of microbial ligands because of the mixed population of bacteria invading the uterus at calving. In our study, we identified mixed populations of bacterial species *in utero* at 15, 30 and 60 DPP. Although none of

the cows developed uterine disease, *E. coli* and *A. pyogenes*, the bacteria commonly cultured in clinical cases of metritis or endometritis (Foldi *et al.*, 2006; Williams *et al.*, 2007), were isolated. From these findings, even though all animals were exposed to similar levels of microbial contamination with bacteria from the environment, cows successfully cleared the bacteria from the uterus and conceived.

Histopathological examination of biopsy sections provides accurate and reliable characterisation of the uterus (Ahmadi *et al.*, 2005; Frazier *et al.*, 2001; Moraitis *et al.*, 2004), and as such, was used to classify uterine biopsy samples from each cow during the postpartum period. Morphological differences were observed between the differing grades of inflammation. The initial cellular response to an infection of the uterine wall is an influx of polymorphonuclear leukocytes (PMN) and macrophages (Bondurant, 1999). At 15 DPP, the severe uterine inflammation detected in all the cows was consistent with findings in other studies (Azawi *et al.*, 2008a; Bondurant, 1999; Hartigan *et al.*, 1974). At 30 DPP, the quantity of bacteria identified on culture, and leukocytes detected on histology were reduced, highlighting the efficiency of uterine defence mechanisms in clearing infection and resolving inflammation. Furthermore, 53% of the cows had cleared all bacteria from the uterus. At 60 DPP, the uterine tissue had completely involuted as evidenced by glandular-rich mucosae with small numbers of inflammatory cells detected. There were no observable differences in the histological structure of the uteri of the cows showing that the uteri were ready for the next pregnancy. The findings here suggest that endometrial biopsies have value for assessing reproductive performance in cows. Previously the technique was associated with deleterious effects on fertility (Bonnett & Martin, 1995 ; Zaayer & van der Horst, 1986). From the findings of the present study, it is recommended to use endometrial biopsy in postpartum cows because the technique is safe and causes no adverse effects on fertility. The lesions resulting from the biopsy healed rapidly with no complications. On gross examination of the female genital tracts, no lesions attributable to biopsy were detected.

The histological changes observed in the uterine tissue are preceded and underpinned by gene expression alterations at a molecular level (Greenfield *et al.*, 2000). We previously described upregulated expression of cell markers, TLRs and cytokines as well β -defensins and AMPs, based on samples collected from dairy cows at two weeks after calving (Chapwanya *et al.*, 2009). In the present study, increases in relative expression of genes encoding cell surface markers, TLRs, cytokines, AMPs and APPs were

detected at 15 and 30 DPP, compared to 60 DPP levels, in the same animal. The overall gene expression pattern of the immune response during uterine involution in postpartum beef cows depicts a dynamic inflammatory environment. These findings are similar to other studies where proinflammatory cytokines and AMPs were significantly induced (Pioli *et al.*, 2004; Riollet *et al.*, 2000; Roosen *et al.*, 2004; Schaefer *et al.*, 2004). These previous studies focussed generally on changes of the mammary glands infected naturally or experimentally in cattle, and the genital tracts in humans. Here we focus on the identification of differentially expressed genes in the bovine endometrium during the course of uterine involution in recently calved cows, in particular, between the early and late postpartum stages. Upregulation of cell-surface receptor markers *CD45* and *CD14* at 15 DPP indicates leukocyte cell recruitment to the postpartum uterus of all cows during involution which was detected histologically. The striking upregulation of mRNAs for proteins of the innate immune response and for proteins involved in APR at this stage indicates changes in the composition of the uterine tissue during involution, similar to other reports (Kim *et al.*, 2005b). Despite the cows being exposed to a wide variety of bacterial species after calving, the identical severity of inflammation (all the cows being classified as histology category 3) suggests that infection and inflammation of the uterus after parturition are physiological processes in cattle. The genes of known function were also analysed in the context of different roles in the involuting uterus where they may perform immuno-regulatory roles in addition to antimicrobial or bacteriostatic activities. A characteristic up-regulation of cytokines, APPs and AMPs was in line with our earlier finding that the postpartum uterine environment is inflammatory.

The unique finding that bovine innate immune gene expression patterns mirror histological change in uterine tissues during the postpartum period strengthens our belief that uterine involution is an inflammatory process. The physiological, immunological or pathological status of the uterus can therefore be accurately studied using the endometrial biopsy technique described in the present study. These data are in agreement with other studies that concluded that if biopsy is performed with due care, informative data on the status of the endometrium can be obtained (Bauersachs *et al.*, 2008; Herath *et al.*, 2009; Mitko *et al.*, 2008), including the composition of uterine cell populations. It is worth noting the contribution of both migrant and resident uterine cells during this period. This relationship may be the reason for successful orchestration of

local uterine responses which resolve inflammation and prepare the uterus for the next pregnancy.

In general terms, infection and inflammation can have devastating consequences to fertility and pregnancy in mammals (Horne *et al.*, 2008; King *et al.*, 2003a). The present study is the first to collate innate immune and histological changes in bovine endometrium during the postpartum period in cows which do not develop clinical uterine disease. Therefore, in cattle, the infection and inflammation of the uterine wall during and after parturition must be accepted as a physiological process. A number of innate immune molecules were identified, most prominently the APPs and AMPs, involved in the marked involutionary functional and structural changes of the endometrium. To complete the analysis of gene expression during the postpartum period relevant to fertility, further analyses need to be performed at the time of insemination, and the function of candidate genes, e.g. *SAA3* and *HP* characterised. Furthermore, the differentially regulated genes identified in this study represent an important basis for future studies, e.g. pathogenesis of uterine disease during the postpartum period. In addition, differential diagnosis of fertility problems in cattle via array-based analysis of endometrial biopsies may be possible.

The endometrium is a complex tissue composed of many different cell types. For the interpretation of differential gene expression there is a need to identify the distinct cell types elaborating the molecules detected. Endometrial epithelial cells which line the entire uterine mucosal surface are known to have the capacity to produce cytokines and chemokines (Wira *et al.*, 2005b). Based on the fact that the genes encoding APPs and AMPs have increased in expression in severely inflamed uterine tissue, and that *E. coli* is a common cause of metritis, there is a need to investigate the role of bovine endometrial epithelial cells during the local uterine proinflammatory response to *E. coli* infection.

Chapter 4

Bovine endometrial epithelial cells: sentinels of uterine innate immune response to *E. coli*

4.1 Introduction

Infection of the uterus is an important cause of reduced fertility in dairy cows (Foldi *et al.*, 2006; LeBlanc *et al.*, 2002). Chronic infection and inflammation of the uterus is thought to be the primary cause of sub-fertility (Azawi, 2008; LeBlanc, 2008), the common aetiological agents of which are bacteria (Williams *et al.*, 2005). Gram negative coliform bacteria such as *E. coli* frequently cause acute inflammation of the uterus.

The bovine uterus is protected from infection by physical barriers as well as innate and adaptive immune mechanisms (Entrican & Wheelhouse, 2006). Endometrial epithelial cells line the entire uterine mucosal surface forming a physical barrier and contributing to local innate immunity (Fahey *et al.*, 2006). The innate defence of the endometrium against bacteria is dependent on uterine immunity, including AMPs, and APPs. Owing to their location, endometrial epithelial cells are the first cells to contact and recognise microbial insults. They can be considered innate immune cells as they detect pathogenic molecules *via* surface receptors such as TLRs (Davies *et al.*, 2008a; Pioli *et al.*, 2004; Schaefer *et al.*, 2004). TLR 4, for which *NFkB1* is the principal downstream signalling transcription factor, is expressed by endometrial cells stimulated with the LPS ligand *in vitro* in humans (Wira *et al.*, 2005b) and cattle (Herath *et al.*, 2006b). Furthermore TLR 4 is thought to be the principal recognition pathway through which endometrial cells detect the LPS ligand or *E. coli*, the Gram-negative bacterial pathogen commonly isolated from infected uterine tissue (Mateus *et al.*, 2002). Endometrial epithelial cells also secrete proinflammatory cytokines which are important for the activation of macrophages and other classical immune cells involved in the orchestration of the innate immune response (Quayle, 2002).

Human uterine epithelial cells have been shown to produce AMPs upon sensing microbial pathogens (King *et al.*, 2003a), and also initiate the innate immune response *via* *IL-1*, *TNF- α* and *IL-6* cytokines (Eriksson *et al.*, 2006; Kopf *et al.*, 1994). Another important immunological role for endometrial cells described in humans is the linking of the innate and adaptive immune responses (Wira *et al.*, 2005a).

In cattle, APPs are thought to be strongly induced during the APR (Huntoon *et al.*, 2008). The two classes of APP molecules are classically induced to restore homeostasis or limit pathogen growth (Baumann & Gauldie, 1994; Streetz *et al.*, 2001). Type 1 APPs such as *SAA* are induced by *IL-1* and *IL-6* type cytokines while type 2 APPs e.g *HP* are induced by *IL-6* cytokines.

APPs were traditionally thought to be hepatically derived during the APR and have a role against infection or injury, and maybe systemic markers of inflammation (Baumann & Gauldie, 1994; Chan *et al.*, 2004; Horadagoda *et al.*, 1999; Huntoon *et al.*, 2008). Production of *SAA3* and *HP* in cattle is a sensitive method of monitoring health status (Ganheim *et al.*, 2007; Nielsen *et al.*, 2004). Although the liver is the primary site for the production of the APPs, extrahepatic expression of *SAA3* has been observed (Larson *et al.*, 2005). APPs were also detected in postpartum cows showing signs of uterine disease (Chan *et al.*, 2009; Huzzey *et al.*, 2009). During the mammalian APR *in vivo*, a correlation between inflammation and systemic induction of *HP* expression was described (Suojala *et al.*, 2008; Wang *et al.*, 2001) providing evidence of an anti-inflammatory role for the APPs. The physiological function of *HP* is interaction with free Hb, thereby neutralizing and restricting its oxidative damage to various organs (Wang *et al.*, 2001). *HP*, like other APPs, is strongly induced by inflammatory mediators including *IL-6*.

There are no studies in cows examining local production of APPs by endometrial epithelial cells. The reports on endometrial epithelial cell elaboration of AMPs are mainly in human or mouse models (King *et al.*, 2003b; Pioli *et al.*, 2006). An earlier study in endometrial biopsies showed production of important innate immune effector molecules; AMPs and APPs (Chapwanya *et al.*, 2009), but their source in the bovine uterus is not clear. Little is known about extrahepatic intrauterine production of *SAA* and *HP*. In this *in vitro* study endometrial epithelial cells are stimulated with *E. coli* and the expression of TLRs, cytokines, AMPs and APPs examined using qRT-PCR.

4.2 Aims

- i. To examine production of innate immune effector molecules by primary cultures of endometrial epithelial cells

- ii. To investigate production of effector molecules by endometrial epithelial cells in response to *E. coli* stimulation

4.3 Methods and Materials

4.3.1 Bovine endometrial epithelial cells

Uteri from postpubertal non-pregnant cows were collected at a local abattoir immediately after slaughter and kept on ice until further processing in the laboratory. These cows had no evidence of genital disease based on visual inspection. The physiological stage of the reproductive cycle for each genital tract was determined by observation of the uterine and ovarian structures, particularly the corpus luteum (CL) (Ireland *et al.*, 1980). Tracts with an early CL (Stage 1) were selected for endometrial tissue and cell culture. Cell isolation was performed as previously described using only the endometrium from the horn ipsilateral to the CL while maintaining sterility at all times (Herath *et al.*, 2006b). Briefly, the external surfaces of the tracts were washed in 70% ethanol (EtOH) and the endometrium dissected from the horn. The endometrial strips were placed in 60 ml of Endo Strip wash. The strips were then transferred into a second container with 25 ml Hanks Balanced Salt Solution (HBSS) prior to being sliced into smaller pieces measuring approximately 3 – 5 mm³ and washed in 70% EtOH. The tissue was then incubated for 10 minutes at 37°C in HBSS. The HBSS was decanted and the tissue digested in 25 ml sterile filtered digestive solution, which was made by dissolving 50 mg trypsin III (Roche, Lewes, UK), 50 mg collagenase II (Sigma, Poole, UK), 100 mg BSA (Sigma) and 10 mg DNase I (Sigma) in 100 ml phenol-red-free (HBSS; Sigma). After 1 h incubation in an agitating water bath at 37°C, the cell suspension was filtered through a 40-µm mesh (Fisher Scientific, Loughborough, UK) to remove undigested material and the filtrate was resuspended in phenol-red-free HBSS containing 10% fetal bovine serum (FBS; Sigma) and 3 µg/ml trypsin inhibitor (Sigma) (washing medium). The suspension was centrifuged at 700 × *g* for 7 min and after two further washes in washing medium, the cells were resuspended in RPMI 1640 medium (Sigma) containing 10% fetal bovine serum (PAA laboratories), 50 IU/ml penicillin, 50 µg/ml streptomycin and 2.5 µg/ml amphotericin B (Sigma). The cells were plated at a density of 6 × 10⁵ cells in 2 ml per well using 24-well plates (Nunc, Rochester, NY, USA). To obtain separate stromal and epithelial cell populations, the cell suspension was removed 17 h after plating, which allowed selective attachment of

stromal cells, and the removed cell suspension was then replated and incubated allowing epithelial cells to adhere. Stromal and epithelial cell populations were distinguished by cell morphology and the purity was greater than 95% as determined by microscopy and the differential production of prostaglandins – stromal cells do not produce prostaglandin F2 α (Herath *et al.*, 2006b). The culture medium was changed every 48 h until the cells reached confluence. All cultures were maintained at 37°C, 5% CO₂ in air, in a humidified incubator. Purified populations of epithelial cells were collected and stored immediately in RNeasy lysis buffer for mRNA isolation to examine gene expression.

4.3.2 *Escherichia coli* bacterial cells

A strain of *E. coli* (MS360) was isolated 7 days postpartum from the uterus of a cow with metritis and pyrexia suggesting that the isolate was pathogenic. The animal subsequently developed endometritis that persisted at least until 28 days postpartum. The MS360 isolate was identified using standard laboratory techniques as a B1 strain, serotype O73:H16, which adhered to and invaded bovine endometrial epithelial and stromal cells *in vitro* (Sheldon, unpublished data). The bacteria were heat killed as described (Herath *et al.*, 2006b). Briefly, MS360 was grown in LB broth (Sigma) at 37°C overnight and the number of colony forming units was determined by serial dilution in PBS and plate count. For the preparation of heat-killed *E. coli*, bacteria were placed on a heat block at 80°C for 30 minutes and diluted to a final working concentration equivalent to 10⁶ CFU/ml aliquots of cell culture medium containing DMEM, 10% foetal calf serum at 300 μ l in 30 ml of medium. The optimum concentration of heat killed *E. coli* was determined previously for epithelial and stromal cells (Herath *et al.*, 2006b).

4.3.3 Stimulation of bovine endometrial epithelial cells

To examine elaboration of AMP and APP effector molecules by endometrial epithelial cells, heat inactivated *E. coli* was used to stimulate 90% confluent epithelial cells (6 x 10⁵ cells in each well) which were stimulated for 6 and 24 h with 10⁶ *E. coli* colony forming units (CFU) in a 30 mm-diameter tissue dish (Sigma, UK) using the experimental protocol shown in Figure 4.1. Control cells were not treated. The endometrial cells were preserved in RNeasy lysis buffer and stored at -80°C until required for

RNA extraction and qRT-PCR analysis. The culture supernatants were harvested and frozen at -20°C until used to measure prostaglandin E2 as previously described (Herath *et al.*, 2006b). Briefly, samples were diluted in 0.05 M Tris buffer containing 0.1% gelatin and 0.01% sodium azide. Standards and tritiated tracers for the prostaglandin assays were purchased from Sigma and Amersham International PLC (Amersham, Little Chalfont, Buckinghamshire, UK), respectively. The antiserum was a generous gift from Professor N. L. Poyser (University of Edinburgh, Edinburgh, UK), the limit of detection was 2 pg/tube, and intra-assay and inter-assay coefficients of variation were 4.4 and 7.8%, respectively.

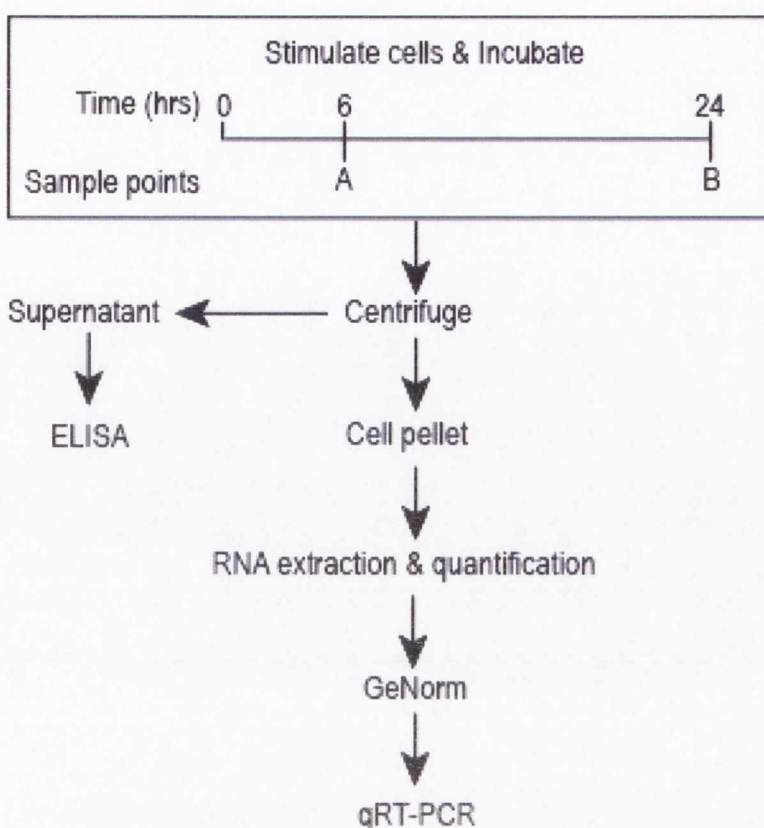


Figure 4.1: Schematic representation of the bovine endometrial epithelial cell stimulation study using heat-killed *E. coli* including sample harvesting and processing. Endometrial cells were transferred into 12 well plates and incubated for 24 hours. At time point 0, media was decanted from 24 h wells and replaced with the media alone in control wells, or medium plus heat-killed *E. coli* in stimulated wells. After 18 hours, media alone or medium plus heat-killed *E. coli*, was added to the 6 h wells. Supernatant and cell pellets were collected from all wells and stored at -80°C until required for ELISA analysis or RNA isolation. The study was performed using duplicate cell samples from 3 animals (N=3).

4.3.4 RNA Extraction, quantification and cDNA synthesis

Total RNA was extracted from cultured endometrial epithelial cells using the Qiagen RNeasy® method (Qiagen Ltd) and DNase treated according to the manufacturer's instructions. To ensure integrity and stability of RNA samples, yield and quality were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). One µg of total RNA from each sample was reverse transcribed into cDNA using an OmniScript III® first-strand synthesis kit using oligo (dT) primers according to the manufacturer's recommendations (Invitrogen Ltd, Paisley, UK). cDNA was quantified using a Nanodrop™ spectrophotometer and then diluted to 40 ng/µl for qRT-PCR analyses.

4.3.5 qRT-PCR

Gene expression was measured by qRT-PCR using the Mx3000P QPPCR System (Stratagene, UK). qRT-PCR analyses were performed with 12.5µl of quantitative PCR mix (Brilliant® SYBR® Green Master Mix) which contains SureStart® *Taq* DNA polymerase, MgCl₂, nucleotides, optimised reaction buffer components and SYBR® Green I dye (Stratagene: www.stratagene.com), 2.5µl of the forward primer together with 2.5 µl of the reverse primer (final concentrations 300 nM, 600 nM or 900 nM), and 80ng of cDNA template making a total of 25µl per reaction. All reactions were performed in duplicate under the following conditions: 95 °C for 15 minutes, followed by 40 cycles of 94 °C for 15 seconds, 55 °C for 20 seconds and 72 °C for 15 seconds. A non template control (NTC) was run for all genes with every plate to ensure the amplified product was due to the bovine endometrial epithelial cDNA and not contamination. Dissociation curves were assessed to confirm presence of single PCR product and absence of primer dimer formation.

4.3.6 GeNorm Analysis

All gene amplifications were normalised to expression of *GAPDH*, selected as the most stable normaliser gene across all endometrial culture samples. A panel of potential normalisers (*ACTB*, *GAPDH*, *MRPS6*, *MRPS9*, *MRPS15*, *H3F3A*, *PPIA*) were analysed

using GeNORM (Vandesompele *et al.*, 2002). The primer sequences of the normaliser genes are presented in Table A1 (Appendices).

4.3.7 Statistical analysis

Fold changes for qRT-PCR were calculated using the $2^{-\Delta\Delta C_t}$ method (Schmittgen & Livak, 2008) as the expression of the gene of interest in stimulated bovine endometrial epithelial cells, normalised to expression of the normaliser gene (*GAPDH*), and shown relative to the mean of the non-stimulated epithelial cells (n=3). Statistical analyses were performed using Mann-Whitney U tests as implemented in version 5.01 of StatView (SAS Institute Inc. Cary, NC, USA) and presented +/- SEM (Standard Error of the Mean).

4.4 Results

4.4.1. Normaliser gene selection

As shown in Figure 4.2, *GAPDH* and *RPS6* were the two most stable normaliser genes. *GAPDH* was selected on the basis of standard efficiency curves generated using serial dilutions (1:2 to 1:100) of cDNA samples.

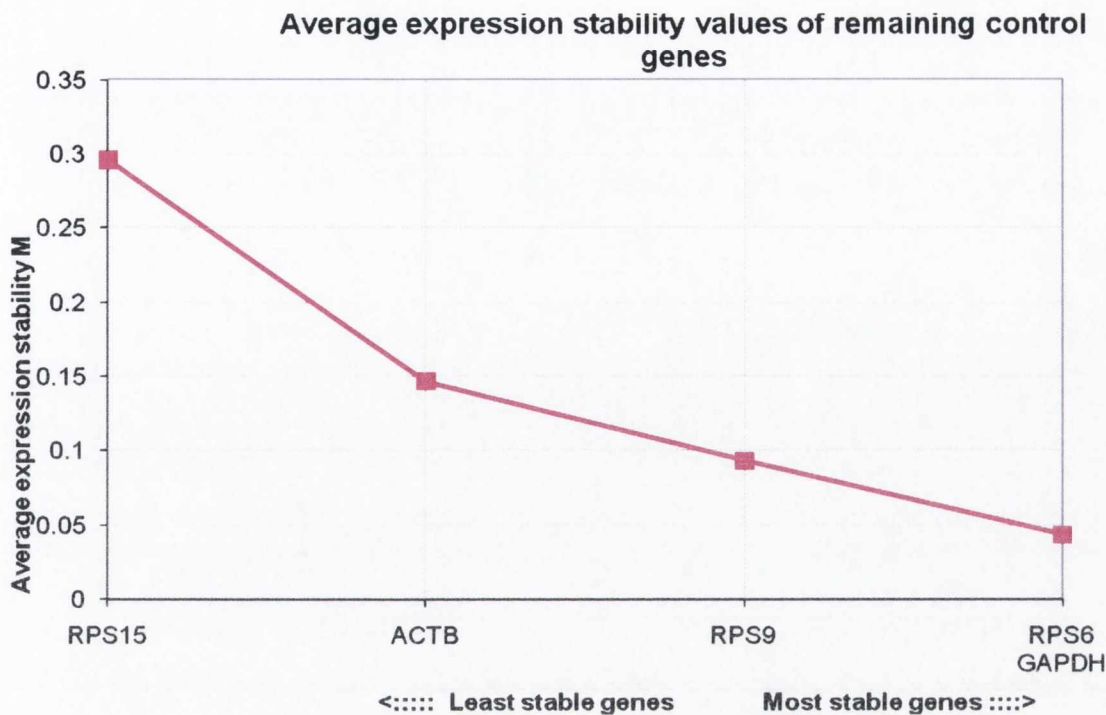


Figure 4.2: GeNorm calculation showing *RPS6* and *GAPDH* as the two most stable normaliser genes in endometrial epithelial cultures. Gene expression stability measure for the reference gene is determined as the average pair-wise variation, for that gene with all other tested reference genes. *GAPDH* was selected on the basis of superior PCR efficiency.

4.4.2 Expression of innate immune gene expression in endometrial cells

Gene expression levels were directly compared between stimulated and unstimulated cells. Gene transcripts for TLRs, cytokines, AMPs and APPs were detected in endometrial epithelial cells challenged with heat-killed *E. coli* for 6 h and 24 h. Of the 23 genes evaluated by qRT-PCR, significant differences were detected in 18. Abundances of mRNA for the 18 genes encoding innate immune and inflammatory molecules in three animals stimulated with heat-inactivated bacteria and normalised to *GAPDH* are presented as means ($n = 3$) in Table 4.1. Expression ratios are presented as fold increases. Most strongly induced genes after 6 h were the APPs involved in inflammatory response and defence against pathogens.

Table 4.1: Mean mRNA fold changes in endometrial epithelial cells challenged with *E. coli* 6 h and 24 h after stimulation compared to unstimulated cells. The significance of each fold change estimate is indicated (*P* value).

Class	Gene	6 h	P value	24 h	P value
Sensor					
	<i>TLR2</i>	2	0.001	4	0.001
	<i>TLR3</i>				
	<i>TLR4</i>	3	0.05	15	0.01
Mediator	<i>IL-1α</i>	82	0.001	26	0.002
	<i>IL-1β</i>	116	0.003	8	0.03
	<i>IL-6</i>	16	0.05	5	0.05
	<i>IL-8</i>	16	0.04	9	0.04
	<i>TNF-α</i>	161	0.002	167	0.002
	<i>NFκB1</i>	2	0.05	2	0.05
Effector	<i>DEFB1</i>	2	0.05	2	0.05
	<i>DEFB5</i>	2	0.05	2	0.05
	<i>BNBD4</i>	2	0.05	2	0.05
	<i>LAP</i>	66	0.001	17	0.05
	<i>TAP</i>	58	0.005	189	0.001
	<i>HP</i>	3	0.05	3	0.05
	<i>SAA3</i>	1700	0.001	1800	0.001

4.4.3 Analysis of TLRs gene expression by endometrial epithelial cells

Of the 10 TLR genes examined by qRT-PCR, 8 were significantly upregulated in stimulated endometrial epithelial cells – *TLR1* to *TLR7* and *TLR9* at 6 h and 24 h. The level of expression did not differ significantly between the stimulated and unstimulated cells for *TLR8* and *10*. *TLR4* was the most significantly induced TLR, showing a 3 fold increase after 6 h and a 15 fold after 24 h (Figure 4.3a; *P* < 0.05) Expression of the other TLRs is presented in Figure 4.3b.

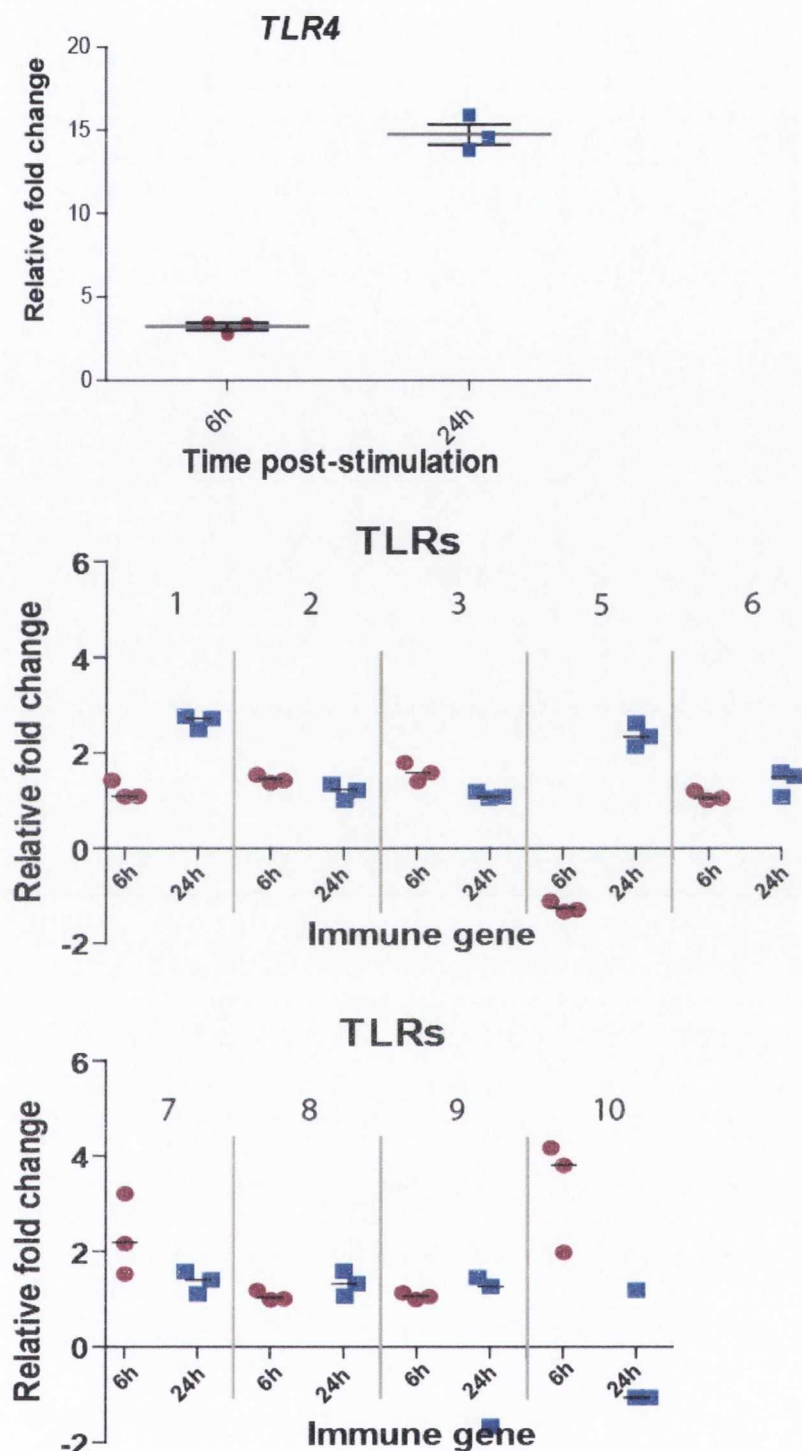


Figure 4.3: Mean gene expression profiles for TLRs from qRT-PCR analyses of endometrial epithelial cells from postpubertal non-pregnant cows ($n = 3$). All C_t values were corrected using the reference gene *GAPDH*. Each point on the graph represents the mean fold change in gene expression ($\pm$ SEM). The circles represent expression 6h and the squares 24 h post-stimulation. Level of significance is indicated relative to unstimulated cells ($P < 0.05$).

4.4.4 Analysis of mediator gene expression by endometrial epithelial cells

The gene encoding the chemokine *IL-8* was also differentially expressed, showing a significant increase in the stimulated cells (16 fold after 6h and 9 fold after 24h; $P < 0.05$). *IL-6* was similarly expressed in the stimulated cells as shown in Figure 4.4. *IL-6* was upregulated by 16 and 5 fold after 6h and 24 h, respectively ($P < 0.05$). Although significantly upregulated in stimulated cells, *NFκB1* gene expression was increased by 2 and 1 fold after 6h and 24 h as shown in Figure 4.5a ($P < 0.05$). The expression of genes encoding *IL-1*, *IFNG* and *TNF-α* were increased (*IL-1α* 82 and 26 fold; and *IL-1β* 116 and 8 fold; $P < 0.05$). *TNF* gene expression was similarly significantly increased in stimulated endometrial epithelial cells by 167 fold after 6 h and 24 h (Figure 4.5b; $P < 0.05$). Also significantly increased in expressed was the *IFNG* gene (53 and 26 fold at 6h and 24 h).

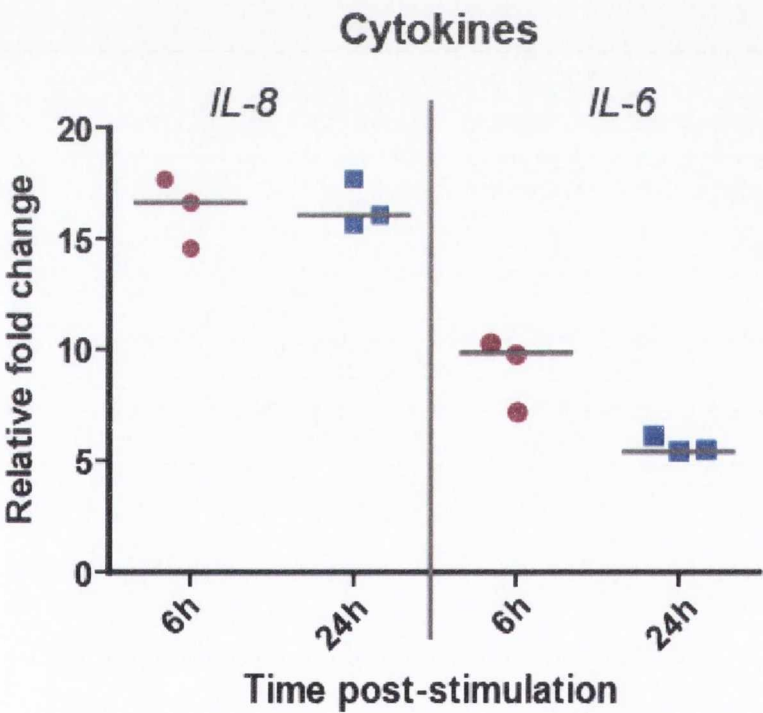


Figure 4.4: Mean gene expression profiles for *IL-8* and *IL-6* from qRT-PCR analyses of endometrial epithelial cells from postpubertal non-pregnant cows stimulated with *E. coli*. ($n = 3$). All C_t values were corrected using the reference gene *GAPDH*. Each point on the graph represents the mean fold change in gene expression ($\pm$ SEM). The circles represent expression 6h and the squares 24 h post-stimulation. Level of significance is indicated relative to unstimulated cells ($P < 0.05$).

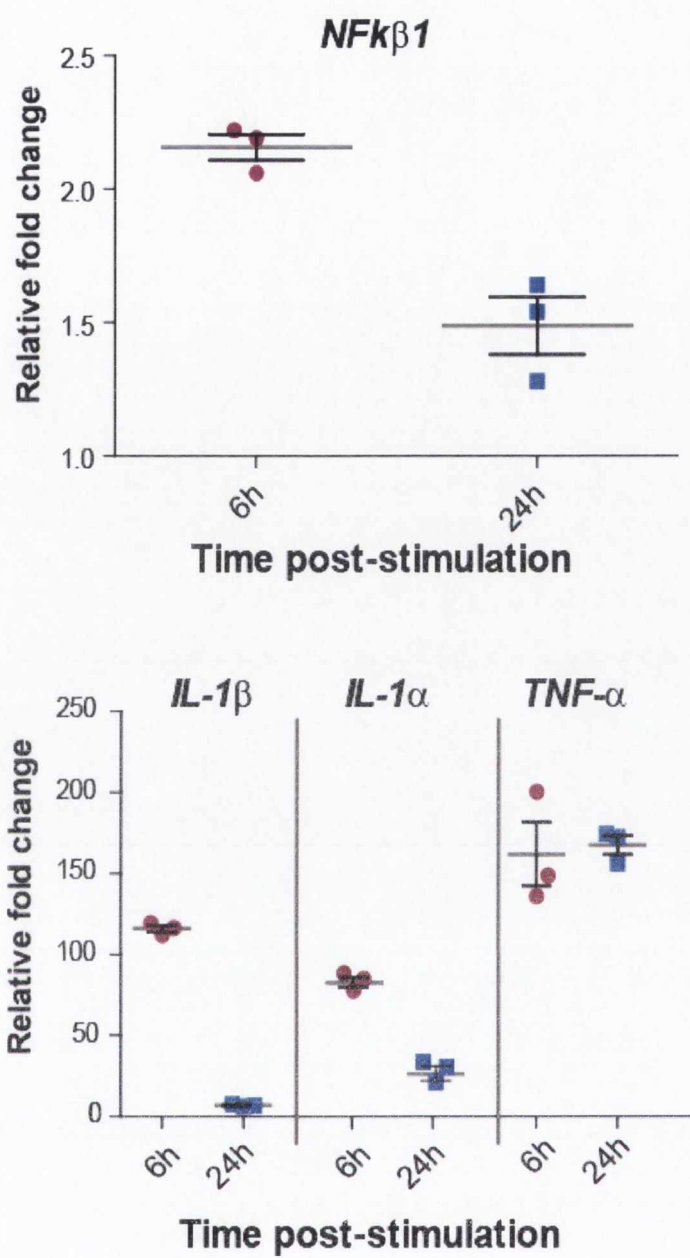


Figure 4.5 Mean gene expression profiles for *NFκB1*, *IL-1α*, *IL-1β* and *TNF-α* from qRT-PCR analyses of endometrial epithelial cells from postpubertal non-pregnant cows stimulated with *E. coli*. (n = 3). All *C_t* values were corrected using the reference gene *GAPDH*. Each point on the graph represents the mean fold change in gene expression (± SEM). The circles represent expression 6h and the squares 24 h post-stimulation. Level of significance is indicated relative to unstimulated cells (*P* < 0.05).

4.4.5 APPs gene expression by endometrial epithelial cells

Differential expression of six APP genes - *HP*, *SAA3*, *SAA4*, *CP*, *LPB* and *CRP* was analysed using qRT-PCR in endometrial epithelial cells. Upregulated expression for 2 of the 6 APP genes - *SAA3* and *HP* was detected (Figure 4.6). The expression of the *CRP*, *CP*, *LPB* and *SAA4* genes was not significantly changed. *SAA3* was significantly increased at 6h by 1700 fold ($P < 0.05$). The elevated expression was sustained until 24 h (Figure 4.7a). *HP* was similarly increased significantly by 3 fold in expression in the stimulated cells at 6 h and 24 h (Figure 4.7b; $P < 0.05$).

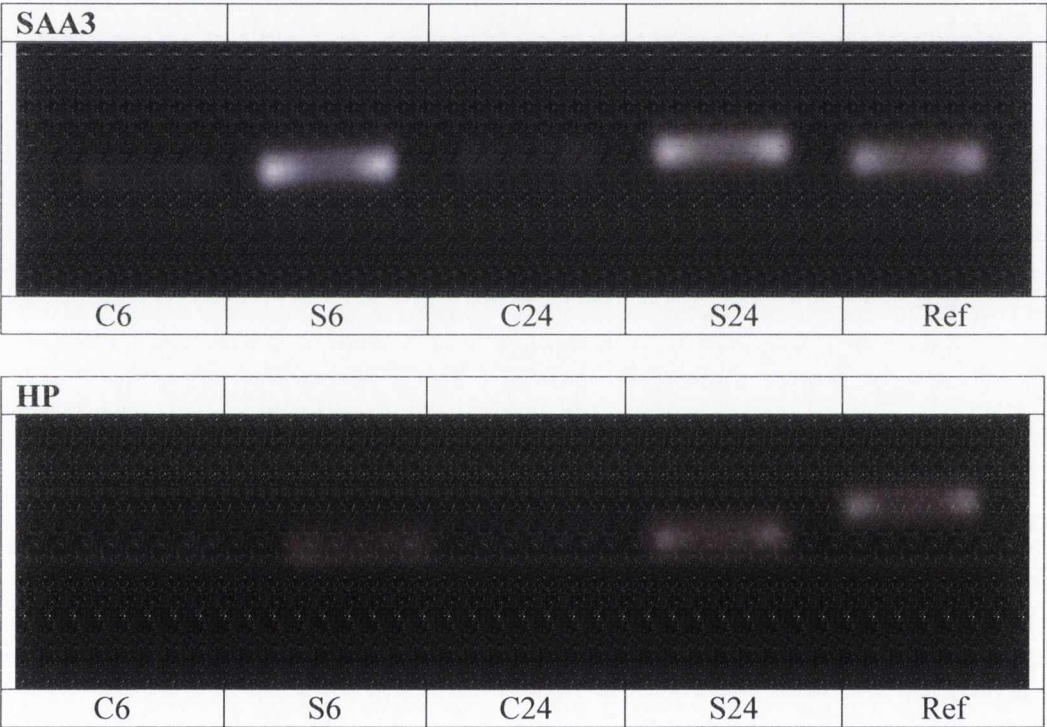


Figure 4.6: *SAA3* and *HP* gene expression of endometrial epithelial cells from postpubertal non-pregnant cows stimulated with *E. coli*. (n = 3). Also shown is the normaliser gene *GAPDH* (Ref) expression for comparison.

- C6 Nil stimulated, 6 hours
- S6 Stimulated, 6 hours
- C24 Nil stimulated, 24 hours
- S24 Stimulated, 24 hours
- REF Normaliser gene (*GAPDH*)

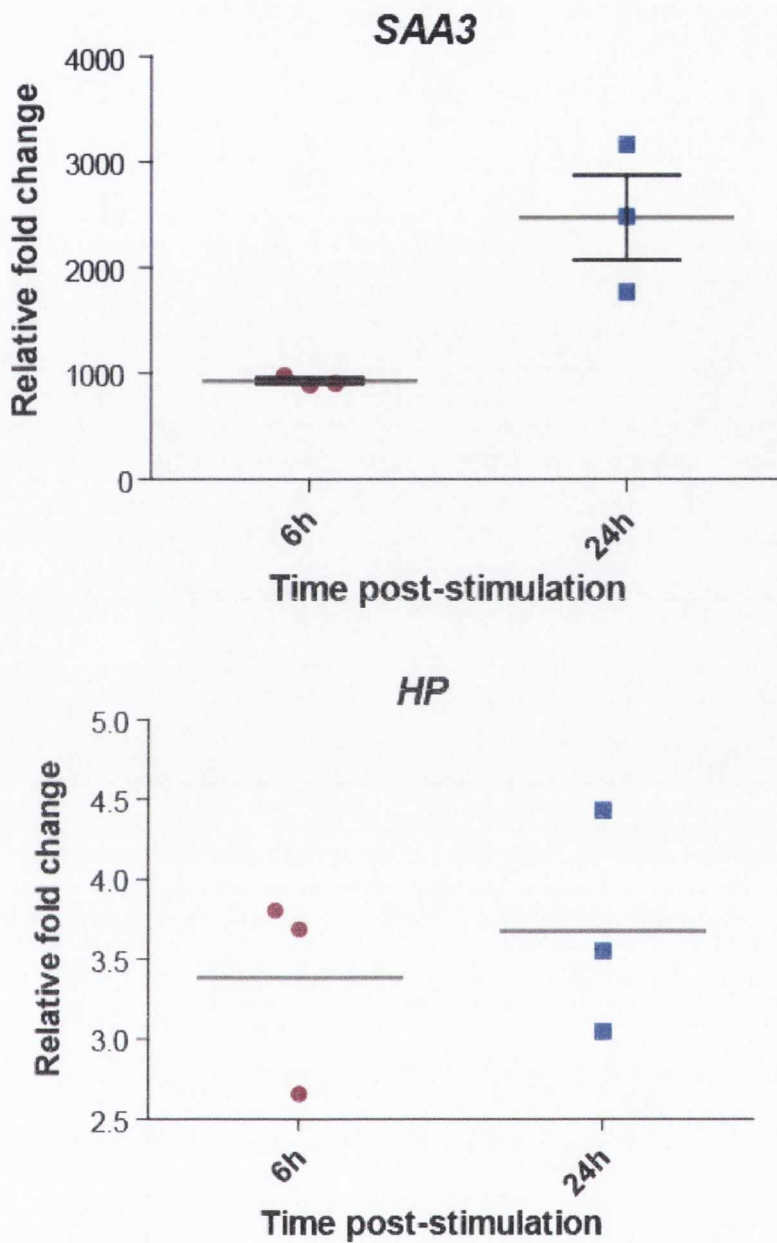


Figure 4.7: Mean gene expression profiles for Acute phase proteins (APPs) *SAA3* and *HP* from qRT-PCR analyses of endometrial epithelial cells from postpubertal non-pregnant cows stimulated with *E. coli*. ($n = 3$). All C_t values were corrected using the reference gene *GAPDH*. Each point on the graph represents the mean fold change in gene expression ($\pm$ SEM). The circles represent expression 6h and the squares 24 h post-stimulation. Level of significance is indicated relative to unstimulated cells ($P < 0.05$)

4.4.6 Analysis of AMPs gene expression by endometrial epithelial cells

The differential expression of five AMPs genes was compared using qRT-PCR. The 5 AMPs genes analysed are *LAP*, *TAP*, *BNBD4*, *DEFB1* and *DEFB5*. Of the five genes, *TAP* and *LAP* were highly significantly increased (Figure 4.8). *TAP* gene expression was significantly increased in all the animals by 58 and 189 fold after 6 h and 24 h ($P < 0.05$). *LAP* gene expression was significantly different between the stimulated and control cells, showing a 66 fold increase at 6 h and 17 fold at 24 hours ($P < 0.05$). The expression pattern for *LAP* was opposite to that of *TAP*, showing higher expression after 6 h (Figure 4.9a). *BNBD4*, *DEFB1* and *DEFB5* were similarly significantly upregulated 2 and 1 fold after 6 h and 24 h, respectively (Figure 4.9b).

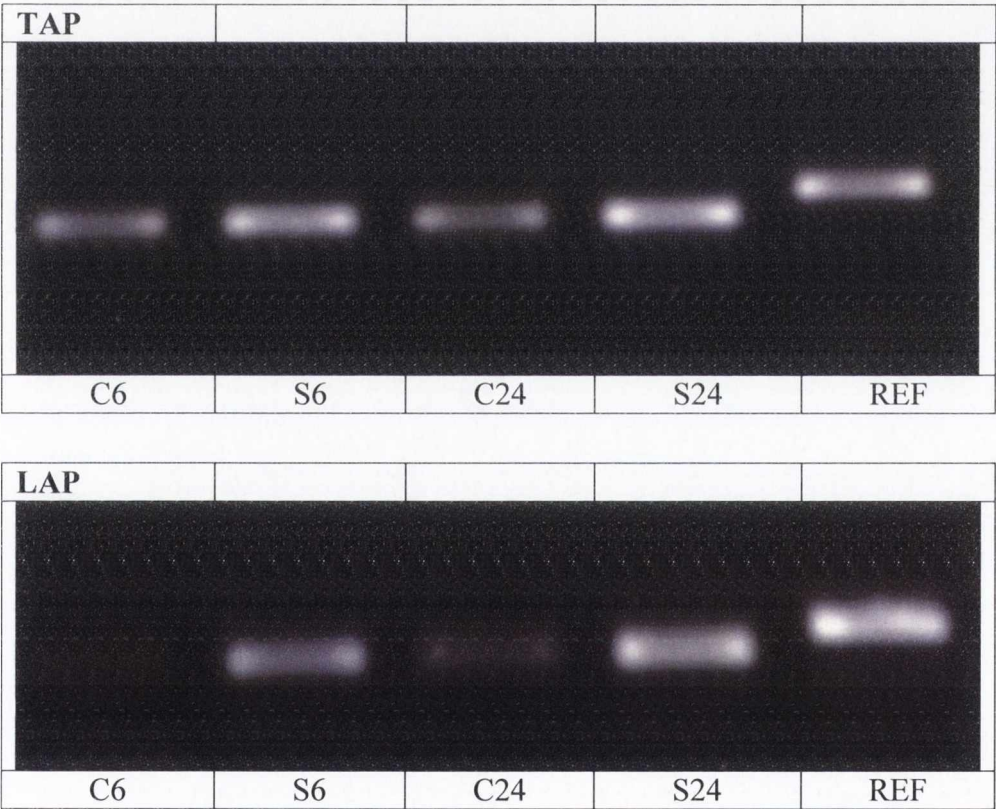


Figure 4.8: Antimicrobial peptides (AMPs) *TAP* and *LAP* gene expression of endometrial epithelial cells from postpubertal non-pregnant cows stimulated with *E. coli*. (n = 3). Also shown is the normaliser gene *GAPDH* (Ref) expression for comparison.

- C6 Nil stimulated, 6 hours
- S6 Stimulated, 6 hours
- C24 Nil stimulated, 24 hours
- S24 Stimulated, 24 hours
- REF Normaliser gene (*GAPDH*)

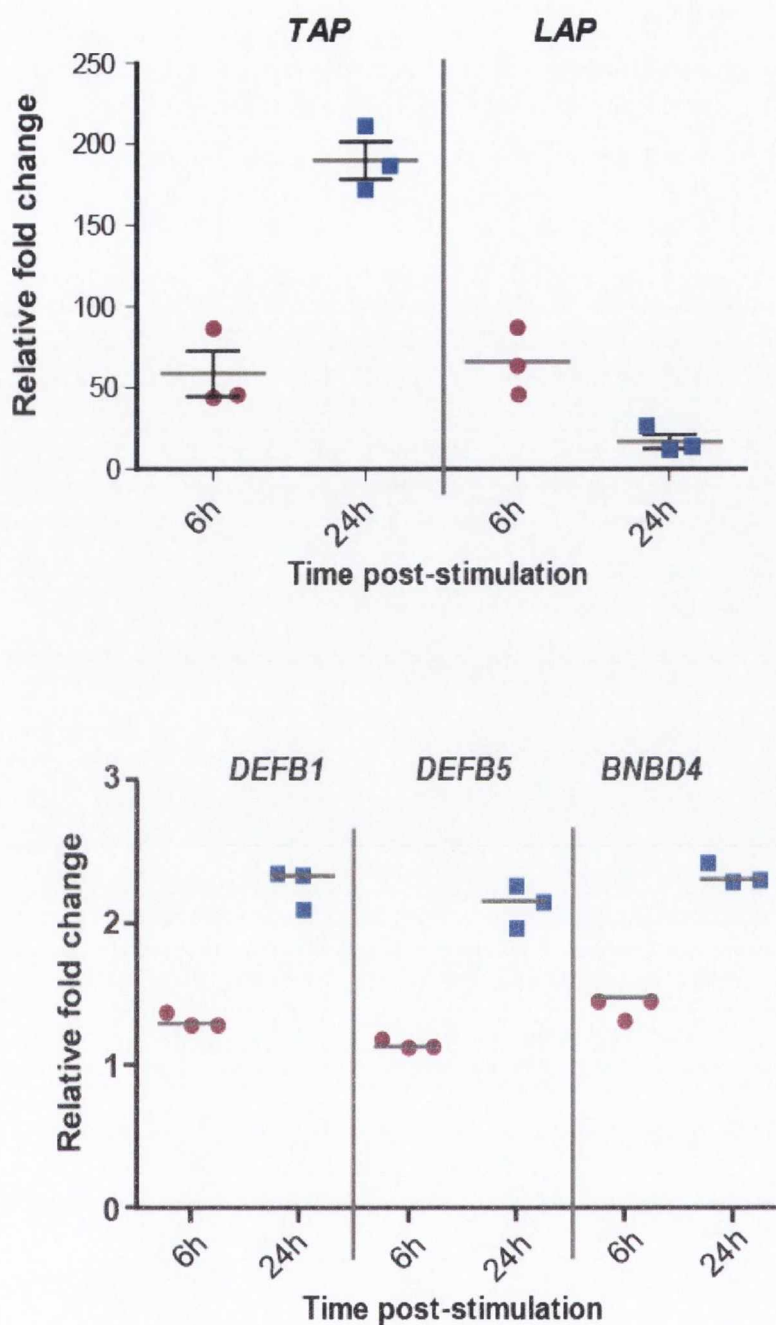


Figure 4.9: Mean gene expression profiles for *TAP*, *LAP*, *DEFB1*, *DEFB5* and *BNBD4* from qRT-PCR analyses of endometrial epithelial cells from postpubertal non-pregnant cows stimulated with *E. coli*. (n = 3). All C_t values were corrected using the reference gene *GAPDH*. Each point on the graph represents the mean fold change in gene expression ($\pm$ SEM). The circles represent expression 6h and the squares 24 h post-stimulation. Level of significance is indicated relative to unstimulated cells ($P < 0.05$).

4.5 Discussion

This study confirms previous findings of increased expression of APPs and proinflammatory cytokines by bovine endometrial cells when stimulated with LPS (Davies *et al.*, 2008b). For the first time, we have shown that these cells are also potential producers of APPs, in particular SAA3 in response to *E. coli* stimulation. Although the role of APPs has been described in cattle with inflammatory diseases (Eckersall *et al.*, 2006; Ganheim *et al.*, 2007; Huzzey *et al.*, 2009), to date, these molecules have not been studied in the bovine endometrial epithelial cell model. Bacterial infection of the female genital tract is common in cattle particularly after parturition, causing considerable disease, sub-fertility or death (LeBlanc, 2008). As evolutionarily conserved effector molecules of the innate immunity, the temporal expression of APPs post-infection may have significant consequences for the subsequent immune response and outcome of infection. These findings suggest that APPs are an important arm of the innate immune defence against bacterial infection of the uterus. The concentrations of APPs, including haptoglobin and serum amyloid A, are substantially increased in the peripheral plasma of a cow with bacterial infection of the endometrium (Chan *et al.*, 2009; Huzzey *et al.*, 2009). A more comprehensive characterisation of the local uterine innate response *in vivo* would elucidate the mechanisms regulating clearance of infection and resolution of inflammation during the postpartum period. Because APPs, and SAA in particular (Urieli-Shoval *et al.*, 2000), play an important role in the host's innate immune response, it is tempting to speculate that temporal changes after infection also indicate a key role of APPs in defence mechanisms of the uterus. This hypothesis remains to be addressed in future studies. SAA has the greatest role in bacterial and pyogenic infections and increases in common infectious diseases in sheep (Winter *et al.*, 2006) such as metritis.

The *E. coli*-induced increase of *HP* mRNA suggests a link between uterine infection and APP synthesis in the uterus. Normally, *HP* gene expression is induced by an *IL-6*-mediated process, which inevitably occurs in the liver as part of inflammatory reactions (Conner *et al.*, 1986; Ganheim *et al.*, 2007; Hiss *et al.*, 2004). Recent studies reported that HP was detectable in serum samples of cattle with metritis (Chan *et al.*, 2009; Huzzey *et al.*, 2009; Sheldon *et al.*, 2001). Here we provide evidence that the haptoglobin detected originates from extrahepatic sites such as the uterus. HP measurements may provide a potential additional tool for the investigation of

inflammation in the uterus and can be used as an early diagnostic marker for metritis or endometritis.

The other effector immune molecules detected here are AMPs, which are key components of the mammalian innate host defense against infection (Palffy *et al.*, 2009). AMPs are widely expressed and induced at mammalian intestinal mucosal surfaces as defence mechanisms against microorganisms (Elsbach, 2003; Ganz, 2003; Lai & Gallo, 2009). Of interest is the role of the AMPs – *TAP* and *LAP*, the production of which was significantly increased in the *in vitro* study, similar to reports in *in vivo* studies in the mammary gland (Roosen *et al.*, 2004; Swanson *et al.*, 2004) and in uterine tissue obtained from dairy cows 15 days after calving (Chapwanya *et al.*, 2009). AMPs are known to be greatly induced in expression as a response to infection or injury via signalling mediated by TLRs or release of proinflammatory cytokines (Selsted & Ouellette, 2005; Sorensen *et al.*, 2008). These findings suggest that *TAP* and *LAP* are key defense factors in the uterus against infectious pathogens such as *E. coli*. The results of this study also demonstrate that endometrial epithelial cells respond to *E. coli* stimulation by secreting *TNF- α* , *IL-1 β* , *IFN- γ* , *IL-8* and *IL-6*. The ability of endometrial cells to produce major immuno-regulating cytokines points to a role for bovine endometrial cells during the innate immune response. Such an expression pattern suggests an early induction geared towards professional immune cell recruitment into the uterus to combat infection (Ishikawa *et al.*, 2004).

In conclusion, the present study supports the extrahepatic intrauterine production of *HP* and *SAA* in cattle. In addition, the study highlights the usefulness of haptoglobin concentration which can be evaluated for the early detection of bovine postpartum reproductive disorders.

Chapter 5

General Discussion

A dramatic decline in reproductive performance of dairy cows has occurred in recent times (Chagas *et al.*, 2007; Lucy, 2001; Lucy, 2007; Royal *et al.*, 2000; Silke *et al.*, 2002; VanRaden *et al.*, 2004). Although significant progress has been made in identifying the causes, current strategies have not improved fertility. Evidence exists to suggest that the postpartum period is a critical time in the cow's reproductive cycle (Morris *et al.*, 2009; Peter *et al.*, 2009; Roche *et al.*, 2000). In order to devise strategies for reversing the declining trends in fertility, assessment of the local uterine involutionary response in postpartum cows is required. Fundamental to this issue is an understanding of uterine molecular mechanisms underlying clearance of infection and resolution of inflammation. A myriad of bacteria invade the uterus at calving but uterine defence mechanisms eliminate most of the bacteria 2 - 3 weeks postpartum (Foldi *et al.*, 2006; LeBlanc, 2008). Approximately 20% of the cows fail to clear infection resulting in continued uterine inflammation and subfertility. The present thesis investigated the link between histology findings and innate immune gene expression in uterine tissues from postpartum cows. Other techniques (clinical, cytological and ultrasonography) were performed as adjunct methods in order to build an accurate picture of the postpartum uterine involutionary reaction.

The first objective was to optimise techniques for studying uterine postpartum immunity. These data revealed that postpartum uterine involutionary responses are inflammatory. Researchers in the area of bovine reproduction have been reluctant to exploit endometrial biopsy sampling because it has been suggested that the procedure can impair subsequent fertility in the cow by inducing uterine pathology (Zaayer & van der Horst, 1986). In addition, in the individual cow undergoing a clinical evaluation, it was proposed that the usefulness of the information provided by an endometrial biopsy was out-weighed by the suggestion that it was considered technically difficult and risky while also requiring expertise to obtain and interpret biopsies (Nielsen, 2005). In Chapter 2, the optimised endometrial biopsy technique facilitated the collection of tissue samples from individual cows which allowed detailed profiling of uterine cellular and molecular changes. The endometrial biopsy technique has the potential to play a key role in assessing endometrial health as well as investigating physiological or disease processes. Using the technique, it is now possible to perform quality microscopic, cellular and molecular assessment on endometrial tissue obtained (Bonnett *et al.*, 1991b; Rhoads *et al.*, 2008). In this study, significant upregulation in expression of proinflammatory genes in severely inflamed tissue was revealed. The design could have

been improved by performing microbial culture in all the cows, and by selecting contemporary cows as controls. Unfortunately, for logistical reasons, microbial swabs could not be obtained and transported to the laboratory at 4°C within 24 hours. Therefore on certain sampling days, no microbial culture samples were obtained. Although important, no comparison was made between histopathology or immune gene expression with bacterial cultures in the present study because the main focus was to describe the inflammatory events of the postpartum uterus. Despite this, distinct histopathological classification of the cows was possible. Secondly, standardisation of animals is a major issue in experimental models in the field. In this study, the control cows selected were not uniformly matched to the experimental cows. This was due to non-availability of first parity cows for biopsy at 60 DPP. These cows were earmarked for insemination 7 to 9 weeks after calving and it was not possible to obtain ethical approval to perform biopsy at that stage. Nevertheless, histopathology and uterine gene expression analysis grouped the cows into 3 distinct classes.

The second objective was to provide insight into the innate immune gene expression and related histological changes in uterine tissues during the postparturient period, and to investigate opportunities to use information on uterine immune responses to improve selection for reproductive traits in cattle. Although histological evaluations of uterine tissue in cows has been performed in non-pregnant cows (Bonnett *et al.*, 1991b) most studies were in metritic or repeat breeder cows (Katagiri & Takahashi, 2004; Katagiri & Takahashi, 2006). Analysis of clinical data, ultrasonographic images or microbial culture profiles in the present study did not reveal significant differences between the cows. Whilst 30 years ago the potential value of uterine biopsy may have not been realised, the increased knowledge of immunity coupled with omics technologies, mean that the technique optimised in the present study offers significant potential in facilitating research in bovine reproduction. Due to perceived problems associated with it, the use of endometrial biopsy has not become mainstream, and researchers preferentially utilise *ex vivo* tissues instead (Gabler *et al.*, 2009; Herath *et al.*, 2006b). However, contrary to reports that uterine biopsy perturbs fertility (Zaayer & van der Horst, 1986) or induce pathology (Simon & McNutt, 1957), the present study demonstrates that repeated endometrial biopsy did not unduly affect health, oestrus cyclicity or fertility in postpartum cows. Furthermore, coupled with recent technological developments including qRT-PCR, microarray analysis and Next Generation Sequencing techniques, which require small amounts of high quality DNA and RNA (Wang *et al.*, 2009), the

endometrial biopsy offers unparalleled potential for understanding the molecular mechanisms regulating the postpartum uterine immunity. In Chapter 3, the data obtained from histological assessment of biopsy samples revealed inflammation at 15 DPP based on the leucocyte cellular infiltration in all uterine tissues. At a molecular level, a total of 18 innate immune genes were upregulated including those involved in classical inflammation such as chemokines and cytokines. The significant upregulation of proinflammatory cytokines, AMPs and APPs mirrored the inflammation detected histologically. Fertility is considered to be an important trait to select for in cattle. Future studies can investigate the manipulation of the unique gene expression patterns in endometrial tissue for good fertility as well as validate if these gene combinations would be possible candidates to use for selecting cows with superior fertility. A novel finding in this study was the extrahepatic intrauterine production of APPs. These data help to define uterine molecular mechanisms underlying clearance of infection and resolution of inflammation postpartum. Given that APPs were upregulated in uterine tissues at 15 and 30 DPP, it seems that the APPs play a central role in direct or indirect uterine protection during the physiological involutionary inflammatory immune response to infection (Figure 5.1). The initiation of the APR locally results in the increase in capillary permeability and infiltration of leucocytes to the uterus. As a result, a local inflammatory cascade is initiated by mononuclear cells. Furthermore, a broad spectrum of inflammatory mediators such as cytokines is released in the uterus which in turn drive the production of APPs in uterine tissue. Therefore, in postpartum cows, the APPs are potential biomarkers for the early and accurate identification of cows at risk of subfertility.

The detection and control of endometritis are primary goals for cattle producers. The uterus is a unique organ with complex cellular interactions. At calving, the bovine uterus is invaded by a myriad of bacteria and clears the majority of the microbes, yet less than 50% of cows re-conceive to first insemination between 42 and 60 DPP (Dobson *et al.*, 2007). There are still questions regarding the role of the specific uterine cells involved in innate immune response after calving. Endometrial tissue samples contain several cell types including immune cells. Of particular interest is the role of endometrial epithelial cells which are located at the uterine mucosal interface. The third objective was to investigate endometrial epithelial cell-specific aspects of inflammation in a monotype cell culture model in response to *E. coli* stimulation. In Chapter 4, *in vitro* studies investigated the production of APPs by endometrial epithelial cells, which

confirmed extrahepatic production of APPs during the APR. The endometrial epithelial cells were stimulated with *E. coli*, the commonly isolated pathogen from the uterus of cows with metritis. This finding also paves the way for future studies to determine potential diagnostic value of haptoglobin and serum amyloid A in uterine fluid samples in the detection of subclinical endometritis in postpartum cows.

The work in this thesis goes some way to defining uterine molecular mechanisms underlying clearance of infection and resolution of inflammation postpartum. This information will facilitate the identification of biomarkers for the early and accurate detection of cows likely to conceive. Characterisation of variations in these genes using newer techniques such as Next Generation Sequencing will provide important tools for devising breeding programmes to improve cattle fertility.

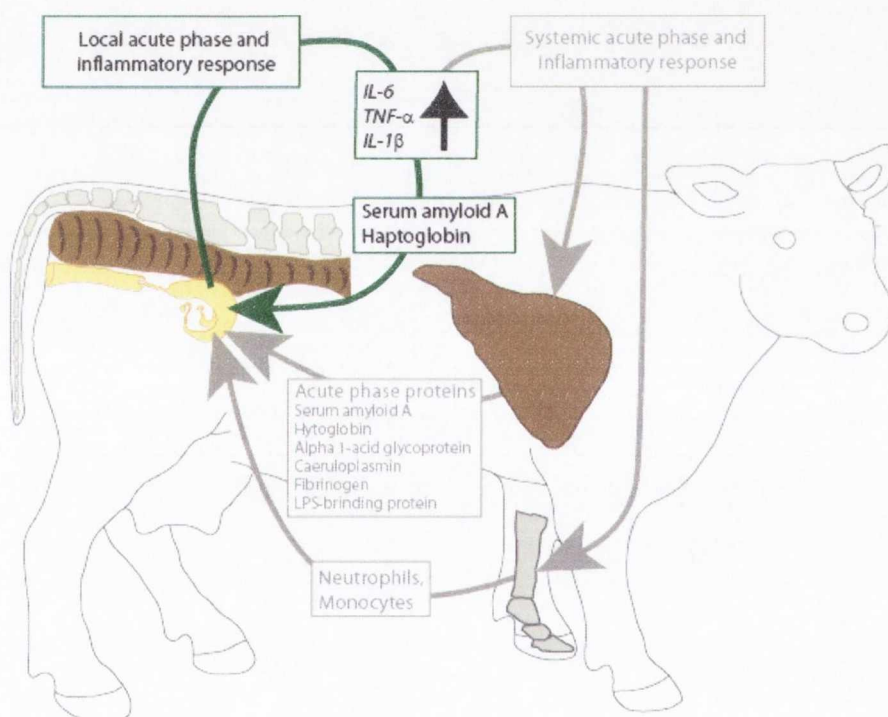


Figure 5.1: Endometrial acute phase reponse. The initiation of the APR locally results in the increase in capillary permeability and infiltration of leucocytes to the uterus. As a result, a local inflammatory cascade is initiated by mononuclear cells.

Chapter 6

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Chapter 7

Appendices

Table A1 Primer sequences used to assay gene expression in uterine tissues and endometrial epithelial cells.

Gene Symbol	Gene Name	Primer Sequence	Amplicon Length (bp)	Genbank accession number	Concentration
<i>ACTB</i>	Actin, beta	F - AGATGACCCAGATCATGTTCTGA R - TGACCCCGTCACCGGAGTCCATCACGAT	126	NM_173979	600
<i>GAPDH</i>	Glyceraldehyde-3-phosphate Dehydrogenase	F - CTCCCAACGTGCTGTGTGTG R - TGAGCTTGACAAAGTGGTCG	222	NM_001034034	900
<i>PPIA</i>	Peptidylprolyl isomerase A	F - CCACCGTGTCTTCGACAT R - TCTGTGAAGCAGGAACCTTT	155	NM_178320	600
<i>H3F3A</i>	H3 histone, family 3A	F - CATGGCTCGTACAAGCAGA R - ACCAGGCGTGTAAACGATGAG	136	NM_001014389	900
<i>MRPS6</i>	Mitochondrial ribosomal protein S6	F - CGCACGGCTTCCTATAAGAT R - TTCACTTCCTGGGTACAGAGG	176	NM_001040584	600
<i>RPS9</i>	Ribosomal protein S9	F - GATTACATCCTGGGCGTGAA R - ATGAAGGACGGGATGTTAC	161	NM_001101152	300
<i>MRPS15</i>	Mitochondrial ribosomal protein S15	F - GCAGCTTATGAGCAAGGTCGT R - GCTCATCAGCAGATAGCGCTT	151	XM_585783	900
<i>TLR1</i>	Toll-like receptor 1	F - ACTTGGAATTCCTTCTTACGA R - GGAAGACTGAACACATCATGGA	176	NM_001046504	900
<i>TLR2</i>	Toll-like receptor 2	F - GGTTTAAAGGCAGAATCGTTTG R - AAGGCACTGGGTAAACTGTGT	190	NM_174197	600
<i>TLR3</i>	Toll-like receptor 3	F - GATGTATCACCTGCAAGACA R - TGCATATTCAAAGTCTCTGCT	195	NM_001008664	600
<i>TLR4</i>	Toll-like receptor 4	F - CTTGCGTACAGGTGTTCTCTAA R - CTGGGAAGCTGGAGAAGTTATG	153	NM_174198	600
<i>TLR5</i>	Toll-like receptor 5	F - CCTCCTGCTCAGTTCACATAT R - TATCTGACTTCCACCCAGGTCT	172	AY634631	600
<i>TLR6</i>	Toll-like receptor 6	F - CCTGTTTTTCACCCAAATAGC R - TAAGGTTGGTCTCCAGTGAGT	154	NM_001001159	600
<i>TLR7</i>	Toll-like receptor 7	F - TCTTGAGGAAAGGACTGGTTA R - AAGGGGCTTCTCAAGGAATATC	205	DQ333225	600
<i>TLR8</i>	Toll-like receptor 8	F - TAACCTTCGGAATGTCTCCAGT R - GTGGGAAATCTGTTTCGACTC	232	NM_001033937	600
<i>TLR9</i>	Toll-like receptor 9	F - CTGACACCTTCAGTCACCTGAG R - TGGTGGTCTTGGTGATGTAGTC	156	NM_183081	900
<i>TLR10</i>	Toll-like receptor 10	F - ATGGTGCCATTATGAACCTAC R - CACATGTCCCTCTGGTGTCTAA	248	NM_001076918	600
<i>NOD1</i>	Nucleotide-binding oligomerization domain containing 1	F - TCTCGCTCTGGCTGTGAAGAA R - TGAATGCAAGACTCAGGTTGGT	140	XM_598513	600
<i>NOD2</i>	Nucleotide-binding	F - CGCCAAAGGACTTGCAAGA	135	NM_001002889	600

Gene Symbol	Gene Name	Primer Sequence	Amplicon Length (bp)	Genbank accession number	Concentration
	oligomerization				
	domain containing 2	R - CCTCGGAGCCAGACTTCCA			
<i>NLRP1</i>	NLR family, pyrin domain containing 1	F - GGCCACTGGTTGAAACATCT R - TGACCTCAATCAAATGCCCT	231	XM_001257103	900
<i>NLRP3</i>	NLR family, pyrin domain containing 3	F - TGTGGGACTGAGGCATCTATTCT R - CTGGTCAGGGAATGGTTGGT	120	NM_001102219	600
<i>TNFA</i>	Tumor necrosis factor (TNF superfamily, member 2)	F - CCATCAACAGCCCTCTGGTT R - TCACACCGTTGGCCATGA	150	NM_173966	600
<i>IL1B</i>	Interleukin 1, beta	F - CCCTGCAGCTGGAGGAAGTA R - CTTCGATTGAGAAGTCTGATGT	150	NM_174093	600
<i>IL6</i>	Interleukin 6	F - TAAGCGCATGGTCGACAAAA R - TTGAACCCAGATTGGAAGCAT	150	NM_173923	900
<i>IFNG</i>	Interferon, gamma	F - TTGAATGGCAGCTCTGAGAAAC R - TCTCTTCCGCTTTCTGAGGTTAGA	150	NM_174086	300
<i>IL8</i>	Interleukin 8	F - AGAAGCTCGATGCCAATGCAT R - GGGTTTAGGCAGACCTCGTTT	150	NM_173925	600
<i>TGFBI</i>	Transforming growth factor, beta 1	F - TGCTTCAGCTCCACAGAAAAGA R - AGGCAGAAATTGGCGTGGT	116	XM_592497	900
<i>IL10</i>	Interleukin 10	F - AGAACCACGGGCTGACA R - ACCGGCTTGCTCTGTTTTTC	121	NM_174088	300
<i>DEFB1</i>	Defensin, beta 1	F - TCTTCCTGGTCCTGTCTGCT R - TTACCTCCACCTGCAGCATT	164	NM_175703	600
<i>BNBD4</i>	Bovine neutrophil beta-defensin 4	F - ATCACCTGCTCCTGCGAGT R - CCTACACGGCACAAGAACG	127	NM_174775	600
<i>DEFB5</i>	Defensin, beta 5	F - ACCAGCATGAGGCTCCAT R - TTGCCAGGGCAGGAGAT	143	NM_001130761	900
<i>TAP</i>	Tracheal antimicrobial peptide	F - TCCTGGTCCTGTCTGCTTC R - CTACAGCATTTTACTGCCCCG	151	NM_174776	600
<i>LAP</i>	Lingual antimicrobial peptide	F - GACAGCATGAGGCTCCATC R - CTCCTGCAGCATTTTACTTGGGCT	194	NM_203435	600
<i>DEFB119</i>	Defensin, beta 119	F - GTTCTTGCCATCCTTCTGG R - CATAGAAAGGGAGGCAGCAG	161	EU036209	600
<i>DEFB120</i>	Defensin, beta 120	F - TTTCTGTTTCTTGCCATCC R - GCAGCGTGTGACACTGTCTT	115	EU036210	600
<i>DEFB122</i>	Defensin, beta 122	F - CTCTCATCCAGGTCATTCC R - GCGGATGTATTGGCTGAAA	155	EU036211	600
<i>DEFB122a</i>	Defensin, beta 122a	F - TGCTAACTTTGGCTGCACTG R - TGGTAAGCCATGTGGTTGGTAC	179	EU036212	600
<i>DEFB123</i>	Defensin, beta 123	F - AGCTCCTCTCGCTGATTCTG R - CAGCACAGTTATTATTGTGCAGTA	154	EU036213	600
<i>DEFB124</i>	Defensin, beta 124	F - TGACACAGCTGCTCTGCTT	190	EU036214	600

Gene Symbol	Gene Name	Primer Sequence	Amplicon Length (bp)	Genbank accession number	Concentration
<i>SAA3</i>	Serum amyloid A 3	R - TTGACAGGCTTGAGTCCGTA F - CTCAGGAAGCTGGTCAAGG	240	NM_181016	900
<i>HP</i>	Haptoglobin	R - CTTGGAATCCTCCGTACCT F - TGGTCTCCCAGCATAACCTC	185	NM_001040470	900
<i>LBP</i>	Lipopolysaccharide binding protein	R - AGGGTGGAGAACCACCTTCT F - TCAGCTCTACGACCCCATTC	151	NM_001038674	600
<i>AGP</i>	Alpha I acid glycoprotein	R - AAGTGCTGCACATCTGGAAA F - GTGCATAGGCATCCAGGAAT	192	NM_001040502	900
<i>CP</i>	Ceruloplasmin	R - CCGAGGGAACTGAGTTACAAA F - TTCATGCACATGGAATGACTT	236	XM_592003	600
<i>CD45</i>	Protein Tyrosine Phosphatase receptor type C	R - TAAAGGCCCAATGAGTCCTG F - TGCAACCGCTCTCTCAACCATA	845	AJ400864	600
<i>CD14</i>	Cluster of differentiation 14	R - CTTGCTTGGCTTGGCTGGATCT F - GACGACGATTCCGTTGTGT	120	NM_174008	900
<i>IL2</i>	Interleukin 2	R - TGCCTAGCGCTAGATATTGGA F - GGGAAACACAATGAAAGAAGTGAAGT	120	M13204	600
<i>IL12A</i>	Interleukin 12	R - GGGCGCGTAAAAGTCAAATG F - CAGCAACACGCTACAGAAGG	120	U14416	900
<i>UBQ</i>	Ubiquitin	R - CCTGCATCAGCTCAGCAATA F - GGCAAGACCATCACCTGGAA	254	NM_174133	900
<i>IL1A</i>	Interleukin 1	R - TGGCAACCATCACCACATTCTC F - TGCCACCATCACCACATTCTC	120	M37211	600
<i>NFKB1</i>	Nuclear factor kappa B	R - GGGAACACAATGAAAGAAGTGAAGT F - TAACCTGTGTTTTGCACCTCGCT	131	NM_001076409	600
		R - TGGCTACATGGATGGCATTG			

Table A2: mRNA relative fold changes in genes encoding cell surface markers *CD45* and *CD14* in uterine tissues from postpartum cows. For presentation of the data, 15 and 30 DPP values are expressed relative to 60 DPP values in the same animal.

	15DPP	30DPP	60DPP
COW	CD45		
1	164.28	9.92	1.00
2	30.81	4.79	1.00
3	177.19	3.09	1.01
4	29.32	19.30	1.00
5	53.79	3.39	1.00
6	247.28	92.11	1.00
7	34.78	2.93	1.00
8	135.35	7.74	1.00
9	18.19	5.26	1.00
10	29.28	15.21	1.00
11	346.84	3.45	1.00
12	51.27	5.43	1.00
13	38.46	1.39	1.00
Mean	104.37	13.39	
Median	51.27	5.26	

	15DPP	30DPP	60DPP
COW	CD14		
1	33.40	20.66	1.00
2	6.37	1.78	1.00
3	5.49	1.93	1.01
4	1.86	1.21	1.00
5	4.40	-1.18	1.00
6	4.86	2.49	1.00
7	4.07	1.97	1.00
8	16.92	2.72	1.00
9	6.78	2.64	1.00
10	11.53	5.99	1.00
11	5.30	2.36	1.00
12	2.72	1.44	1.00
13	6.74	3.48	1.00
Mean	8.50	3.65	
Median	5.49	2.36	

Table A3: mRNA relative fold changes in genes encoding *TLR4* and *TLR3* in uterine tissues from postpartum cows. For presentation of the data, 15 and 30 DPP values are expressed relative to 60 DPP values in the same animal.

	15DPP	30DPP	60DPP
COW	TLR4		
1	8.75	1.93	1.00
2	1.90	1.13	1.00
3	59.85	10.06	1.00
4	6.79	10.21	1.00
5	1.64	-1.01	1.00
6	3.84	1.79	1.00
7	4.86	3.96	1.00
8	14.34	1.87	1.00
9	1.96	-15.16	1.00
10	22.34	1.27	1.00
11	14.78	2.36	1.00
12	42.43	17.98	1.00
13	24.47	1.51	1.00
Mean	16.00	2.92	
Median	8.75	1.87	

	15DPP	30DPP	60DPP
COW	TLR3		
1	1.91	1.29	1.00
2	6.42	2.33	1.00
3	4.08	2.46	1.00
4	5.57	3.75	1.00
5	5.76	2.03	1.00
6	5.86	1.75	1.00
7	2.18	1.28	1.00
8	3.02	1.22	1.00
9	1.69	-7.77	1.00
10	4.89	1.71	1.00
11	2.61	2.53	1.00
12	16.99	1.65	1.00
13	4.94	1.67	1.00
Mean	5.07	1.22	
Median	4.89	1.71	

Table A4: mRNA relative fold changes in genes encoding anti-inflammatory cytokines *IL-10* and *TGfB* in uterine tissues from postpartum cows. For presentation of the data, 15 and 30 DPP values are expressed relative to 60 DPP values in the same animal.

	15DPP	30DPP	60DPP
COW	<i>IL-10</i>		
1	8.68	3.04	1.00
2	3.27	-3.86	1.00
3	-20.17	-9.59	1.00
4	322.17	86.02	1.00
5	1.42	-1.86	1.00
6	1.38	-1.83	1.00
7	-19.38	1.04	1.00
8	1.76	9.64	1.00
9	112.29	1.03	1.00
10	-1.35	-2.33	1.00
11	40.95	4.35	1.00
12	69.55	12.19	1.00
13	83.88	5.12	1.00
Mean	46.50	7.92	
Median	3.27	1.04	

	15DPP	30DPP	60DPP
COW	<i>TGfB</i>		
1	-16.67	1.90	1.00
2	1.23	-4.94	1.00
3	-22.54	-32.66	1.00
4	4.18	12.61	1.00
5	-1.03	-5.80	1.00
6	-1.84	-68.10	1.00
7	2.61	2.84	1.00
8	-3.29	1.89	1.00
9	1.31	1.12	1.00
10	-11.96	-6.63	1.00
11	33.96	1.38	1.00
12	-49.16	-4.71	1.00
13	-2.11	-2.58	1.00
Mean	-5.02	-7.98	
Median	-1.84	-2.58	

Table A5: mRNA relative fold changes in genes encoding cytokines *TNF-α* and *IFNG* in uterine tissues from postpartum cows. For presentation of the data, 15 and 30 DPP values are expressed relative to 60 DPP values in the same animal.

	15DPP	30DPP	60DPP
COW	TNF-α		
1	15.58	2.08	1.00
2	7.45	5.07	1.00
3	136.90	2.52	1.00
4	62.48	19.58	1.00
5	10.67	5.51	1.00
6	75.61	29.56	1.00
7	6.69	5.36	1.00
8	62.95	3.13	1.00
9	36.03	1.20	1.00
10	26.27	7.16	1.00
11	50.70	12.13	1.00
12	36.27	7.79	1.00
13	24.94	2.59	1.00
Mean	42.50	7.97	
Median	36.03	5.36	

	15DPP	30DPP	60DPP
COW	IFNG		
1	146.19	7.14	1.00
2	4.95	2.01	1.00
3	78.64	19.91	1.00
4	125.40	46.96	1.00
5	2.92	-1.57	1.00
6	18.57	11.78	1.00
7	11.27	5.04	1.00
8	179.17	2.40	1.00
9	24.85	9.45	1.00
10	80.33	11.20	1.00
11	100.43	3.34	1.00
12	69.55	12.19	1.00
13	50.75	2.56	1.00
Mean	68.69	10.19	
Median	69.55	7.14	

Table A6: mRNA relative fold changes in genes encoding transcription factor *NFκB1* in uterine tissues from postpartum cows. For presentation of the data, 15 and 30 DPP values are expressed relative to 60 DPP values in the same animal.

	15DPP	30DPP	60DPP
COW	<i>NFκB1</i>		
1	32.59	11.96	1.00
2	9.23	4.07	1.00
3	144.02	8.20	1.00
4	16.12	8.51	1.00
5	33.22	1.21	1.00
6	20.76	12.34	1.00
7	88.76	19.68	1.00
8	40.51	15.91	1.00
9	14.02	4.83	1.00
10	5.72	5.08	1.00
11	41.36	6.17	1.00
12	14.17	5.62	1.00
13	5.63	2.03	1.00
Mean	35.85	8.12	
Median	20.76	6.17	

Table A7: mRNA relative fold changes in genes encoding chemokine *IL-8* in uterine tissues from postpartum cows. For presentation of the data, 15 and 30 DPP values are expressed relative to 60 DPP values in the same animal.

	15DPP	30DPP	60DPP
COW	<i>IL-8</i>		
1	96.30	23.86	1.00
2	100.78	12.24	1.00
3	68.57	9.65	1.00
4	191.51	90.62	1.00
5	34.91	4.73	1.00
6	344.10	98.87	1.00
7	17.47	2.82	1.00
8	77.27	39.76	1.00
9	252.50	18.84	1.00
10	11.93	1.25	1.00
11	75.75	16.36	1.00
12	138.78	2.57	1.00
13	117.97	35.66	1.00
Mean	117.53	27.48	
Median	96.30	16.36	

Table A8: mRNA relative fold changes in genes encoding APPs *SAA3* and *HP* in uterine tissues from postpartum cows. For presentation of the data, 15 and 30 DPP values are expressed relative to 60 DPP values in the same animal.

	15DPP	30DPP	60DPP
COW	SAA3		
1	36.13	1.00	1.00
2	6.53	3.01	1.00
3	20.41	2.88	1.00
4	16.18	10.11	1.00
5	199.52	7.42	1.00
6	69.58	3.34	1.00
7	-3.18	-1.77	1.00
8	11.09	24.26	1.00
9	16.49	-1.41	1.00
10	5.08	-1.20	1.00
11	66.14	157.37	1.00
12	7.41	17.54	1.00
13	33.95	9.21	1.00
Mean	37.33	17.83	
Median	16.49	3.34	

	15DPP	30DPP	60DPP
COW	HP		
1	6.07	7.34	1.00
2	143.07	1.08	1.00
3	234.87	9.26	1.00
4	2.07	1.79	1.00
5	1.40	-1.97	1.00
6	9.42	3.00	1.00
7	13.13	2.32	1.00
8	4.45	34.20	1.00
9	2.91	1.71	1.00
10	15.58	-7.21	1.00
11	34.66	2.73	1.00
12	2.55	1.79	1.00
13	3.13	1.29	1.00
Mean	36.41	4.41	
Median	6.07	1.79	

Table A9: mRNA relative fold changes in genes encoding proinflammatory cytokines *IL-1β* and *IL-6* in uterine tissues from postpartum cows. For presentation of the data, 15 and 30 DPP values are expressed relative to 60 DPP values in the same animal.

	15DPP	30DPP	60DPP
COW		<i>IL-1β</i>	
1	24.01	4.86	1.00
2	21.93	7.59	1.00
3	62.04	2.51	1.00
4	26.10	4.81	1.00
5	27.84	1.79	1.00
6	108.06	15.29	1.00
7	32.09	7.54	1.00
8	44.96	2.00	1.00
9	6.40	3.08	1.00
10	-1.28	-1.20	1.00
11	39.88	6.71	1.00
12	-1.46	-1.11	1.00
13	17.65	3.22	1.00
Mean	31.40	4.39	
Median	26.10	3.22	

	15DPP	30DPP	60DPP
COW		<i>IL-6</i>	
1	9.78	4.43	1.00
2	2.34	1.26	1.00
3	25.40	4.35	1.00
4	10.62	3.41	1.00
5	1.99	1.35	1.00
6	13.93	8.18	1.00
7	17.99	22.00	1.00
8	121.03	2.99	1.00
9	2.50	1.28	1.00
10	5.70	3.10	1.00
11	7.37	3.01	1.00
12	12.56	2.14	1.00
13	5.32	3.28	1.00
Mean	18.20	4.68	
Median	9.78	3.10	

Table A10: mRNA relative fold changes in genes encoding AMPs *LAP* and *TAP* in uterine tissues from postpartum cows. For presentation of the data, 15 and 30 DPP values are expressed relative to 60 DPP values in the same animal.

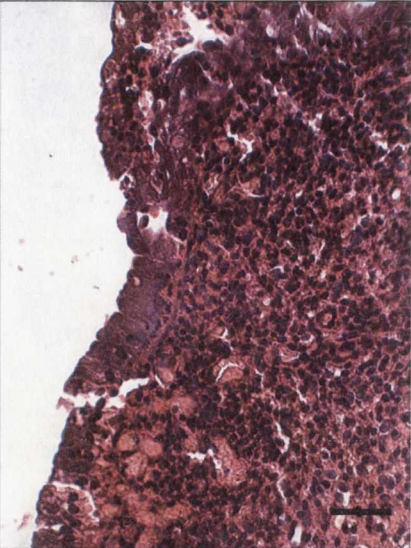
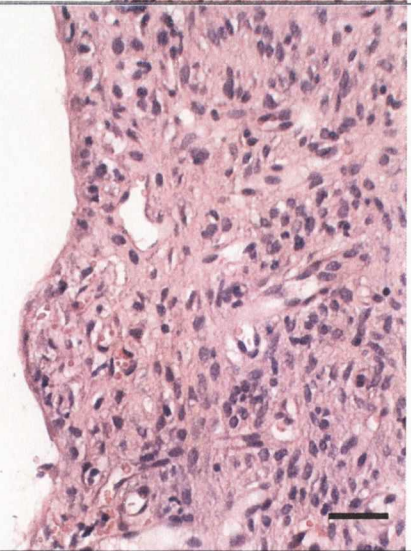
	15DPP	30DPP	60DPP
COW		LAP	
1	49.36	9.28	1.00
2	21.26	1.06	1.00
3	3.61	1.80	1.01
4	2.16	4.29	1.00
5	5.96	1.25	1.00
6	3.57	5.11	1.00
7	4.64	2.56	1.00
8	1.21	0.97	1.00
9	20.70	2.63	1.00
10	1.49	112.73	1.00
11	9.43	4.26	1.00
12	1.25	-1.44	1.00
13	16.99	7.55	1.00
Mean	10.89	11.70	
Median	4.64	2.63	

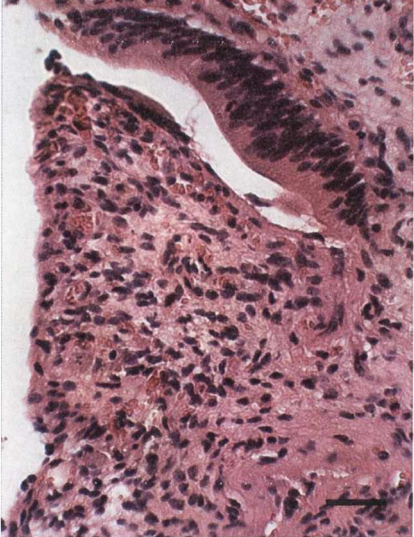
	15DPP	30DPP	60DPP
COW		TAP	
1	27.12	8.31	1.00
2	5.28	1.35	1.00
3	54.19	5.84	1.00
4	114.56	2.92	1.00
5	1.80	-1.54	1.00
6	26.83	5.03	1.00
7	21.22	8.47	1.00
8	48.35	3.28	1.00
9	474.46	38.58	1.00
10	74.59	39.56	1.00
11	8.91	9.14	1.00
12	344.67	2.44	1.00
13	235.71	11.47	1.00
Mean	110.59	10.37	
Median	48.35	5.84	

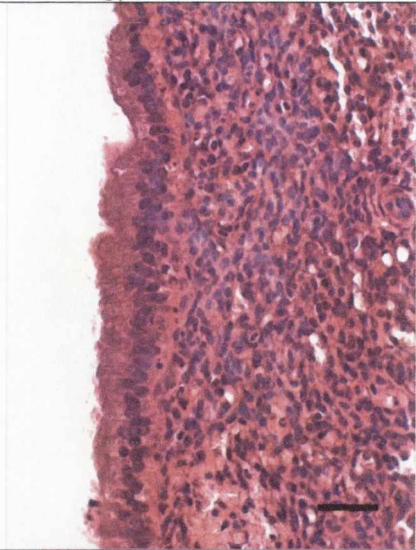
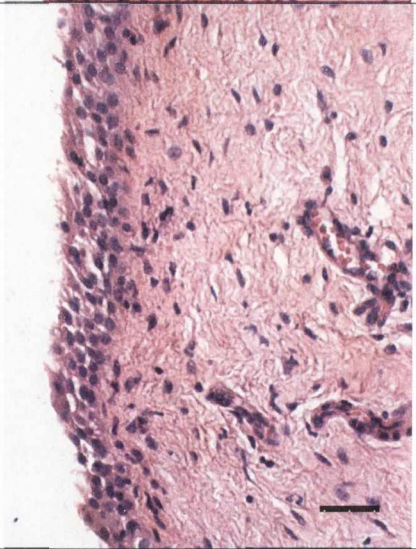
Table A11: mRNA relative fold changes in genes encoding AMPs *BNBD4* and *DEFB5* in uterine tissues from postpartum cows. For presentation of the data, 15 and 30 DPP values are expressed relative to 60 DPP values in the same animal.

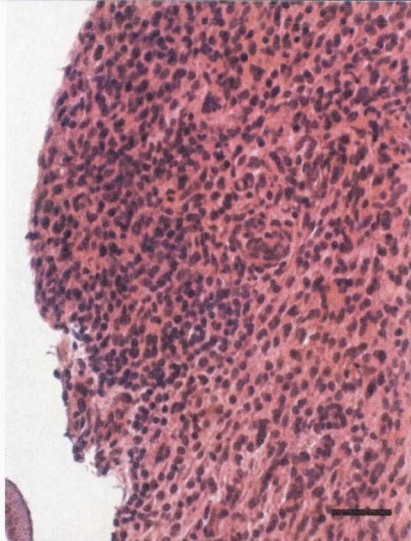
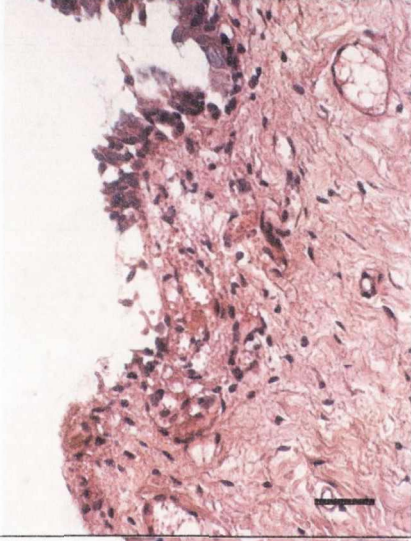
	15DPP	30DPP	60DPP
COW	<i>BNBD4</i>		
1	140.15	5.32	1.00
2	19.08	1.19	1.00
3	-22.09	-23.26	1.00
4	102.14	29.98	1.00
5	1.20	-1.38	1.00
6	6.14	3.41	1.00
7	1.39	1.91	1.00
8	44.67	3.92	1.00
9	1.98	15.63	1.00
10	1.24	2.56	1.00
11	12.69	-2.25	1.00
12	-10.88	-7.16	1.00
13	2.82	2.25	1.00
Mean	23.12	2.47	
Median	2.82	2.25	

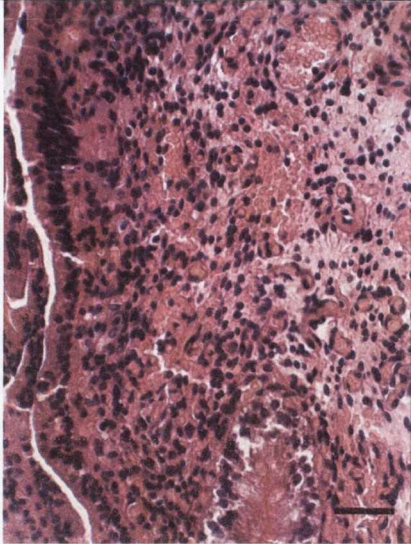
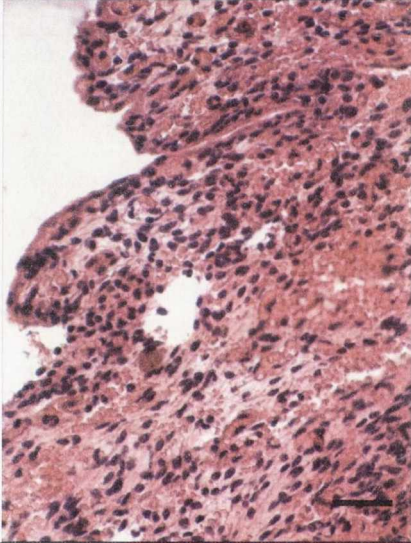
	15DPP	30DPP	60DPP
COW	<i>DEFB5</i>		
1	131.60	11.88	1.00
2	15.68	2.47	1.00
3	7.22	3.57	1.00
4	80.49	67.24	1.00
5	3.27	1.73	1.00
6	4.45	1.77	1.00
7	76.64	72.70	1.00
8	256.85	41.81	1.00
9	12.31	8.11	1.00
10	21.14	1.24	1.00
11	24.35	6.39	1.00
12	126.77	1.39	1.00
13	2.58	2.54	1.00
Mean	58.72	17.14	
Median	21.14	3.57	

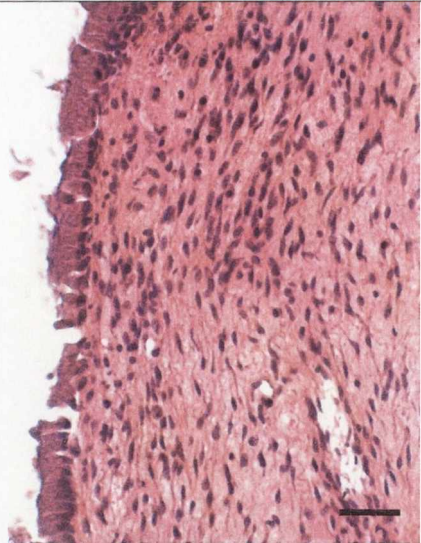
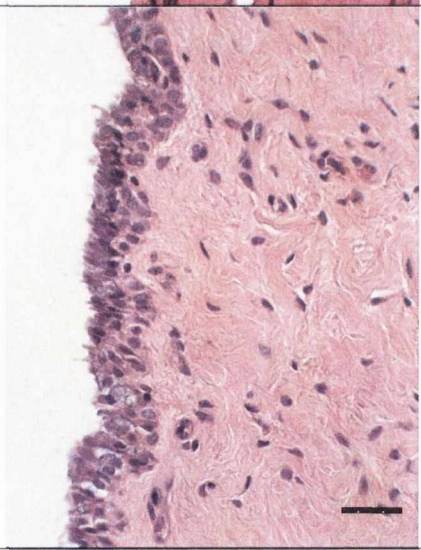
Time point	Histology	Feature
15 DPP		Columnar epithelial covering with prominent vascular congestion. A mixed lymphocyte and neutrophil infiltration. Neutrophil exocytosis. 92 cells/hpf Category: 3 Bar = 250 μm
30 DPP		Columnar to cuboidal epithelium with mononuclear cell infiltration. Some lymphocyte aggregations. 67 cells/hpf Category: 2 Bar = 250 μm
60 DPP		Pseudostratified epithelium (some sloughed off) with a low-grade mononuclear cell infiltration 50 cells/hpf Category: 1 Bar = 250 μm

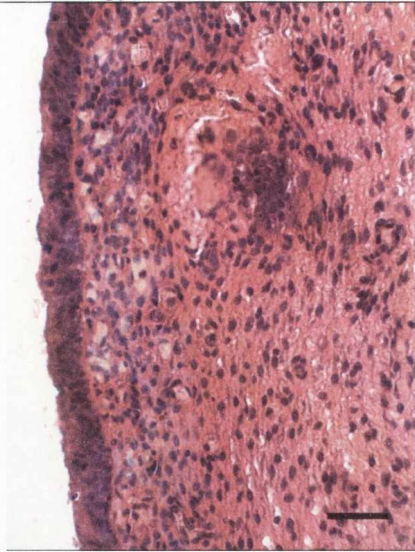
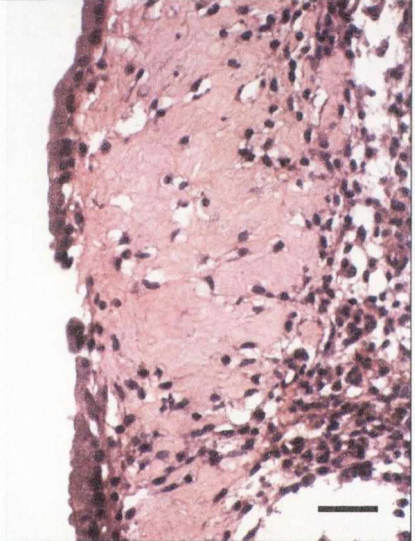
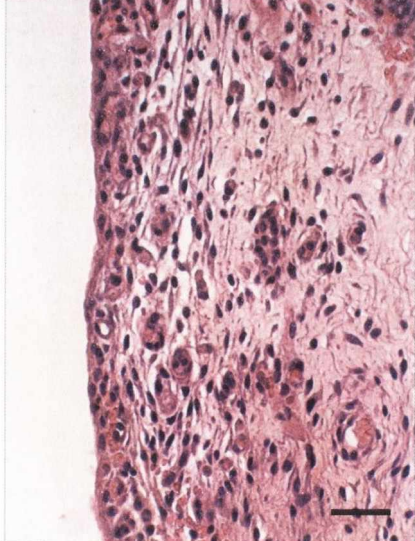
Time point	Histology	Feature
15 DPP		Prominent columnar epithelium, oedema and moderate lymphocyte-rich. Some vascular congestion and occasional neutrophils. 110 cells/hpf Category: 3 Bar = 250 μm
30 DPP		Columnar epithelium, rare cell infiltrates, and some muscular tissue. 42 cells/hpf Category: 2 Bar = 250 μm
60 DPP		Columnar epithelium with mononuclear cells. Prominent glands. 19 cells/hpf Category: 0 Bar = 250 μm

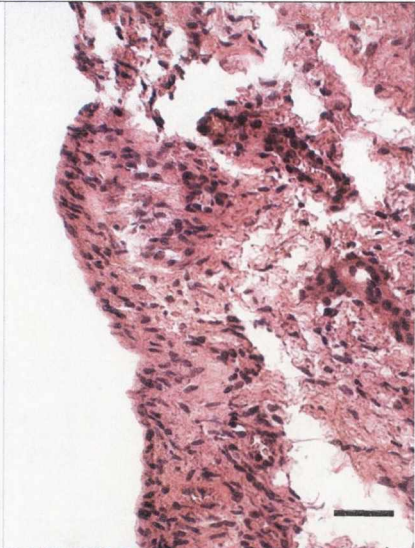
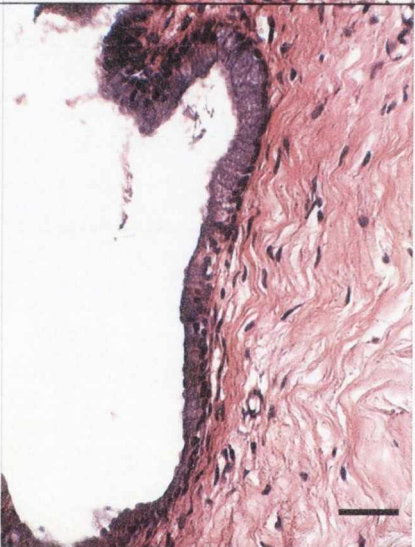
Time point	Histology	Feature
15 DPP		Columnar epithelium. Prominent lymphocyte and neutrophil infiltration accompanied by vascular congestion. 114 cells/hpf Category: 3 Bar = 250 μm
30 DPP		Columnar epithelium (partial sloughing in areas), low-grade mononuclear cell infiltration.. 104 cells/hpf Category: 3 Bar = 250 μm
60 DPP		Pseudostratified non-keratinising ciliated epithelium on a mature collagen-rich non-inflamed stroma. 29 cells/hpf Category: 1 Bar = 250 μm

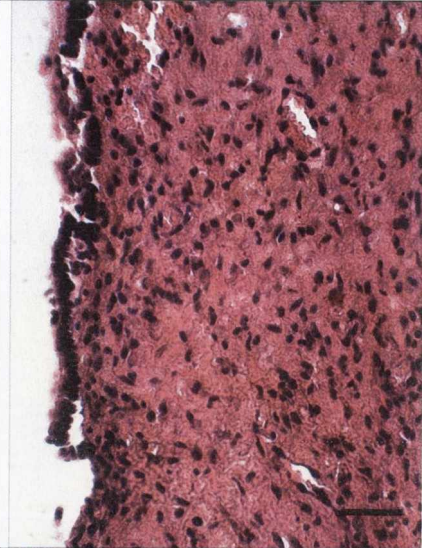
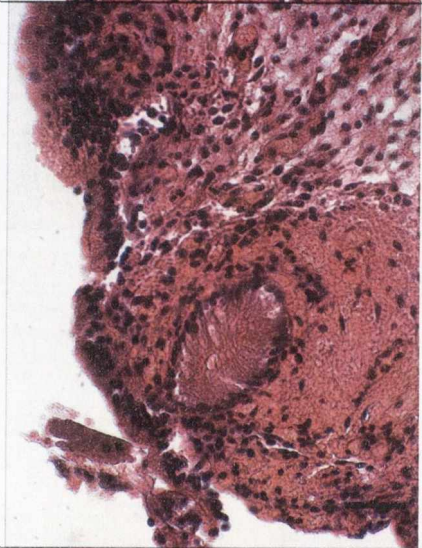
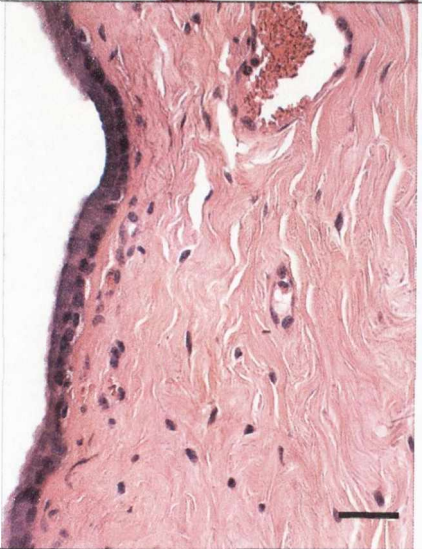
Time point	Histology	Feature
15 DPP		Epithelial loss, some columnar epithelium present in places. Prominent inflammatory infiltration. Vascular congestion. 123 cells/hpf Category: 3 Bar = 250 μm
30 DPP		Columnar epithelium (partial sloughing), low-grade mononuclear cell infiltration. 42 cells/hpf Category: 1 Bar = 250 μm
60 DPP		Pseudostratified ciliated epithelium on a mature collagen-rich non-inflamed stroma. 18 cells/hpf Category: 0 Bar = 250 μm

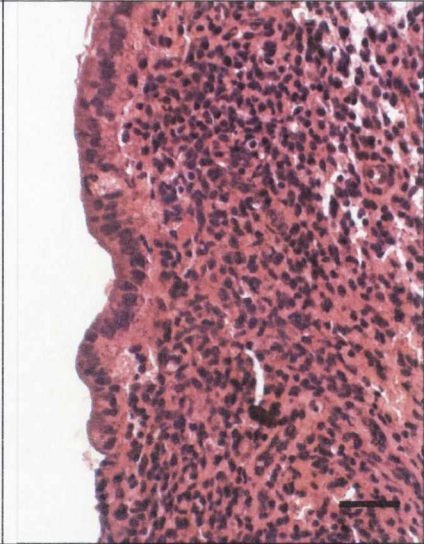
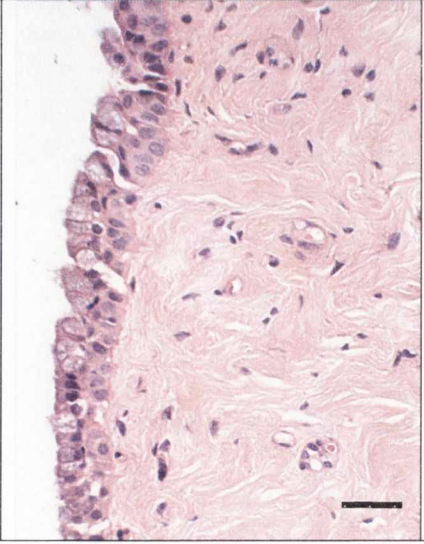
Time point	Histology	Feature
15 DPP		Columnar epithelial covering with prominent vascular congestion. A mixed lymphocyte and neutrophil infiltration, with neutrophil exocytosis. 97 cells/hpf Category: 3 Bar = 250 μm
30 DPP		Columnar epithelium, some sloughing off. Moderate mononuclear cell infiltration and vascular congestion. 92 cells/hpf Category: 3 Bar = 250 μm
60 DPP		Epithelium sloughed. Mild mononuclear cell infiltration. 48 cells/hpf Category: 1 Bar = 250 μm

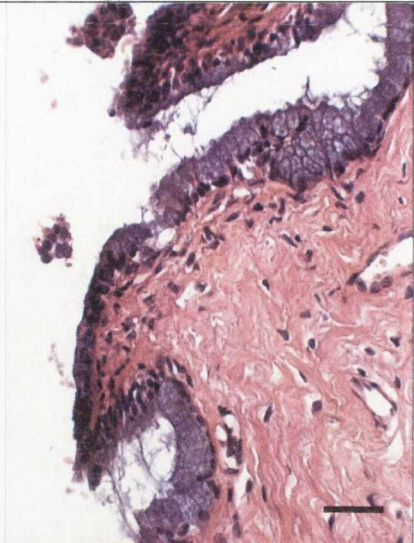
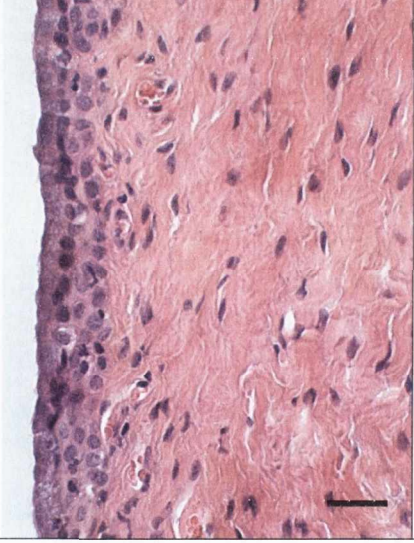
Time point	Histology	Feature
15 DPP		Columnar epithelium with prominent mononuclear cell infiltration. Neutrophils present. Vascular congestion. 120 cells/hpf Category: 3 Bar = 250 μm
30 DPP		Columnar epithelium, low-grade lymphocyte (and occasional neutrophil) infiltration. 106 cells/hpf Category: 2 Bar = 250 μm
60 DPP		Pseudostratified to columnar ciliated epithelium on a mature collagen-rich non-inflamed stroma. 16 cells/hpf Category: 0 Bar = 250 μm

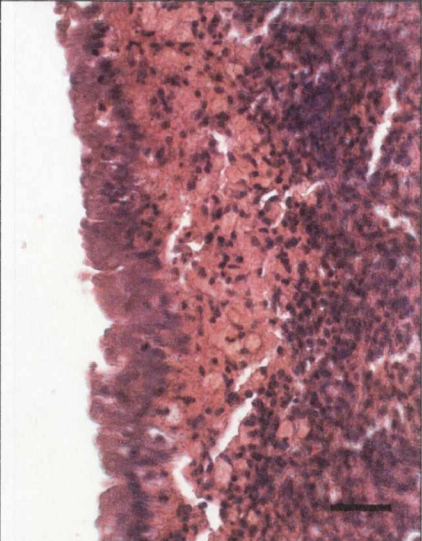
Time point	Histology	Feature
15 DPP		Prominent vascular congestion in and a mixed lymphocyte and neutrophil infiltration, with neutrophil exocytosis. Endometrium mostly intact. 119 cells/hpf Category: 3 Bar = 250 μm
30 DPP		Columnar epithelium with a low-grade mononuclear cell infiltration. 47 cells/hpf Category: 1 Bar = 250 μm
60 DPP		Columnar epithelium with mild mononuclear cell infiltration. 21 cells/hpf Category: 0 Bar = 250 μm

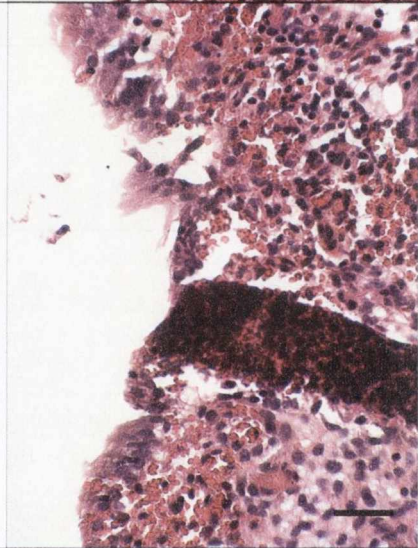
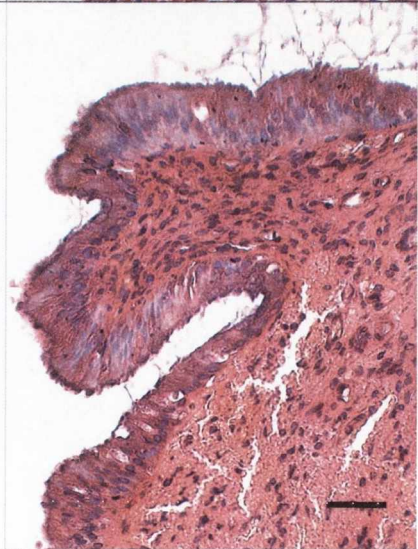
Time point	Histology	Feature
15 DPP		Columnar epithelium. Prominent mononuclear cell infiltration. Neutrophils present. Vascular congestion. 122 cells/hpf Category: 3 Bar = 250 μm
30 DPP		Columnar epithelium with a low-grade mononuclear cell infiltration and occasional neutrophils with exocytosis. 33 cells/hpf Category: 1 Bar = 250 μm
60 DPP		Pseudostratified to columnar epithelium on a mature collagen-rich non-inflamed stroma. 24 cells/hpf Category: 0 Bar = 250 μm

Time point	Histology	Feature
15 DPP		Prominent inflammatory influx. Epithelial, muscular and connective tissue. Vascular congestion. 147 cells/hpf Category: 3 Bar = 250 μm
30 DPP		Columnar epithelium with a low-grade mononuclear cell infiltration and occasional neutrophils. 46 cells/hpf Category: 1 Bar = 250 μm
60 DPP		Pseudostratified to columnar epithelium on a mature collagen-rich non-inflamed stroma. 17 cells/hpf Category: 0 Bar = 250 μm

Time point	Histology	Feature
15 DPP		Epithelial loss in places. Columnar epithelium present. Intense lymphocyte and neutrophil infiltration 127 cells/hpf Category: 3 Bar = 250 μm
30 DPP		Columnar epithelium with a mononuclear cell infiltration and neutrophils. 99 cells/hpf Category: 3 Bar = 250 μm
60 DPP		Pseudostratified to columnar epithelium on a mature collagen-rich non-inflammed stroma. 22 cells/hpf Category: 0 Bar = 250 μm

Time point	Histology	Feature
15 DPP		Epithelial loss in places, columnar epithelium present. Lymphocyte and neutrophil infiltration. 89 cells/hpf Category: 3 Bar = 250 μm
30 DPP		Columnar epithelial cells on a mature connective tissue stroma with focal low-grade mononuclear cell infiltrations. Sloughed epithelial cells in places and acellular proteinaceous material. 33 cells/hpf Category: 1 Bar = 250 μm
60 DPP		Pseudostratified to columnar epithelium on a mature collagen-rich non-inflamed stroma. 38 cells/hpf Category: 1 Bar = 250 μm

Time point	Histology	Feature
15 DPP		Prominent leukocyte infiltration, mainly lymphocytes. 213 cells/hpf Category: 3 Bar = 250 μm
30 DPP		Columnar epithelium with a low-grade mononuclear cell infiltration 65 cells/hpf Category: 2. Bar = 250 μm
60 DPP		Pseudostratified to columnar epithelium on a mature collagen-rich non-inflamed stroma. 13 cells/hpf Category: 0 Bar = 250 μm

Time point	Histology	Feature
15 DPP		Mucus and inflammatory cells accumulation within the uterine wall. 115 cells/hpf Category: 3 Bar = 250 μm
30 DPP		Columnar epithelium. Vascular congestion and a low-grade neutrophil rich infiltrate along base of epithelium with exocytosis. 74 cells/hpf Category: 2 Bar = 250 μm
60 DPP		Columnar and pseudostratified epithelium with a low-grade mononuclear cell infiltration. Aggregates of lymphocytes. 46 cells/hpf Category: 1 Bar = 250 μm