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BOVINE BLOOD NEUTROPHIL GENE EXPRESSION AND THE EFFECTS OF PARTURITION

By

Sally Ann Madsen

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ABSTRACT

BOVINE BLOOD NEUTROPHIL GENE EXPRESSION AND THE EFFECTS OF PARTURITION

By

Sally Ann Madsen

Neutrophil dysfunctions and increased occurrences of production diseases are often observed during periparturition. The objectives of the current study were to reveal and characterize differential gene expression in neutrophils from periparturient dairy cows, determine if altered gene expression relates to fluctuating concentrations of steroid hormones, and obtain putative identities of the differentially expressed genes. Altered gene expressions were identified using differential display reverse transcription polymerase chain reaction (DDRT-PCR). Fourteen cDNAs from DDRT-PCR were analyzed by dot blot hybridization for initial confirmation of altered mRNA abundance. Two of the most dramatically altered genes were quantitatively assessed by Northern and slot blot analyses using multiple pre- and post-parturition neutrophil RNA samples from four Holstein cows. Sera were also harvested from these animals to assay cortisol, progesterone, and estradiol by RIA. Results showed that the two pursued DDRT-PCR products had DNA sequences similar to genes for bovine mitochondrial cytochrome b and rig/ribosomal protein S15 involved in energy metabolism and translation regulation, respectively. Both genes showed significant ($P \leq 0.02$) repression between parturition and approximately seven days into lactation, and significant positive correlations with serum progesterone and (or) estradiol concentrations. Thus, parturition induces changes in mRNA abundance of neutrophil genes consistent with known defects in cell function during the periparturient period.

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LIST OF ABBREVIATIONS

ACD = Acid Citrate Dextrose

AML-1 = (a transcription factor)

BLASTn = Basic Local Alignment Search Tool – nucleotide

bp = Base Pair

C/EBP-α = (a transcription factor)

CAP-18 = 18-kDa Cationic Antibacterial Protein

CD = Cluster of Differentiation (e.g. CD11a, CD11b, CD14, CD15, CD16, CD18, CD68)

CD62L = L-selectin

cDNA = Complementary DNA

cytb = Bovine Mitochondrial cytochrome b

d = Day

DNA = Deoxyribonucleic Acid

dNTP = Deoxyribinucleoside Triphosphate

DDRT-PCR = Differential Display Reverse Transcription Polymerase Chain Reaction

EDTA = Ethylenediaminetetraacetic Acid

fMLP = formyl-methionyl-leucyl-phenylalanine

IL = Interleukin (e.g. IL-1β, IL-6, IL-8)

LFA-1 = CD11a/CD18

Mac-1 = CD11b/CD18

MHC = Major Histocompatibility Complex

mRNA = Messenger Ribonucleic Acid

NADPH = Nicotinamide adenine dinucleotide phosphate - hydrogenated

NaOH = Sodium Hydroxide

NGAL = neutrophil gelatinase-associated lipocalin

PBS = Phophate Buffered Saline

PCR = Polymerase Chain Reaction

rig/RPS15 = rig/Ribosomal Protein S15

ROS = Reactive Oxygen Species

rRNA = Ribosomal Ribonucleic Acid

RT = Reverse Transcription

SDS = Sodium Dodecyl Sulfate

SSC = Saline Sodium Citrate

tRNA = Transfer Ribonucleic Acid

INTRODUCTION

Parturition occurs multiple times in the life of an average dairy cow. This event is necessary to maintain a level of milk production beneficial to the dairy industry. Through the periparturient period, dairy cattle experience a multitude of changes including changes to the udder during the transition from non-lactating to lactating (Oliver and Sordillo, 1988), alterations in metabolism and intake (Ingvartsen and Andersen, 2000), as well as dramatic fluctuations of metabolic and steroid hormones (Smith et al., 1973; Goff and Horst, 1997). The periparturient period has also been associated with decreased immune function that has been postulated to be primarily responsible for the increased susceptibility to mastitis and other production diseases observed during this time (Cai et al., 1994; Mallard et al., 1998; Kehrli and Harp, 2001). Numerous investigators have hypothesized that the changes in steroid and metabolic hormones during periparturition are responsible for the decreased immune function leading to this disease susceptible state (Curtis et al., 1985; Goff et al., 1989; Goff and Horst, 1997; Lee and Kehrli, 1998; Kehrli and Harp, 2001; Weber et al., 2002).

Several cell types of the immune system are affected during bovine parturition. For example, the effects of parturition have been studied on blood and milk neutrophils as well as various subsets of blood and milk lymphocytes (i.e., CD4+, CD8+, and γδ TCR+ T cells). In such studies, the T cells from parturient cows have been shown to decrease in numbers within the blood and milk; this is accompanied by decreased *in vitro* proliferative responses to several mitogens compared to T cells from cows in mid-lactation (Shafer-Weaver et al., 1996; Kimura et al., 1999a). However, the actual

relevance of these changes in T cell numbers and *in vitro* functions to immunosuppression and periparturient disease susceptibility has not been established.

In contrast, parturition's negative effects on blood neutrophil counts and functions appear to be of considerable consequence to disease susceptibility of parturient cows. Under normal circumstances, blood derived neutrophils are the first line of innate immune defense against bacteria and other microorganisms that cause acute mastitis, metritis, and other common infectious diseases of parturient dairy cows. Neutrophils originate in the bone marrow and continuously enter the circulation with or without presence of peripheral tissue infections (Kuby, 1997; Janeway et al., 1999). Blood neutrophils spend much of their short life span (10 to 24 hours) adhering to and rolling on the endothelial cells that line the walls of post-capillary venules in peripheral tissues. They do this as a surveillance mechanism to determine the infection status of the underlying peripheral tissues. If rolling neutrophils detect endothelial and soluble signals such as cytokines and chemokines of inflammation and infection, the cells rapidly adhere to the endothelial cell wall and migrate from the blood compartment into the infected tissue (Kansas, 1996). The process of migration activates oxygen dependent and independent mechanisms in neutrophils enabling these cells to become highly efficient phagocytes that ingest and destroy infecting pathogens in tissues.

Periparturition alters many of the functions of neutrophils, resulting in nearly complete dysfunction of the chain of events described above. For example, pronounced reductions have been observed in trafficking, random migration, chemotaxis, and myeloperoxidase activity of blood neutrophils from periparturient cows (Nagahata et al., 1988; Kehrli et al., 1989; Cai et al., 1994; Kimura et al., 1999b; Weber et al., 2002).

Although many of the neutrophils' functions were decreased around parturition, neutrophilia, or an increase in the number of neutrophils in the blood, is generally observed during this time. Several researchers have shown that this could be related to decreased expression of adhesion molecules on the neutrophils' surface (Lee and Kehrli, 1998; Kimura et al., 1999b; Weber et al., 2002). Such molecules are needed for normal interactions with endothelial cells that lead to neutrophil rolling and migration. The reduction in trafficking results in neutrophil accumulation in the circulation leaving the mammary gland and other tissues devoid of first line immunity against opportunistic bacteria.

Despite the fact that researchers have documented observations of decreased neutrophil functions in periparturient dairy cows for several decades, little is known about how or why dysfunctions of these leukocytes occur. The work described in Chapter Two focuses on the possibility that neutrophils of parturient cows respond to the physiology of parturition with altered gene expression. It is widely believed by leukocyte biologists that circulating neutrophils are biosynthetically inactive phagocytes (Bainton et al., 1971). This is in large part due to the fact that these terminally differentiated white blood cells have highly condensed nuclei, store many preformed proteins in their multiple complex granules for rapid killing of pathogens in infected tissues, and have such a short half-life in blood. However, several recent studies challenge this dogma showing that neutrophils actively express hundreds of genes in the resting state and respond to multiple environmental stimuli, such as bacteria and cytokines, with altered expression of numerous genes (Lloyd and Oppenheim, 1992; Itoh et al., 1998; Newberger et al., 2000). These findings substantiate numerous observations from experiments in our laboratory

(Weber et al., 2002), which have shown that bovine blood neutrophils respond to the stress of parturition and to administration of glucocorticoid hormones with inhibited expression of the gene for L-selectin. To our knowledge, however, no studies have been published that explore overall gene expression changes in bovine neutrophils around parturition, nor the potential factors responsible for these changes

The goal of the present study was to begin exploring differences in overall gene expression in blood neutrophils as cows transition from the dry period through parturition. The objective of the first series of experiments in this work was to identify and fully confirm differential expression of at least two bovine neutrophil genes not previously reported from other periparturient dairy cow research. The objective of the second series of experiments was to determine if fluctuations in the three major steroid hormones of bovine parturition (cortisol, progesterone, and estradiol) correlate with changes in expression of the identified neutrophil genes. Steroid hormones were selected for this study as possible contributors to altered gene expression in blood neutrophils because these agents have direct and profound effects upon gene expression in other cell systems (Carson-Jurica et al., 1990; Beato et al., 1995). The final objective was to obtain putative identities of genes demonstrating differential expression in objective one. Results of the experiments reported in this thesis are novel and important because they clearly identify reduced mRNA abundance of two neutrophil genes shortly after parturition. One of these genes is required for energy metabolism in the cell, while the other gene may have a role in translation regulation. Expressions of both genes showed substantial relationships with serum steroid concentrations. Approximately ten additional mRNAs were identified as putatively reduced due to parturition, some of which appear to

encode proteins involved in the citric acid cycle and DNA binding. Further experiments will be required to confirm and discern the contributions of the decreased mRNA abundance of all of these genes to neutrophil dysfunctions and mastitis susceptibility in periparturient cows.

CHAPTER ONE

A Review of Literature

I. THE IMMUNE SYSTEM

The bovine immune system, like that found in all mammals, consists of two branches that work together to provide protection from invading pathogens. These branches are referred to as innate, or nonspecific immunity, and adaptive, or acquired immunity (See **Figure 1.1**) (Kuby, 1997; Janeway et al., 1999). The adaptive immune system is specific in its response to pathogens and is continually developing. This branch of the immune system has two components, cell-mediated and humoral immune responses. The humoral response is mediated through B lymphocytes and their ability to differentiate into antibody producing plasma cells that secrete large amounts of antibodies following exposure to foreign antigens (such as those on bacteria). Antigens presented by MHC molecules on macrophages, dendritic cells, and B lymphocytes are also capable of activating T lymphocytes, which subsequently facilitate the humoral immune responses of B lymphocytes or mediate cytotoxicity of T lymphocytes against virally-infected cells.

Unlike the adaptive immune responses, innate immunity is present at birth, is immediately available through cellular and humoral factors present in the blood at all times, and provides the first line of defense against invading pathogens. It consists primarily of phagocytic leukocytes, called neutrophils and macrophages, and serum factors such as complement proteins, collectins, and acute phase proteins. In the event that the innate immune response is dysfunctional, or an infection overcomes the phagocytes' abilities to clear it, adaptive immunity will become activated over subsequent

days and weeks to provide highly specific and targeted defense against the invading pathogens.

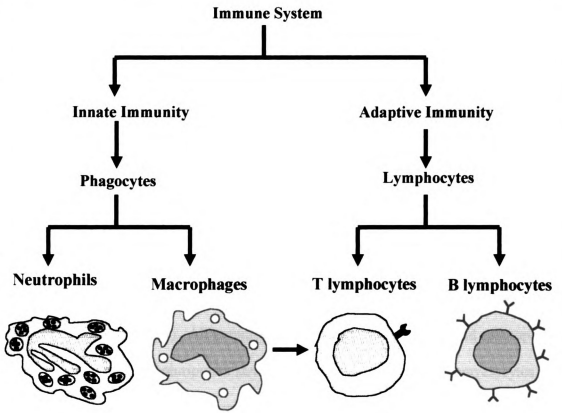


Figure 1.1 – The immune system consists of two branches, innate immunity and adaptive immunity. Phagocytic cells, such as neutrophils and macrophages, are key players in innate immunity. B and T lymphocytes are necessary for the proper function of adaptive immunity. Interactions between innate and adaptive immunity occur when macrophages present antigens to T lymphocytes for activation and when neutrophils phagocytose antigens opsonized by antibodies that were secreted from activated B lymphocytes.

II. NEUTROPHILS: THE FIRST LINE OF IMMUNE DEFENSE

Neutrophils play a key role in innate immunity in cattle. In particular, neutrophils are primarily responsible for clearing the bacteria that infect mammary quarters and cause clinical mastitis in dairy cows (Kehrli and Harp, 2001). The following summary of information about neutrophil development and the five main functions of this leukocyte subset is provided to set the stage for the context under which the current thesis research was performed.

A. Neutrophil Development in the Bone Marrow

Immune cells are produced in the bone marrow, which is also the site of neutrophil maturation. Approximately 60% of all marrow cells are neutrophils at various stages of development (Edwards, 1994). All granulocytes (neutrophils, basophils, and eosinophils) differentiate from a pool of pluripotent stem cells into myeloblasts and promyelocytes via the process of granulopoeisis. Neutrophilic cells then mature in stages into myelocytes, metamyelocytes, band cells, and finally the segmented (mature) neutrophils that are released into the circulating blood (**Figure 1.2**). The maturing cells were named according to appearance or disappearance of specific cellular markers (detected by various staining methods) and cell morphology, which is described below. Development from pluripotent stem cells into segmented neutrophils takes approximately 14 days, 7.5 days of which are spent in proliferation stages (myeloblast, promyelocyte, and myelocyte) and the remaining 6.5 days of which see maturation through metamyelocyte, band cell and segmented neutrophil stages (Bainton et al., 1971).

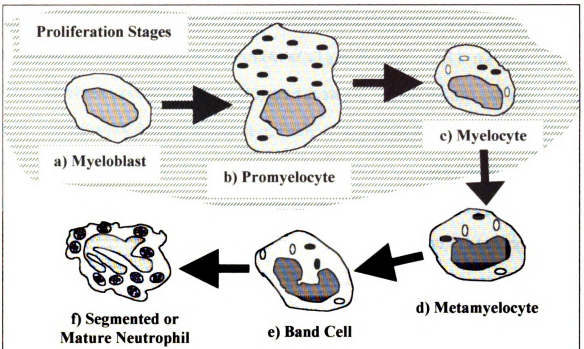


Figure 1.2 – Neutrophil development occurs in the bone marrow. There are a total of six developmental stages, three that are proliferative in which many granules are formed (mitotic; steps a, b, and c in shaded area) and three that mainly consist of changes in the nuclear morphology (steps d, e, and f) resulting in mature cells with large, multi-lobed nucleus and abundant cytoplasmic granules. See text for details.

Myeloblasts, cells in the first stage of granulopoeisis, are characterized by their positive staining for peroxidase in the rough endoplasmic reticulum and Golgi apparatus regions (**Figure 1.2, step a**) (Edwards, 1994). During the transition from myeloblast to promyelocyte, granules are formed within which the peroxidase is packaged (**Figure 1.2, step b**) (Edwards, 1994; Berliner, 1998). Promyelocytes contain a large number of these azurophil, or primary, granules that contain peroxidase and stain a red-purple color with

azure dyes. The majority of the peroxidase within azurophil granules is myeloperoxidase, an enzyme important for the killing functions of all granulocytes. Cells in the next stage of development are called myelocytes (**Figure 1.2, step c**). This is the first cell type committed to become neutrophils (Berliner, 1998). The accumulation of numerous peroxidase-negative granules (named specific, or secondary granules) is characteristic of myelocytes. No additional azurophil granules are synthesized during this stage of development (Edwards, 1994). Myelocytes are the last stage of neutrophil development in which proliferation occurs. Metamyelocytes, band cells, and segmented neutrophils are distinguished by their granule protein content and nuclear morphology (Edwards, 1994; **Figure 1.2, steps d, e, f respectively**). The mature neutrophil is distinct from other leukocytes in that it has a large, multi-lobed nucleus and abundant acidic and basic granules located in the cytoplasm that are packed full with myeloperoxidase, lytic enzymes, and other proteins required for surveillance, migration, phagocytosis, and killing functions of these leukocytes (Edwards, 1994; Kuby, 1997).

Many additional membrane proteins, enzymes, and secretory proteins distinguish stages of neutrophil development in the bone marrow. In 1995, Borregaard et al. hypothesized that sorting of granule proteins is controlled by the timing of their synthesis, not by the targeting of proteins to the various granules after they have already formed. Since then, several groups have been able to support this hypothesis by showing that mRNA for granule contents was only produced and most likely processed during developmental stages in which those granules were formed. Most of the transcripts detected were for matrix proteins (proteinase-3, elastase, defensin, lysozyme, lactoferrin, gelatinase, NGAL, and CAP-18), membrane proteins (CD68), or cell surface markers

(CD11b, CD15, and CD16). For example, myeloblasts are shown to have both mRNA and protein for myeloperoxidase, proteinase-3, neutrophil elastase, CD68 (Cowland and Borregaard, 1999), and CD15 (Terstappen et al., 1990). Promyelocytes also exhibit large quantities of myeloperoxidase (Nagaoka et al., 1998), proteinase-3, neutrophil elastase, CD68, and CD15 as well as defensin (Nagaoka et al.; 1998). In myelocytes there is continued (CD15 and defensin) and decreased (myeloperoxidase, proteinase-3, neutrophil elastase, and CD68) expression of some mRNA although protein levels remain high. At this stage, when peroxidase-negative granules are being formed, expression of lactoferrin and gelatinase increase (Nagaoka et al., 1998) along with NGAL, CAP-18 (Cowland and Borregaard, 1999), and CD11b (Terstappen, et al., 1990). Metamyelocytes are characterized by continued expression of gelatinase (Nagaoka et al., 1998), CD15 and CD11b, and increases in expression of CD16 (Terstappen et al., 1990) and lysozyme (Cowland and Borregaard, 1999). In conjunction with timing of mRNA and protein production, Borregaard et al. (2001) has shown that certain transcription factors are present during specific developmental stages. They demonstrated that transcription factor AML-1 was critical for primary granule protein expression while C/EBP-α was necessary for secondary granule protein expression. All of these data support the hypothesis that granule protein content is controlled by timing of synthesis. The research described above was performed using human neutrophils obtained from bone marrow.

In addition to azurophil and specific granules, bovine neutrophils contain a third type of granule referred to as large granules. These large granules, named due to their relative size, constitute the main storage compartment of bovine neutrophils. They contain oxygen-independent bactericidal agents and lactoferrin but lack enzymes and

proteins typically found in azurophil and specific granules (Gennaro et al., 1983). Zanetti et al. (1990) demonstrated that the large granules are formed after the azurophil granules but before specific granules. Other than the additional large type granules in bovine neutrophils, azurophil and specific granule contents are relatively similar across species. The biggest difference in granule content across species lies in the amount of granule proteins and enzymes stored. In comparison to human neutrophil granules, bovine neutrophil granules contain 15-20% less peroxidase activity (Gennaro et al., 1983) as well as lowered lysozyme and catalase activities (Styrt, 1989). In contrast, concentration and/or activities of alkaline phosphatase, lactoferrin, vitamin B_{12}-binding protein and general protein are greater in bovine neutrophils (Gennaro et al., 1978). Comparisons described by these two groups demonstrate that although there are differences in concentrations of the various proteins, overall granule content and the functions of neutrophils are similar across species (Gennarro et al., 1983; Styrt, 1989).

B. Mature Neutrophil Functions

Upon release from the bone marrow, neutrophils spend 10 to 24 hours in the blood, followed by 1 to 2 days in peripheral tissues. As previously stated, mature neutrophils have large, multi-lobed, segmented nuclei in which the chromatin is coarsely clumped (Gennaro et al., 1978; Edwards, 1994). Other characteristics of circulating neutrophils include numerous cytoplasmic granules (azurophil, specific, and large granules in the bovine), small amounts of Golgi and endoplasmic reticulum, as well as a few mitochondria and ribosomes. This cellular morphology is not surprising because much of

what neutrophils require to function properly in innate immunity against infections is needed for rapid action and thus must be preformed and stored in cytoplasmic granules.

Mature neutrophils must perform five main functions to effect immunity against invading pathogens – surveillance, recruitment, receptor-mediated phagocytosis, respiratory burst, and fusion of the phagosome (created by phagocytosis) with cytoplasmic granules containing lytic enzymes (i.e. lysosomes) to create phagolysosomes. Surveillance and recruitment occur while neutrophils are in the blood. Blood neutrophils are constantly surveying for infections in the peripheral tissue via a process called margination. If an infection is present, neutrophils become recruited to the infected area through the mechanisms of migration and chemotaxis. Margination and migration are known collectively as neutrophil trafficking. The remaining neutrophil functions take place once the cells have successfully migrated into tissues and result in the killing and clearance of invading pathogens.

Researchers have shown that neutrophil trafficking is mediated by a variety of adhesion molecules, such as selectins and integrins, located on the cell's surface. Under normal conditions, neutrophils attach lightly to the blood vessel endothelial cell wall via L-selectin adhesion molecules. The shear force of blood flow facilitates the rolling of L-selectin tethered neutrophils along the vessel wall (margination) (Jutila, 1992; Bargatze et al., 1994; Kansas, 1996), and on other neutrophils already arrested on the endothelium (Bargatze et al., 1994). High expression of integrin adhesion molecules is required for tight adhesions that arrest rolling neutrophils for migration into infected peripheral tissue (Jutila, 1992). When rolling neutrophils detect infection in underlying tissues, the cells up-regulate protein expression of β_2-integrins, such as LFA-1 (CD11a/CD18) and Mac-1

(CD11b/CD18), from preformed stores of these molecules in the peroxidase negative granules. Such up-regulation causes arrest of rolling neutrophils on the inflamed vessel and permits migration through the vessel wall into the infected tissue (Jutila, 1992). Therefore, proper trafficking of neutrophils requires expression of both L-selectin and β_2-integrins (Crockett-Torabi et al., 1995). However, while β_2-integrin expression on activated neutrophils is increased, expression of L-selectin is rapidly shut down via proteolytic cleavage at a membrane proximal site of the molecule. Together, these processes halt the rolling phenotype of neutrophils and promote the arrested phenotype with subsequent migration (Jutila, 1992; Soler-Rodriquez et al., 2000). While migration of arrested neutrophils into infected peripheral tissues requires Mac-1, it is also facilitated by heightened surface expression of chemokine receptors, especially the IL-8 receptor. Migrated neutrophils continue to use Mac-1, IL-8, and other receptors to follow concentration gradients of chemokines, complement components, and multiple cytokines released at high concentration from the infection focus. This concentration-dependent movement of migrated neutrophils into the infection focus is called chemotaxis (Kuby, 1997). If margination, migration and chemotaxis are completed efficiently, neutrophils are properly placed and become highly activated for rapid clearance of the pathogen.

Once in the infection focus, migrated neutrophils begin to clear pathogens by a process called phagocytosis (Kuby, 1997; Janeway et al., 1999). During phagocytosis, neutrophils mount a substantial respiratory burst that generates a variety of highly reactive oxygen species (ROS). The ROS begin to work on the phagocytosed pathogen, causing lipid peroxidation and oxidative damage to proteins, RNA, and DNA (Smith, 1994; Crockett-Torabi et al., 1995; Ward and Lentsch, 1999). The respiratory burst is signaled

when neutrophils bind to pathogens for phagocytosis. This is a receptor-mediated event in which a variety of cell surface receptors (e.g. CD14, CD18, Fc receptors, and complement receptors) bind various bacterial cell wall components and (or) host immune proteins (antibody and complement) that have opsonized the pathogen for enhanced phagocytosis by neutrophils (Janeway et al., 1999). The receptors, located within the neutrophil plasma membrane, transduce signals to the cell interior that initiate respiratory burst activity and the formation of pseudopods that encircle the bound pathogen for internalization into the neutrophil in a vesicle known as a phagosome.

Following phagocytosis, ROS-mediated oxidative degradation of the pathogens begins when incoming phagosomes fuse with outgoing lysosomes to form a phagolysosome. The various cytoplasmic granules, collectively called lysosomes, generate ROS by shuttling electrons across their membrane NADPH oxidase system resulting in the reduction of oxygen to superoxide anions, which is sometimes further converted to hydrogen peroxide. These lysosomes fuse with the incoming phagosome and release ROS into the phagolysosome to assist in the degradation of pathogens (Smith, 1994; Dahlgren and Karlsson, 1999). The various granules also contain myeloperoxidase and proteolytic enzymes (in azurophil granules), lactoferrin and lysozyme (in specific granules) (Smith, 1994), and bactenecins that are specific to the large granules of bovine neutrophils and have non-oxidative microbicidal activity (Zanetti et al., 1990). Myeloperoxidases aid in the oxidative damaging of pathogens. The other agents listed heighten damage already initiated by ROS and participate in complete digestion of the pathogen. For example, proteolytic enzymes aid in the digestion of bacterial structural proteins, lactoferrin sequesters numerous minerals and thus deprives the invading

pathogen of essential nutrients, and lysozyme and collagenase help destroy components of the bacterial envelope (Smith, 1994). In all, the functions of phagocytosis, respiratory burst, and generation of the phagolysosome are highly connected and necessary to mediate successful killing and clearance of invading pathogens by neutrophils.

Work has been performed that demonstrates how several neutrophil functions are affected under various conditions. One *in vitro* study established that older neutrophils express lower levels of surface L-selectin as well as a decreased ability to change shape and chemotactically migrate following exposure to fMLP, a bacterial protein that is commonly used to stimulate neutrophils during *in vitro* studies (Tanji-Matsuba et al., 1998). Aging neutrophils were also shown to degranulate more readily and release higher levels of reactive oxygen species than younger cells. A study in which cortisol and dexamethasone were administered to cows *in vivo* showed that these glucocorticoids lead to complete down regulation of L-selectin and significant reductions in CD18 expression on the surface of blood neutrophils, demonstrating a possible mechanism for the potent anti-inflammatory actions of these steroid hormones (Burton et al., 1995). Glucocorticoid induced down regulation of neutrophil L-selectin also correlated well with neutrophilia and increased mastitis susceptibility in treated cows (Burton and Kehrli, 1995), highlighting a significant pitfall in the leukocyte's sensitivity to steroidal anti-inflammatory agents. Others have shown that administration of epinephrine or cortisol to humans increased blood neutrophil counts (Hetherington and Quie, 1985; Steele et al., 1987), a phenomenon associated with the release of marginating neutrophils from the blood vessel wall. In addition, epinephrine and cortisol may cause premature release of neutrophils from bone marrow because up to 56% of circulating neutrophils have the band

nucleus morphology after administration of these hormones (Burton et al., 1995; Hetherington and Quie, 1985). Together, these studies demonstrate that neutrophils are sensitive to their environment and are capable of altering their phenotype and function under a variety of natural and artificial stimuli. This is in sharp contrast to a widely held but incorrect belief that neutrophils have little capacity to respond to the environment due to their cellular morphology and terminally differentiated status (Bainton et al., 1971).

III. PARTURITION: A TIME OF MANY CHANGES FOR THE BOVINE IMMUNE SYSTEM

Parturition plays an important role in the life of a dairy cow. In order to continue producing milk, this event must occur approximately every thirteen months (to maintain production levels beneficial to producers) and is characterized by numerous dramatic physiological and metabolic changes. Not only does the cow go from the non-lactating to the lactating state, there is significantly reduced dry matter intake and pronounced fluctuations in blood concentrations of metabolic and steroid hormones. This section will present evidence that neutrophils respond to the physiology of parturition with altered phenotypes and functional capacities, possibly in response to integrated changes in blood concentrations of cortisol, estradiol, and progesterone.

A. Neutrophil Dysfunctions During Periparturition

Most metabolic diseases, including milk fever, ketosis, retained placenta, and displacement of the abomasum, occur during the first two weeks following parturition (Goff and Horst, 1997). This is also a time when infectious diseases become clinical,

particularly mastitis. Intramammary infections and clinical mastitis result in altered mammary function, decreased milk production, and altered milk composition costing the dairy industry over $2 billion annually (National Mastitis Council, 1996). Neutrophils are the main immunological defense against mastitis. Thus, several researchers have studied immune functions of periparturient dairy cows as a means to explain the heightened mastitis susceptibility in these animals (**Tables 1.1 and 1.2**). Oliver and Sordillo (1988) showed that clinical mastitis often results from mammary infections that occurred during the dry period. They also demonstrated that the rate of new infections during the dry period is substantially higher (6.25 times) than the rate of infections in the previous lactation. Some hypothesized that this phenomena is a result of immune system malfunction around dry off and during the periparturient period. Numerous investigators have tested this hypothesis and shown that blood leukocyte functions start to change three weeks prior to calving and continue to be dysfunctional through three weeks post partum (reviewed by Mallard et al., 1998). In particular, blood neutrophils of parturient cows have decreased respiratory burst activity when measured *in vitro* and this phenotype associates strongly with increased occurrence of clinical mastitis (Mallard et al., 1998). It has also been demonstrated that neutrophil recruitment into the mammary gland is reduced around parturition, a phenotype well correlated with increased severity of clinical coliform mastitis in periparturient dairy cows (Hill et al., 1979; Shuster et al., 1996; Kehlri and Harp, 2001). Thus, strong evidence supports that increased susceptibility to clinical mastitis during the peripartum period is a direct function of decreased abilities of blood neutrophils to migrate into infected mammary quarters and kill pathogens once the cells have migrated.

Other researchers examined specific aspects of neutrophil function during the periparturient period to discern why these dysfunctions occur. For example, Guidry et al. (1976) found increases in circulating band neutrophils in blood of parturient cows, suggesting release of immature neutrophils from the bone marrow (**Table 1.1**). It was hypothesized by Guidry et al. that band neutrophilia occurs in response to the high levels of blood cortisol at parturition. Other researchers have also shown that the migration capacity of blood neutrophils, measured *in vitro* as random migration of the cells under agarose, is significantly reduced at parturition (Nagahata et al., 1988; Kehrli et al., 1989; Detilleux et al., 1994). Reduced migration could also explain the pronounced increase in circulating mature neutrophils around parturition (Preisler et al., 2000a), possibly as a result of significantly reduced L-selectin and β2 integrin expression on the surface of blood neutrophils in response to the surge in cortisol (Lee and Kehrli, 1998; Kimura et al., 1999b; Weber at al., 2002). In any case, it is clear from these studies that blood and possibly bone marrow neutrophils respond to parturient physiology by altering the expression of their surface adhesion molecules, which changes the trafficking patterns of these cells in favor of reduced migration into infected mammary glands. This alone could explain the increased susceptibility to mastitis in parturient dairy cows.

Table 1.1 – Summary of blood neutrophil responses to parturient physiology: altered trafficking of the cells.

Neutrophil Response	Parturient Alteration	Citation
Migration	Decreased	Nagahata et al., 1988 Kehrli et al., 1989 Detilleux et al., 1994
Blood neutrophil numbers	Increased	Guidry et al., 1976 Preisler et al., 2000a Weber et al., 2002
L-selectin (surface expression)	Decreased	Lee and Kehrli, 1998 Kimura et al., 1999b Weber et al., 2002
β2 integrin (surface expression)	Decreased	Lee and Kehrli, 1998 Kimura et al., 1999b

The functions of neutrophils known to be critical in bacterial clearance and killing in peripheral tissues have also been examined *in vitro* and shown to be markedly affected by parturition (**Table 1.2**). In one study, chemotaxis, detected as directed movement of neutrophils under agarose, was significantly reduced when the neutrophils were collected during the first week post partum versus several weeks prepartum (Nagahata et al., 1988). Cai et al. (1994) also observed decreased chemotaxis of parturient neutrophils and found it to be associated with occurrences of clinical mastitis, retained placenta, and metritis.

Phagocytic ability of neutrophils from parturient cows has also been evaluated *in vitro*. Guidry et al. (1976) showed that the total phagocytic capacity of neutrophils increased at parturition, mainly due to the increased numbers of circulating neutrophils found at this time. However, these researchers also showed that the increase in total phagocytosis was followed by an overall decrease in phagocytosis during the first three weeks post partum. This may be explained by the findings of others who showed that while overall bacterial ingestion was increased around parturition, other aspects of phagocytic activity such as number of bacteria phagocytosed and killed were decreased in parturient cows (Kehrli and Goff, 1989; Kehrli et al., 1989; Cai et al., 1994). Superoxide anion production, which is critical for pathogen damage following the respiratory burst in phagocytosing neutrophils, was demonstrated as significantly reduced during periparturition (Kehrli and Goff, 1989; Detilleux et al., 1994). This was particularly evident in neutrophils from cows that exhibiting clinical mastitis, metritis, and retained placenta (Cai et al., 1994). Myeloperoxidase activity, another important factor in pathogen damage within the phagolysosome, was also deficient in neutrophils from periparturient cows (Kehrli and Goff, 1989; Kehrli et al., 1989; Cai et al., 1994; Detilleux et al., 1994; Kimura et al., 1999b), as was overall oxidative capacity of these phagocytes (Kehrli and Goff, 1989; Kehrli et al., 1989; Detilleux et al., 1994). In summary, researchers have clearly established that neutrophil trafficking (**Table 1.1**) and killing functions (**Table 1.2**) are significantly impaired in periparturient dairy cows. Not only does this show that neutrophils are highly sensitive to the physiology of parturition, results of past research easily explain the increased disease susceptibility to clinical mastitis and other common diseases of periparturient dairy cows.

Table 1.2 – Summary of responses of blood neutrophils to parturient physiology: altered phagocytic and killing functions of the cells.

Neutrophil Response	Parturient Alteration	Citation
Chemotaxis	Decreased	Nagahata et al., 1988
		Cai et al., 1994
Bacterial Ingestion	Increased	Kehrli and Goff, 1989
		Kehrli et al., 1989
		Cai et al., 1994
Super Oxide Production	Decreased	Kehrli and Goff, 1989
Myeloperoxidase Activity	Decreased	Kehrli and Goff, 1989
		Kehrli et al., 1989
		Cai et al., 1994
		Kimura et al., 1999b
Oxidative Capacity	Decreased	Kehrli and Goff, 1989
		Kehrli et al., 1989

B. Steroid Hormones of Parturition – Cortisol, Estradiol, and Progesterone

In order for parturition to occur, concentrations of cortisol, estradiol, and progesterone must fluctuate dramatically yet in a highly coordinated fashion. Several weeks prior to parturition, cortisol and estradiol are at low levels while progesterone concentrations are high (**Figure 1.3**) (Smith et al., 1973; Weber et al., 2002). Several days

prior to parturition, cortisol and estradiol begin to increase reaching peak levels at parturition. Progesterone concentrations drop off precipitously at parturition and stay low throughout the first week postpartum. Estradiol returns to prepartum levels within the first day after parturition and the concentration of cortisol returns to mid-gestation values by the second post partum day. The growing fetus initiates and coordinates these steroid hormone fluctuations in the following manner. As the fetus begins to experience space limitations in the uterus, it releases corticotropin-releasing hormone from the hypothalamus, which stimulates the pituitary gland to secrete adrenocorticotropic hormone (ACTH) (Senger, 1997). The ACTH stimulates production of corticosteroids by the fetal adrenal glands. Fetal cortisol crosses the placenta into the dam's blood stream, promoting enzymatic conversion of maternal progesterone into estradiol. This removes the "progesterone block" of pregnancy, increases blood estradiol concentrations, and together with the cortisol signals initiation of parturition and lactation. Approximately two to three days before parturition, estradiol concentrations begin to rise, peaking at parturition (**Figure 1.3**) (Smith et al., 1973; Weber et al., 2002). The estrogen dominance over progesterone increases contractility of the uterus and secretory activity of the cervix and vagina to aid in the expulsion of the fetus (Senger, 1997). This also causes the characteristic fall in progesterone immediately before parturition, a time when cortisol concentrations also peak. The cortisol spike at parturition is likely due to a combination of fetal and maternal hormone synthesis and plays an important role in the induction of parturition and the initiation of milk secretion and let down (Smith et al., 1973). Thus, the pronounced fluctuations in blood cortisol, estradiol, and progesterone concentrations around parturition are highly coordinated and lead to successful expulsion of the fetus and

subsequent lactation. However, these fluctuating steroid concentrations may also be responsible for the immune dysfunctions of parturient dairy cows (see next section).

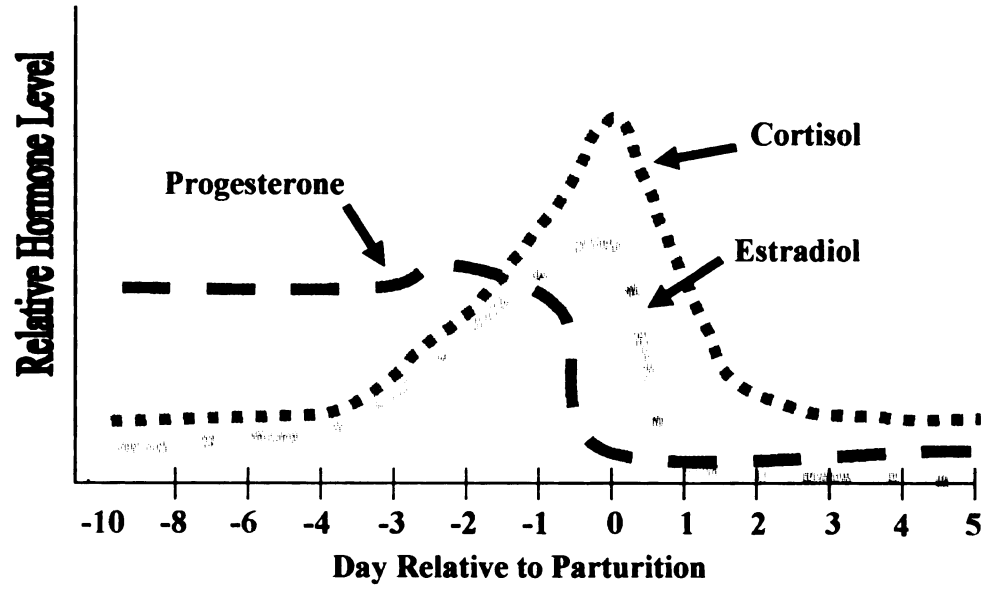


Figure 1.3 – Blood serum profiles of cortisol, estradiol, and progesterone in periparturient dairy cows. Figure adapted from data presented in Smith et al. (1973) and Weber et al. (2002).

C. Parturient Hormones May be Linked to Neutrophil Dysfunctions in Dairy Cows

The hormone fluctuations at parturition have been hypothesized to cause immune dysfunctions and disease susceptibility in dairy cows. For example, elevated blood glucocorticoid concentrations, such as occurs when cortisol spikes at parturition or when animals are treated with the hormone, induce release of bone marrow neutrophils (Hetherington and Quie, 1985). Down-regulation of L-selectin expression on the surface of blood neutrophils (Burton et al., 1995; Lee and Kehrli, 1998; Nakagawa et al., 1999;

Weber et al., 2002) and pronounced neutrophilia (Preisler et al., 2000a; Weber et al., 2002) have also been noted under these conditions. Preisler et al. (2000a) demonstrated that bovine blood neutrophils bind labeled glucocorticoid *in vitro* and that binding is decreased during parturition most likely due to occupation of the receptor by endogenous cortisol. More recently, glucocorticoid receptor mRNA levels in neutrophils have been evaluated and shown to be at highest levels of expression in conjunction with the cortisol spike of parturition (Weber et al., 2002). These studies, combined with observations from other investigators that glucocorticoids suppress many of the functions of neutrophils (Guidry et al., 1976; Detilleux et al., 1994; Burton et al., 1995; Lee and Kehrli, 1998; Nakagawa et al., 1999), strongly argue that the cortisol spike at parturition is partly responsible for the heightened disease susceptibility of parturient dairy cows. However, it is not known how cortisol and its receptor mediate these suppressing effects on the bovine immune system.

The two other steroid hormones that fluctuate dramatically at parturition, progesterone and estradiol, have also been shown to affect the functions of neutrophils in many species. Chemotaxis and migration of human neutrophils were enhanced by progesterone and reduced by estradiol in an *in vitro* study by Miyagi et al. (1992). Migration of neutrophils under agarose was enhanced when the cells were obtained from steers treated with progesterone (Roth et al., 1982). A study of cow neutrophils collected during various stages of the estrous cycle demonstrated increased *in vitro* migration when serum concentrations of progesterone or estradiol were high (Roth et al., 1983). In both studies, neutrophil myeloperoxidase activity, measured *in vitro*, was decreased during high serum progesterone concentrations. Although the presences of receptors for progesterone

and estradiol in bovine neutrophils have not yet been described, biomedical researchers have used estrogen receptor antagonists to show that human neutrophils do express 17β-estradiol receptors (Ito et al., 1995; Stefano et al., 2000). In addition, the presence of progesterone receptors in murine neutrophils has been demonstrated through the generation of progesterone receptor knockout mice (Tibbetts et al., 1999). In this study, progesterone was shown to antagonize the degranulating effects of estrogen on neutrophils in normal mice but this did not occur in the progesterone receptor knockout mice, thus supporting a direct affect of progesterone on neutrophils. Furthermore, preliminary microarray data from our laboratory suggests that bovine leukocytes express progesterone receptor mRNA before parturition and down regulate expression of this receptor during parturition (Burton and Coussens, unpublished). Therefore, it is highly likely that bovine blood neutrophils express receptors for cortisol, progesterone, and estradiol and may have the capacity to respond to parturient physiology through these receptors.

IV. MATURE NEUTROPHILS ARE RESPONSIVE TO THEIR ENVIRONMENT: EVIDENCE FOR ALTERED GENE EXPRESSION

Based on the granule contents and morphological appearance of mature neutrophils, researchers have long believed that these phagocytic cells are biosynthetically inactive (Bainton et al., 1971). The presence of dense nuclei, coarsely clumped chromatin, few ribosomes and endoplasmic reticulum, as well as numerous cytoplasmic granules packed full of oxidative and non-oxidative antimicrobial agents has been used to support this belief. However, difficulty accepting this view begins to occur when one considers the five functions (margination, migration, phagocytosis, respiratory burst, and

fusion of the phagolysosome) neutrophils must be able to perform to protect the host from infection. In addition, dramatic alterations of these functions are clearly demonstrated in research performed on blood neutrophils from parturient dairy cows (**Tables 1.1 and 1.2**). Evidence indicates that the steroid hormone fluctuations during periparturition may play a role in altering the phenotypes and functions of bovine blood neutrophils (Guidry et al., 1976; Preisler et al., 2000a; Weber et al., 2002). On the other hand, little published research is available showing that the neutrophil dysfunctions at parturition occur at the level of gene expression.

For just over a decade, researchers have examined what mRNAs and proteins are synthesized in mature, circulating neutrophils. Some have chosen the candidate gene approach while others have used global gene expression analysis (i.e. analysis of all mRNA present in control versus treatment sample neutrophils). Using the candidate gene approach, mRNA for MHC class I and actin as well as proteins such as Fc receptor, CD18, MHC class I, and actin were shown to be synthesized in human blood neutrophils (Jack and Fearon, 1988). Others demonstrated that neutrophils are able to synthesize a wide variety of cytokines, including interleukin-1β (IL-1β), IL-6, IL-8, and interferon-alpha (IFN-α) (review by Lloyd and Oppenhein, 1992; Cassatella, 1999). In 1998, Itoh et al. identified approximately 750 genes that are active in resting blood neutrophils. These genes encoded for DNA-binding proteins, cytokines, MHC proteins and receptors, and cell surface membrane proteins. More recently, global gene expression analysis approaches have been used to document altered gene expressions in blood neutrophils activated with cytokines (Cowling and Birnboim, 2000) or various pathogenic and non-pathogenic bacteria (Newberger et al., 2000). These studies have not only demonstrated

that blood neutrophils are biosynthetically active but that global gene expression changes relate to the five functions key to neutrophil mediated immune defense against pathogens. In retrospect, it makes perfect sense that a cell so integral to host defense against infection has full synthetic capacities.

To date, altered gene expression in bovine neutrophils during the periparturient period has only been reported in one study (Weber et al., 2002). This study was performed to elucidate the mechanism behind altered neutrophil trafficking in parturient dairy cows (**Table 1.1**). Weber et al. (2002) demonstrated rapid down regulation of L-selectin mRNA abundance and more gradual down-regulation of glucocorticoid receptor mRNA abundance in blood neutrophils of periparturient Holstein cows. Furthermore, these investigators showed that repressed L-selectin gene expression was highly correlated with the surge in blood cortisol concentrations, down-regulation of surface L-selectin on the neutrophils, and neutrophilia. Weber et al. (2002) thus concluded that bovine neutrophils respond to parturient cortisol with altered gene expression that influenced the phenotype and trafficking functions of the cells. Thus, it is feasible that cortisol and the other steroids of bovine parturition have significant effects on expression of multiple genes in bovine blood neutrophils. If true, these altered gene expression profiles could certainly begin to explain why dairy cows become immunosuppressed and highly susceptible to mastitis and other diseases around parturition.

The overall hypothesis of the current thesis research is that parturition induces changes in bovine blood neutrophil gene expression. The following three objectives were put forth to test this hypothesis:

Objective 1: Identify and characterize differential gene expression in bovine blood neutrophils from periparturient dairy cows;

Objective 2: Determine relationships between altered mRNA abundance of neutrophil genes and concentrations of blood steroids (cortisol, estradiol, and progesterone) around parturition;

Objective 3: Obtain putative identities and functions of differentially expressed genes discovered in objective 1.

The research presented in Chapter Two of this thesis is novel because it demonstrates that multiple neutrophil genes respond to parturition with reduced mRNA abundance, potentially due to changes in serum steroid concentrations. These results may aid in the understanding of why dysfunction of normal neutrophil functions (including surveillance, recruitment, receptor-mediated phagocytosis, respiratory burst, and fusion of the phagolysosome) occur in periparturient dairy cows. The importance of these observations is that upon further research, the identified genes could become targets for new drug development or genetic selection strategies aimed at bolstering innate immune defenses and disease resistance in dairy cows during the periparturient period.

CHAPTER TWO

Altered Blood Neutrophil Gene Expression in Periparturient Dairy Cows

I. ABSTRACT

The periparturient dairy cow undergoes a plethora of changes, including changes in the immune system, that lead to profound effects on animal health. Of the immune cells affected at parturition, the neutrophil has been of particular interest due to its primary role in innate immune defense against mastitis. Neutrophil function around parturition has been shown by assay of cellular functions and protein and/or enzymatic activities to be reduced, but it has not been discerned at what level these altered functions occur, including the possibility that there are alterations in neutrophil gene expression during periparturition. The hypothesis of the present study was that global gene expression in circulating neutrophils is altered during periparturition. The main objectives of the study were to detect and characterize parturition-induced changes in neutrophil mRNA abundance, to determine if altered mRNA levels are associated with changes in the main steroid hormones of bovine parturition, and to obtain identities of the genes exhibiting altered expression during the periparturient period. Preliminary assessment of gene expression was done using differential display reverse transcription polymerase chain reaction (DDRT-PCR) followed by high throughput cDNA dot blot analysis. mRNA size and number of transcripts were determined using Northern blot analysis. Detailed mRNA profiles were then characterized using quantitative slot blot analysis. Putative identities for two cDNAs with pronounced repression of mRNA following parturition ($P \leq 0.02$) had high DNA sequence homology with genes for

bovine mitochondrial cytochrome b (cytb) and rig/ribosomal protein S15 (rig/RPS15), which are genes of known importance in energy metabolism and translation of mRNA into protein (respectively). Cytb mRNA abundance profiles were significantly correlated with blood progesterone profiles ($r = 0.44$) and rig/RPS15 mRNA abundance with both progesterone and estradiol profiles ($r = 0.35$ and $r = 0.36$, respectively). Results of this study show for the first time that mRNA abundance of genes regulating basic functional machinery of bovine neutrophils is depressed by parturition, possibly due to the influences of steroid hormones on gene expression.

II. INTRODUCTION

Parturition is a complex time for dairy cows, nutritionally, hormonally, and immunologically. It is during this time that cows experience an increased susceptibility to intramammary infections and clinical mastitis, quite possibly as a result of decreased immunocompetence observed during periparturition (Oliver and Sordillo, 1988; Cai et al., 1994, Mallard et al., 1998). Altered functions of blood neutrophils in periparturient cows are of particular interest to researchers due to their importance in innate mammary defense against mastitis (Guidry et al., 1976; Kehrli and Harp, 2001). In an attempt to explain immune dysfunctions of parturition, investigators have speculated that fluctuating metabolic and reproductive hormones are involved in disease susceptibility because immune dysfunction profiles tend to associate temporally with fluctuating hormone profiles (Goff and Horst, 1997; Ingvartsen and Andersen, 2000).

As a part of the first line of immune defense, mature blood neutrophils perform five main functions to successfully clear pathogens that infect peripheral tissues.

Circulating neutrophils marginate along blood vessel walls, after which they rapidly migrate into tissues if infection is detected. Once in the tissue, neutrophils engage in the seeking out and ingestion of pathogens using energy-requiring, receptor mediated phagocytosis. Phagocytosis stimulates neutrophils to undergo massive respiratory burst, producing multiple reactive oxygen species and myeloperoxidase that initiate oxidative damage to ingested bacteria. Finally, vesicles containing the phagocytosed bacteria fuse with vesicles containing lysosome to complete pathogen killing via enzymatic lysis. Several of these functions are altered during parturition in dairy cows. For example, neutrophil margination and migration, known together as trafficking, are profoundly decreased due to reduced expression of key adhesion molecules on the surface of blood neutrophils (Cai et al., 1994; Lee and Kehrli, 1998; Weber et al., 2002). Similarly, neutrophil chemotaxis, myeloperoxidase activity, superoxide production, and general phagocytic ability are dramatically altered around parturition (Guidry et al., 1976; Nagahata et al., 1988; Kehrli et al., 1989; Cai et al., 1994; Detilleux et al., 1994; Lee and Kehrli, 1998; Kimura et al., 1999b). Each of these altered neutrophil functions could help explain the increased rates of mastitis that occur in periparturient cows.

Although the studies above have clearly demonstrated the presence of multiple neutrophil dysfunctions around parturition, few report mechanisms describing why or at what level these dysfunctions occur. Indeed, most of the studies have focused on measuring altered protein levels and enzyme activities because leukocyte researchers have generally believed that blood neutrophils are terminal cells with little to no capacity to synthesize new molecules (Bainton et al., 1971). Recently, however, several studies using human neutrophils have begun to challenge this belief by showing that

approximately 700 neutrophil genes are active during the resting state (Itoh et al., 1998). Others have begun to explore global gene expression changes in neutrophils presented with bacteria and cytokine stimuli (Lloyd and Oppenhein, 1992; Newberger et al., 2000). To date, only one study demonstrated gene expression changes in neutrophils in response to parturition (Weber et al., 2002).

During bovine parturition, blood neutrophils are exposed to a rapidly changing hormonal environment to which they appear to be extremely sensitive. For example, observational studies have described relationships between changing corticosteroid levels and altered neutrophil phagocytic ability during parturition (Guidry et al., 1976), as well as generally altered neutrophil functions in association with changing estradiol and progesterone levels (Roth et al., 1982; Roth et al., 1983; Goff and Horst, 1997). Another study demonstrated that chemotaxis and migration increase while blood progesterone concentrations are high and decrease when neutrophils are exposed to high levels of blood estradiol (Miyagi et al., 1992). Still others have demonstrated that the dramatic increase in blood cortisol concentrations at parturition have pronounced down-regulating effects on neutrophil L-selectin expression (Lee and Kehrli, 1998; Weber et al., 2002), which correlates well with altered trafficking of these cells (Preisler et al., 2000a; Weber et al., 2002). In addition, Preisler et al. (2000a) began to address the issue of mechanism by showing that bovine neutrophils express glucocorticoid receptors that appear to become fully occupied by cortisol around parturition. This substantiates arguments by many researchers that bovine neutrophils are sensitive to hormones of parturition and respond to them with altered functional capacities.

To our knowledge, few studies have been published that describe alterations in the expression of genes that regulate neutrophil functions around parturition, nor whether such changes may relate to fluctuating blood steroid hormone concentrations. Our overall hypothesis is that parturition induces changes in bovine blood neutrophil gene expression. The current study was designed to begin testing this hypothesis. The objectives were to determine if parturition induces changes in blood neutrophil mRNA abundance of multiple genes, to determine if altered mRNA levels relate to blood concentrations of cortisol, estradiol, and (or) progesterone, and to obtain putative identities of neutrophil genes with altered mRNA abundance. Several experiments were used to complete the first objective. These included a primary screening for differential gene expression using differential display reverse transcription polymerase chain reaction (DDRT-PCR) of total leukocyte RNA, followed by secondary screening of altered neutrophil gene expression using high throughput cDNA dot blot analysis. Confirmation of differential mRNA abundance in neutrophils was performed using Northern blot analysis and characterization of changing mRNA levels by quantitative slot blot analysis (**Figure 2.1**). For objective 2, relationships between mRNA abundance profiles (from slot blot analysis) and steroid hormone profiles were determined statistically by correlation analysis. Finally, objective 3 was completed by sequencing the cDNAs encoding for mRNAs determined in objective 1 to be differentially expressed in neutrophils during parturition. These sequence data were subjected to BLASTn analysis to obtain putative identities of the differentially expressed neutrophil genes.

III. MATERIALS AND METHODS

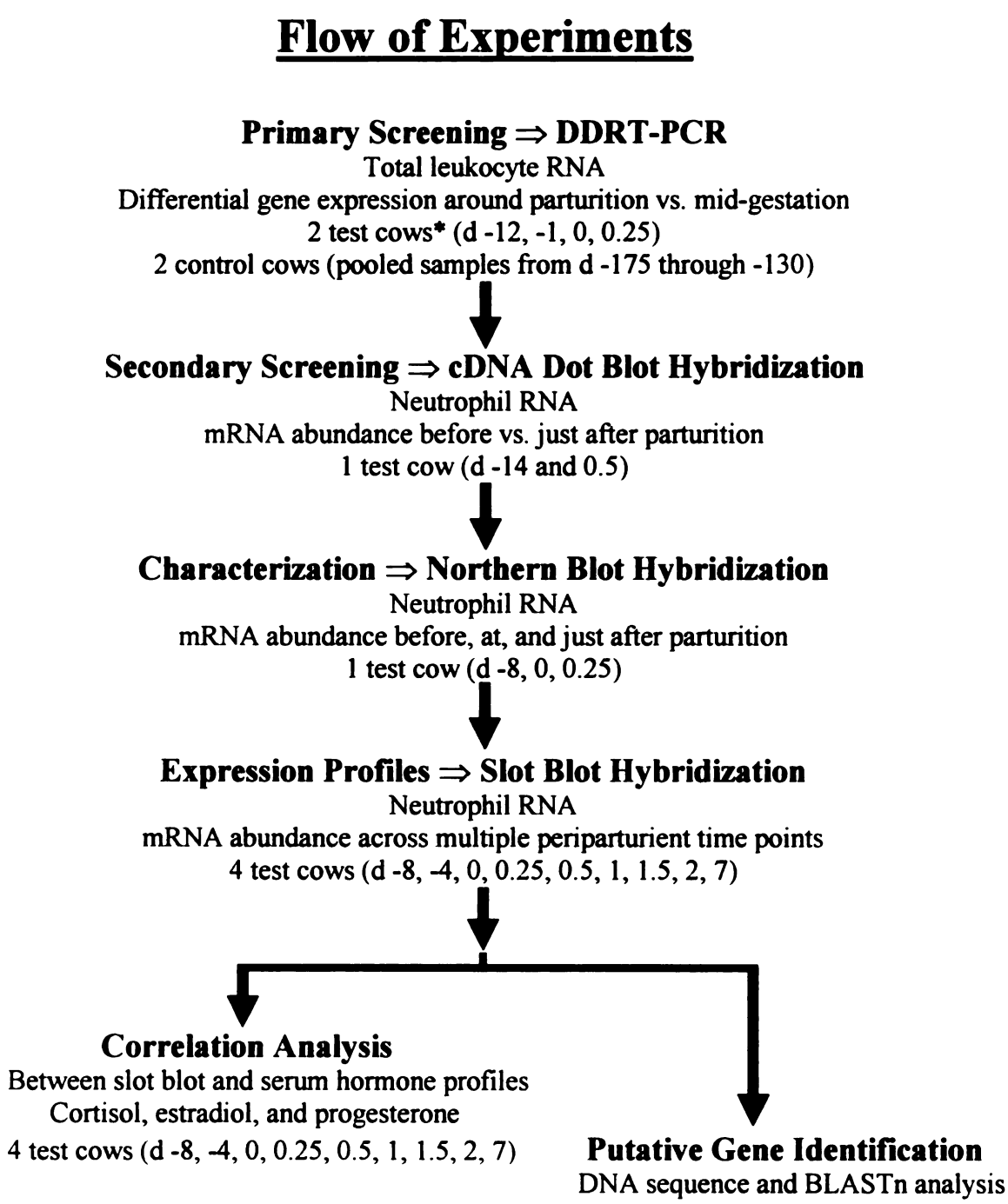
A. Animals and Sample Collection

This work was performed using five primiparous, parturient Holstein cows as the test animals. Samples from several of these cows were utilized in multiple experiments (**Figure 2.1**). Two primiparous, mid-gestation cows were sampled to serve as controls for DDRT-PCR. All animals were fed and housed according to standard operating procedures at the Michigan State University Dairy Teaching and Research facility, a seven minute drive from our laboratory. Use of these animals for the experiments described below was approved by the All University Committee for Animal Use and Care of Michigan State University.

Samples were collected at various times for use as described in **Figure 2.1**. Parturition is called day (d) 0. The control cows were sampled once each day that a periparturient test cow was sampled to provide ample amounts of control leukocyte RNA for DDRT-PCR. Test cows were sampled as needed on d -14, -12, -8, -4, -1, 0, 0.25, 0.5, 1, 1.5, 2, and 7 relative to parturition for leukocyte and neutrophil test samples to complete all experiments shown in **Figure 2.1**. All samples were obtained as described in Weber et al. (2002). Briefly, 60 to 80 ml of blood from the coccygeal (tail) vein was collected into 6-ml evacuated tubes containing 1.0 ml of acid citrate dextrose (ACD) anti-coagulant using 20-gauge, 2.5-cm multi-sample needles (Fisher Scientific; Pittsburgh, PA). All samples were placed immediately on ice and transported back to the laboratory for processing. A 10-ml evacuated tube without anti-coagulant was also collected at each sampling for serum harvesting.

Figure 2.1 - Several methods were utilized to address our hypothesis that parturition induces changes in bovine blood neutrophil gene expression. Shown are the techniques, sample types, cows, and sample times we used to begin testing this hypothesis. Fourteen leukocyte genes were identified as differentially expressed upon primary screening, twelve of which were decreased in neutrophils of periparturient cows upon secondary screening. In the current study, two of these genes were pursued through the entire flow of experiments described in the figure.

Figure 2.1


Flow of Experiments
Primary Screening ⇒ DDRT-PCR
Total leukocyte RNA
Differential gene expression around parturition vs. mid-gestation
2 test cows* (d -12, -1, 0, 0.25)
2 control cows (pooled samples from d -175 through -130)
Secondary Screening ⇒ cDNA Dot Blot Hybridization
Neutrophil RNA
mRNA abundance before vs. just after parturition
1 test cow (d -14 and 0.5)
Characterization ⇒ Northern Blot Hybridization
Neutrophil RNA
mRNA abundance before, at, and just after parturition
1 test cow (d -8, 0, 0.25)
Expression Profiles ⇒ Slot Blot Hybridization
Neutrophil RNA
mRNA abundance across multiple periparturient time points
4 test cows (d -8, -4, 0, 0.25, 0.5, 1, 1.5, 2, 7)
Correlation Analysis
Between slot blot and serum hormone profiles
Cortisol, estradiol, and progesterone
4 test cows (d -8, -4, 0, 0.25, 0.5, 1, 1.5, 2, 7)
Putative Gene Identification
DNA sequence and BLASTn analysis
*Test cows = Parturient
Control cows = Mid-gestation
Day (d) = Day relative to parturition

B. Blood Leukocyte Purification and RNA Isolation

Total leukocytes for preliminary DDRT-PCR screening of differential gene expression (**Figure 2.1**) were obtained from ACD-anticoagulated blood samples on d -12, -1, 0, and 0.25 from two test cows, and from the two control cows as described in Burton et al. (2001). Briefly, each 6-ml tube of whole blood was decanted into a 50-ml conical tube containing 24 ml cold hypotonic lysis solution (10.56 mM Na_2HPO_4, 2.67 mM NaH_2PO_4, pH 7.3) and agitated for 90 seconds. A 12 ml volume of cold hypertonic restore solution (10.56 mM Na_2HPO_4, 2.67 mM NaH_2PO_4, 0.43 M NaCl, pH 7.3) was added to restore isotonicity. Leukocytes were pelleted by centrifugation (1000 X g for 5 minutes at 4°C) followed by aspiration of the supernatants. The cells were then washed in 15 ml ice cold PBS and centrifuged (1000 X g for 5 minutes at 4°C). Supernatants were aspirated and discarded and cell pellets lysed in 4 ml TRIzol reagent (Life Technologies; Rockville, MD) for 10 minutes at room temperature. The time from blood sampling to lysis of leukocyte pellets in TRIzol was approximately 1.5 hours.

Blood neutrophil samples for cDNA dot blot, Northern blot, and slot blot analyses (**Figure 2.1**) were obtained from test cows at d -14, -8, -4, 0, 0.25, 0.5, 1, 1.5, 2, and 7 for gene expression analysis. Upon arrival in the laboratory, ACD blood tubes were centrifuged at 1000 X g for 20 minutes at 4°C to separate plasma and buffy coat from the red cell pack. Plasma, buffy coat, and approximately two-thirds of the red cell pack were discarded while the remaining red cell pack was transferred to a 50-ml conical tube containing 20 ml cold PBS. Additional cold PBS was added to a final volume of 35 ml. This was then under layered with 12 ml of 1.084 g/ml Percoll (Sigma Chemical Company; St. Louis, MO). Neutrophils were pelleted through the Percoll by

centrifugation at 400 X g for 40 minutes at 22°C. Supernatant, mononuclear layer, and Percoll were aspirated and discarded. Lysis of erythrocytes was performed as described above for total leukocyte lysis. Neutrophil pellets from a single cow and sample time were pooled for washing with 20 ml cold PBS. Following a final 5 minute centrifugation (1000 X g, 4°C), neutrophil pellets were lysed in 8 ml TRIzol reagent (Life Technologies; Rockville, MD) for 10 minutes at room temperature. Total processing time for neutrophil isolation from collection to TRIzol was less than three hours. Neutrophil purity was analyzed by flow cytometry (see **Appendix One, Figure A.1**). Prepartum samples were greater than 80% neutrophils while post partum samples exhibited greater than 90% neutrophils.

All samples, total leukocytes and purified neutrophils, were stored at –80°C in TRIzol reagent. Total RNA was isolated from each cell preparation according to the manufacturer's instructions. Following isolation, quantity and quality of RNA was evaluated in each sample using 260 and 280 nm spectrophotometry readings (DU-650 spectrophotometer; Beckman, Schaumberg, IL).

C. Primary Screening of Differential Gene Expression in Total Leukocytes of Periparturient Cows using DDRT-PCR

DDRT-PCR was utilized in initial screening for differences in gene expression in all blood leukocytes (Liang and Pardee, 1992). Total leukocyte RNA samples from two test cows on d -12, -1, 0, and 0.25, as well as samples from both control cows (pooled over multiple days), were treated with DNase I (Life Technologies, Rockville, MD) to remove contaminating genomic DNA prior to reverse transcription. A total of 18

differential display primer pairs were utilized in this study. Fourteen of the primer pairs were generated by the United States Pig Genome Coordination Program (provided by Dr. Max Rothschild, U.S. Pig Genome Coordinator, and donated for use in this project by Dr. Catherine Ernst; Department of Animal Science, Michigan State University). The remaining four primer pairs were obtained from the Heiroglyph mRNA kit Profile System for Differential Display Analysis (Beckman Coulter, Inc.; Fullerton, CA) and were donated by Dr. George Smith (Department of Animal Science, Michigan State University).

To start, reverse transcription (RT) was performed on total leukocyte RNA (0.1 μg/ul) using 1 unit Superscript II reverse transcriptase (Life Technologies; Rockville, MD), 1X First Strand Buffer, 0.1 M DTT, 250 μM of each dNTP, and 2.0 μM of various 3' anchored primers. The cDNAs generated through RT were amplified by polymerase chain reaction (PCR) using Taq DNA Polymerase (Life Technologies; Rockville, MD), 10X PCR Buffer, 50 mM $MgCl_2$, 250 μM of each dNTP, 2.0 μM of the 3' anchored primer used in the RT reaction, and 2.0 μM of various 5' arbitrary primers. Also included in these PCR reactions was α-^{33}P-dATP for subsequent visualization of differentially expressed bands. All PCR products and a negative control (no RNA added) for each primer pair were electrophoresed on 5.2% polyacrylamide denaturing gels. Following electrophoresis, gels were dried to 3MM Chr Whatman chromatography paper (Fisher Scientific; Pittsburgh, PA) with a Slab Gel Dryer SDG2000 (ThermoQuest Corp.; Marietta, OH). Autoradiographs were produced for band visualization by 24 hours of exposure of the dried gels to BioMax MR X-ray film (Fisher Scientific; Pittsburgh, PA). Those differential display products (or amplicons) with putative changes in expression

were cut out and eluted from the gel matrix for probe generation and DNA sequence analysis.

D. Secondary Screening of Differential Gene Expression in Neutrophils using Dot Blot Hybridization

Excised amplicons were eluted from the gel matrix with Tris-EDTA (pH 7.4). Reamplification was performed by PCR using the same primer pairs and PCR conditions that created the amplicons. The reamplified amplicons were analyzed by agarose gel electrophoresis (1.2% gel) and visualized by ethidium bromide staining to confirm single bands. The amplicons were then ligated into the pGEM-T Easy vector (Promega; Madison, WI) and resulting recombinant plasmids were transformed into JM109 competent cells (Promega; Madison, WI). Positive clones containing amplicons were selected by blue/white colony screening. Plasmids from white colonies were isolated using the Mini-prep Plasmid DNA Isolation kit (Promega; Madison, WI) and subjected to *Eco* RI restriction enzyme digestion (Life Technologies; Rockville, MD) to release the amplicons for confirmation of their presence by agarose gel electrophoreses and ethidium bromide staining. Finally, PCR was performed using M13 Forward and Sp6 sequencing primers (Promega; Madison, WI) to amplify a large quantity of each amplicon for validation of altered gene expression in neutrophils using cDNA dot blot hybridization.

For cDNA dot blot hybridizations, PCR reamplified amplicons were denatured in a 0.4 M NaOH, 0.01 M EDTA solution for 10 minutes at 95°C and placed immediately on ice. Positively charged nylon membranes (Life Technologies; Rockville, MD) were pre-soaked in 6X SSC and each dot was rinsed with sterile water. The membranes were

then dotted in quadruplicate with 0.50 μg of each amplicon (determined by serial dilution and gel electrophoresis using a molecular ruler containing known concentrations of DNA at specific sizes) using a dot blot manifold (The CONVERTIBLE Filtration Manifold System, Life Technologies; Rockville, MD). The hybridization positive control was β-actin (described in Weber et al., 2002), 0.05 μg of which was also dotted in quadruplicate on both membranes. All dots were rinsed with 0.4 M NaOH followed by UV cross-linking of the amplicon to the membrane. The duplicate membranes were dotted on the same day and prehybridized overnight at 42°C using a solution containing 50% formamide, 5X Denhardt's, 6X SSC, 0.1% SDS, 0.05 M phosphate buffer (pH 6.8), 1.0 mM EDTA, and 0.15 mg per ml yeast tRNA. Hybridizations were performed using α-^{32}P-dCTP (NEN Life Science Products, Inc.; Boston, MA) labeled cDNA created by reverse transcription of 5.0 μg neutrophil RNA from a single test animal sampled on d –14 and 0.5 relative to parturition. Each labeled cDNA (d –14 versus d 0.5) was hybridized for 18 hours with one of the two dotted membranes. Following hybridization, membranes were washed twice at room temperature in 2X SSC, 0.1% SDS for 15 minutes followed by twice at 65°C in 0.2X SSC, 0.1% SDS for 15 minutes prior to exposure to BioMax MS film (Fisher Scientific; Pittsburgh, PA) for 96 hours at –80°C with an intensifying screen (Fisher Scientific; Pittsburgh, PA). Relative hybridizations of labeled cDNA from test samples to the spotted amplicons were estimated using a scanning densitometer (GS-710 Calibrated Imaging Densitometer and Multi-Analyst Software; BioRad; Hercules, CA) and recorded as dot density units.

E. Northern Blot Characterization of Neutrophil Genes

Those neutrophil genes represented by our amplicons as being differentially expressed before and after parturition in the dot blot analysis were further analyzed by Northern blot hybridization to determine size and number of mRNA transcripts in blood neutrophils (**Figure 2.1**). A portion of the PCR amplified amplicon (described above) was gel purified to remove residual plasmid DNA using the Wizard PCR Prep kit (Promega; Madison, WI). These were then used as probes for Northern blot analyses. Approximately 100 ng of PCR products were used for dual labeling with α-^{32}P-dCTP and α-^{32}P-dATP (NEN Life Science Products, Inc.; Boston, MA) using the random prime method (Feinberg and Vogelstein, 1983). A neutrophil Northern blot was generated using three RNA samples from a parturient test cow (d –8, 0, and 0.25) as described in Weber et al. (2002). Briefly, 10 μg of RNA from each time point was electrophoresed on a 1.2% denaturing agarose gel and transferred to a nylon membrane using a Turbo Blotter (Schleicher and Schuell; Keene, NH). RNA was UV cross-linked to the membrane. Prehybridization was carried out for 4 hours at 42°C in the same pre-hybridization cocktail described for the dot blots above. Hybridization was carried out for ~16 hours at 42°C in the same buffer using the purified, labeled amplicon. Membranes were washed once at room temperature with 2X SSC, 0.1% SDS for 15 minutes and 3 times with 0.1X SSC 0.1%SDS at 60°C prior to exposure using BioMax MS film (Fisher Scientific; Pittsburgh, PA). Time of exposure at –80°C varied from one to four days depending on the amplicon. Membranes were stripped between hybridizations with different amplicons and reprobed with α-^{32}P-labeled β-actin cDNA, (obtained from Dr. L. Kedes; Stanford University School of Medicine; Palo Alto, CA), for normalization purposes (Weber et al.,

2002). Approximate sizes of the mRNA transcripts hybridized by the various amplicons were estimated using the 28S and 18S rRNA bands (4718 and 1874 base pairs (bp), respectively) as markers from an ethidium bromide stained lane of the gel (Current Protocols in Molecular Biology, 1995).

F. Characterization of Neutrophil mRNA Abundance Profiles from Periparturient Cows

Neutrophil slot blots were used to quantify mRNA abundance of genes confirmed to be differentially expressed by Northern blot, as described in Weber et al. (2002). In brief, 5 μg of neutrophil RNA from 4 test cows on d -8, -4, 0, 0.25, 0.5, 1, 1.5, 2, and 7 (**Figure 2.1**) were spotted on a nylon membrane using a slot blot manifold (PR600 slot blot filtration manifold; Hoefer Scientific; San Francisco, CA) and cross-linked to the membrane using UV-light. Blots were then prehybridized, hybridized, and washed as outlined above for Northern blots. Autoradiographs were obtained after various hours of blot exposure to BioMax MS film (Fisher Scientific; Pittsburgh, PA) at –80°C, depending on the hybridized amplicon probe. The slot blot was stripped between each hybridization with a new amplicon probe and, finally, with the β-actin probe. Relative mRNA abundance was quantitated using scanning densitometry units as described above, normalized to the relative abundance of β-actin mRNA, and recorded as ratio values.

G. Radioimmunoassay of Serum Cortisol, Estradiol, and Progesterone

Serum collections and assay of serum cortisol, estradiol, and progesterone levels (**Figure 2.1**) is described in Weber et al. (2002). Briefly, sera collected from the four test

cows sampled on d -8,-4, 0, 0.25, 0.5, 1, 1.5, 2, and 7 was harvested from clotted blood (10-ml draw with no anti-coagulant) following centrifugation of the blood tubes at 1000 X g at 4°C for 20 minutes. Sera were stored at –20°C until assayed. Serum cortisol and progesterone concentrations were assayed in duplicate using commercially available Coat-A-Count RIA kits (Diagnostic Products Corporation; Los Angeles, CA). Sera for the estradiol RIA assay were extracted with diethyl ether before use in duplicate in the assay (Coat-A-Count RIA kit; Diagnostic Products Corporation; Los Angeles, CA).

H. Identification of Differentially Expressed Neutrophil Genes

Amplicon nucleotide sequences were obtained using fluorescent ABI dye-Terminator cycle sequencing kits (Perkin-Elmer/ABI; Palo Alto, CA) in conjunction with M13 forward and Sp6 sequencing primers. Prepared samples were loaded onto a 4.75% polyacrylamide sequencing gel and analyzed using an ABI 373 Automated DNA Sequencer (Perkin-Elmer/ABI; Palo Alto, CA). Putative identities were obtained by comparing sequences to sequences available in DNA databanks using BLASTn (basic local alignment search tool - nucleotide), available through the National Center for Biotechnology Information (NCBI) web page (http://www.ncbi.nlm.nih.gov/BLAST) and The Institute for Genomic Research (http://www.tigr.org/tdb/tgi.shtml).

I. Statistical Analysis

Ratio data (mRNA abundance/β-actin) from the slot blots were analyzed using the PROC MIXED function of SAS (SAS Institute Inc., 1996) and the following statistical model:

$$Y_{ij} = \mu + \rho_i - \alpha_j + e_{ij}$$

where Y_{ij} is the observation of the i^{th} cow on the j^{th} day relative to parturition, μ is the overall mean, ρ_i is the random effect of the i^{th} cow, α_j is the fixed effect of the j^{th} day relative to parturition, and e_{ij} is the normal error ij. Statistical analysis was performed on data collected from the slot blot analysis of neutrophil mRNA abundance from four parturient test cows (d -8, -4, 0, 0.25, 0.5, 1, 1.5, 2, and 7). The effect of day relative to parturition was tested first by analysis of variance, assuming a compound symmetry correlation of the residuals, and then by pair wise comparisons between least squares means of post-parturient samples and prepartum test samples (d –8 and –4). Significance between these means was declared when $P \leq 0.05$. Analysis assuming a first-order antedependence correlation of the residuals resulted in an even more significant effect of day relative to parturition ($P < 0.0001$; statistical model and data not shown).

The cortisol, estradiol, and progesterone data were natural logarithm-transformed prior to analysis of variance using the same statistical model presented above to test effect of day relative to parturition. The PROC CORR function of SAS (SAS Institute Inc., 1990) was used to determine Pearson Product Correlations between the transformed hormone data sets and normalized gene expression data sets from slot blot analyses. Statistical significance was acknowledged when $P \leq 0.05$.

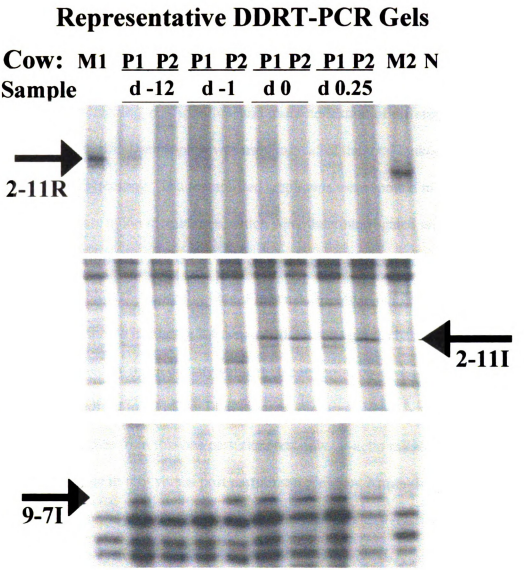
IV. RESULTS

A. Primary Screening for Differential Gene Expression Induced by Parturition in Total Leukocytes

DDRT-PCR identified putative differentially expressed leukocyte genes throughout the periparturient period as well as between parturition and mid-gestation samples. A total of eighteen 3'-5' primer pair combinations were used for DDRT-PCR, from which fourteen differentially expressed cDNA fragments (amplicons) were identified (some examples in **Figure 2.2**). These fourteen amplicons were named based on the 3' and 5' primer numbers used to generate them, and whether the cDNA appeared to be induced (I) or repressed (R) around parturition within test samples or between control and test samples. For example, if 3' anchored primer number 5 and 5' arbitrary primer number 15 yielded an amplicon that appeared to be induced its name would be 5-15I. If more than one amplicon was identified per primer pair, an additional letter (a, b, c, etc.) was added to indicate the relative position of the cDNA from the top of the gel (e.g. 5-15Ia, 5-15Ib, 5-15Ic, etc.). **Figure 2.2** shows three such differentially expressed cDNAs. The first was repressed throughout all periparturient time points evaluated (2-11R), the second was induced at parturition (2-11I), and the third was induced throughout the entire periparturient period (9-7I). Of the fourteen amplicons pursued, a total of eight appeared to be induced in leukocytes during parturition while the remaining six exhibited repressed expression during periparturition.

Figure 2.2 - Primary screening for differential gene expression in total leukocytes from parturient and mid-gestation cows was performed using differential display reverse transcription polymerase chain reaction (DDRT-PCR). Shown are three examples of differentially expressed cDNAs observed when total leukocyte RNA from two mid-gestation control cows (M1 and M2; pooled samples from d -175 to -130 prepartum) and two parturient test cows (P1 and P2; sampled on d -12, -1, 0, 0.25 relative to parturition) were electrophoresed on a 5.2% polyacrylamide gel. The sample in lane N was the DDRT-PCR negative control and received no RNA during reverse transcription. Amplicons were named based on 3' and 5' primer numbers and if they appeared induced (I) or repressed (R) by parturition. In the top panel, amplicon 2-11R was shown to be repressed in leukocytes throughout the periparturient period when compared to mid-gestation. The middle panel shows 2-11I induced at (d 0) and just after parturition (d 0.25) compared to prepartum (d -12, -1) and in mid-gestation. In the bottom panel, 9-7I showed strong induction throughout the entire periparturient period compared to mid-gestation. These amplicons, as well as eleven others, demonstrated differential expression in leukocytes of both parturient cows compared to both mid-gestation cows, thus they were excised from the DDRT-PCR gels and used for secondary screening of differential gene expression in periparturient neutrophils. Additional DDRT-PCR gel pictures in **Appendix One, Figure A.2**.

Figure 2.2


Representative DDRT-PCR Gels
Cow: M1 P1 P2 P1 P2 P1 P2 P1 P2 M2 N
Sample d -12 d -1 d 0 d 0.25
2-11R
2-11I
9-7I

B. Secondary Screening of Differential Gene Expression Induced by Parturition

The fourteen amplicons were successfully cloned and amplified by PCR into crisp, single bands (**Figure 2.3**). Comparison of the reamplified amplicons with a molecular marker showed that the cDNAs ranged from approximately 350 to 1100 bp in length. For secondary screening, confirmation, and characterization of differential gene expression, RNA from purified neutrophils was used instead of the total leukocyte RNA utilized for the primary screening. The reamplified amplicons were spotted in quadruplicate on duplicate nylon membranes, which were then interrogated with neutrophil RNA collected from a single test cow on d –14 and d 0.5 relative to parturition (autoradiographs of dot blots in **Appendix One, Figure A.3**). Mean dot densities for all amplicons analyzed are shown in **Figure 2.4** (separated into two graphs based on levels of dot intensity represented by mean density values). Upon initial observation, amount of labeled cDNA that hybridized to 12 of the 14 amplicons appeared altered between d -14 and d 0.5 neutrophil samples from this one parturient test cow. β-actin mRNA abundance exhibited very little change in pre- versus post partum samples (see **Appendix One, Figure A.4**) and has been shown to not be significantly affected by day relative to parturition by slot blot analysis (Weber et al., 2002). Thus, our secondary screening clearly demonstrated a decrease in mRNA abundance for several neutrophil genes in one parturient cow. Confirmation of these observations were pursued through the use of Northern and slot blot analyses on neutrophil RNA.

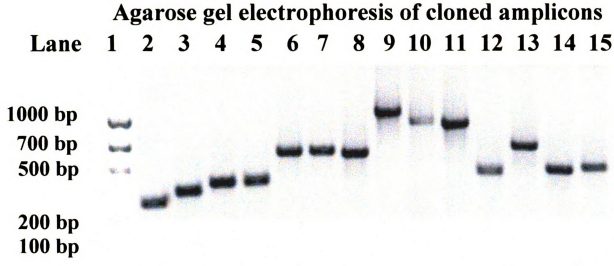
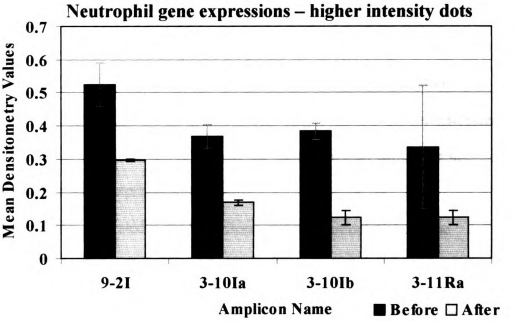
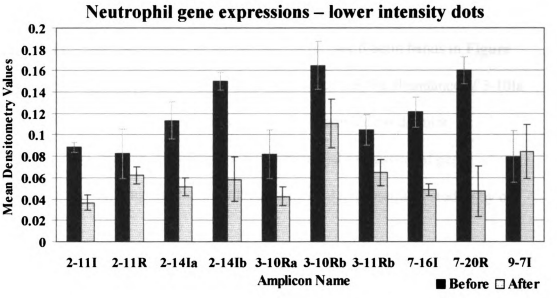


Figure 2.3 - Fourteen amplicons generated during primary screening of differential gene expression in total blood leukocytes were cloned and following PCR reamplification ranged in sizes from 350 to 1100 base pairs (bp). Presented is a 1.2 % agarose checking gel showing distinct, single bands from this reamplification step. Size approximations were made using the EZ Load Precision Molecular Mass Standard (BioRad; Hercules, CA) shown in lane 1 of the gel. **Lane 1:** Molecular ruler, **lane 2:** 2-11I, **lane 3:** 2-11R, **lane 4:** 2-14Ia, **lane 5:** 2-14Ib, **lane 6:** 3-10Ia, **lane 7:** 3-10Ib, **lane 8:** 3-10Ra, **lane 9:** 3-10Rb, **lane 10:** 3-11Ra; **lane 11:** 3-10Rb, **lane 12:** 7-16I, **lane 13:** 7-20R, **lane 14:** 9-2I, **lane 15:** 9-7I.

Figure 2.4 - Secondary screening for differential gene expression in neutrophils of a periparturient test cow using cDNA dot blot hybridization. Briefly, the fourteen amplicons identified in total leukocytes as either induced (I) or repressed (R) around parturition, were spotted on two dot blots in quadruplicate. Radiolabeled cDNA generated from neutrophil RNA sampled at d -14 (dark bars) and d 0.5 (light bars) were used to interrogate the blots. Shown are the mean densitometry units (± SEM) of the quadruplicate dots for each amplicon spotted. Data in this figure demonstrates that post partum neutrophil mRNA hybridized to twelve of the fourteen amplicons at substantially lower levels than prepartum neutrophil mRNA. Genes represented by amplicons 3-10Ia and 9-2I were studied in greater detail in subsequent Northern and slot blot hybridization experiments (**Figures 2.5 and 2.6**).

Figure 2.4


Neutrophil gene expressions – higher intensity dots
Mean Densitometry Values
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
9-2I
3-10Ia
3-10Ib
3-11Ra
Amplicon Name
Before
After


Neutrophil gene expressions – lower intensity dots
Mean Densitometry Values
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
2-11I
2-11R
2-14Ia
2-14Ib
3-10Ra
3-10Rb
3-11Rb
7-16I
7-20R
9-7I
Amplicon Name
Before
After

C. Characterization of Neutrophil mRNA Hybridized by 3-10Ia and 9-2I

To date, two neutrophil genes have been followed through the entire scheme outlined in **Figure 2.1**. The genes encoded by amplicons 3-10Ia and 9-2I showed 57 % and 46 % less mRNA in neutrophils right after parturition compared to prepartum, respectively, as determined by cDNA dot blot analysis (**Figure 2.4**). They were selected for further characterization based on this pronounced repression after parturition in addition to the high level of expression prepartum.

A Northern blot generated using RNA from purified neutrophils from one parturient test cow sampled on d -8, 0, and 0.25 was utilized to characterize size and number of transcripts for mRNA complimentary to 3-10Ia and 9-2I (**Figure 2.5**). Each of these amplicons hybridized to a single transcript, 3-10Ia at approximately 1100 bp and 9-2I at approximately 500 bp. Transcripts hybridized by these two amplicons also demonstrated decreased mRNA abundance at and after parturition, an effect that did not appear to be due to unequal loading of the sample RNAs (see β-actin bands in **Figure 2.5**). This is the second parturient cow in which decreased mRNA abundance of 3-10Ia and 9-2I was observed (the first was the parturient cow sampled for dot blot hybridization). Size, number of transcripts, and differential expression of genes represented by the remaining amplicons will be confirmed in subsequent studies by our group.

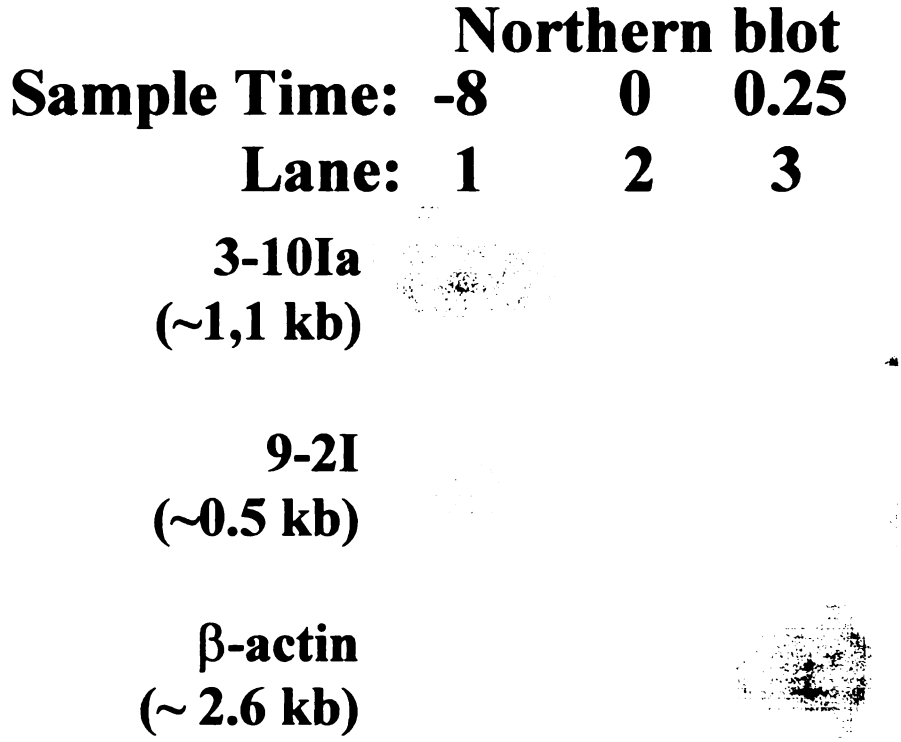


Figure 2.5 – Characterization of size and number of transcripts complimentary to 3-10Ia and 9-2I in neutrophils from a periparturient test cow using Northern blot hybridization. Neutrophil RNAs collected on d -8, 0, and 0.25 were subjected to electrophoresis and the resulting Northern blot was probed with amplicons 3-10Ia (top panel) and 9-2I (middle panel), as well as β-actin (a housekeeping gene used to verify even loading of the lanes; bottom panel). A single transcript was detected at approximately 1100 bp using the 3-10Ia probe while the transcript detected by the 9-2I probe was approximately 500 bp. Data in this figure show that parturition induced down regulation of 3-10Ia and 9-2I, confirming results of our dot blot analysis shown in **Figure 2.4**. The greater intensity of the β-actin band in neutrophil RNA in lane 3 was from overloading of this lane relative to lanes 1 and 2.

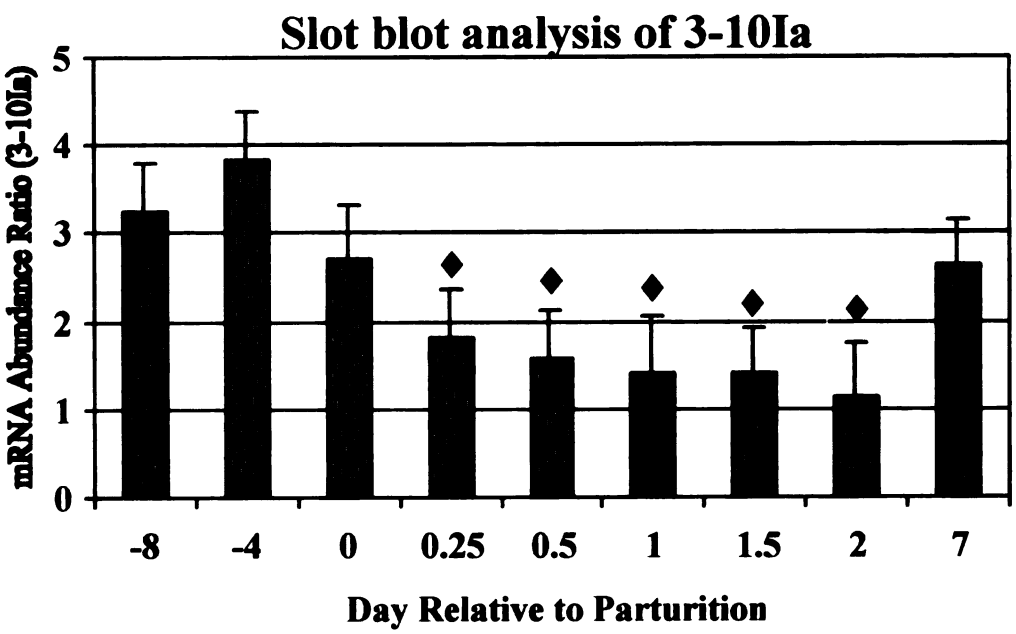
D. Characterization of 3-10Ia and 9-2I mRNA Abundance Profiles in Neutrophils from Periparturient Cows

Quantitative slot blot analysis was utilized to characterize mRNA abundance profiles of 3-10Ia and 9-2I in neutrophils from four periparturient dairy cows. Sample days included d -8, -4, 0, 0.25, 0.5, 1, 1.5, 2, and 7, relative to parturition on d 0. Blots were probed with 3-10Ia, stripped, probed with β-actin, stripped, and probed with 9-2I. Resulting mRNA abundance data were recorded as β-actin normalized abundances of transcripts hybridized by 3-10Ia and 9-2I. Statistical analysis of these data with an assumption of compound symmetry correlation of the residuals showed that day of parturition significantly affected 3-10Ia ($P = 0.018$) and 9-2I ($P = 0.003$) mRNA abundance. When comparing daily least squares means for 3-10Ia mRNA abundance at post partum time points to the d -4 time point (**Figure 2.6a**), significant down-regulation was observed that began on d 0.25 ($P \leq 0.05$) and continued through d 0.5, 1, 1.5, and 2 ($P \leq 0.01$). Relative to d -8, there was significant down regulation of 3-10Ia on d 0.5, 1, 1.5, and 2 post partum ($P \leq 0.05$). Based on the least square means for 9-2I, the gene corresponding to this amplicon appears to have slightly lower mRNA abundance in neutrophils than that corresponding to 3-10Ia (**Figure 2.6b**). However, the changes in mRNA abundance for 9-2I were similar to those for 3-10Ia, with mean decreases at d 0.25, 0.5, 1, 1.5, and 2 compared to d -4 ($P \leq 0.01$). Changes relative to d -8 were significant at $P \leq 0.05$ on d 0.25 and 0.5 while d 1, 1.5, and 2 were significantly different from d -8 at $P \leq 0.01$. Therefore, mRNA abundance of neutrophil genes represented by 3-10Ia and 9-2I was repressed by parturition and remained low for at least the first two days of lactation.

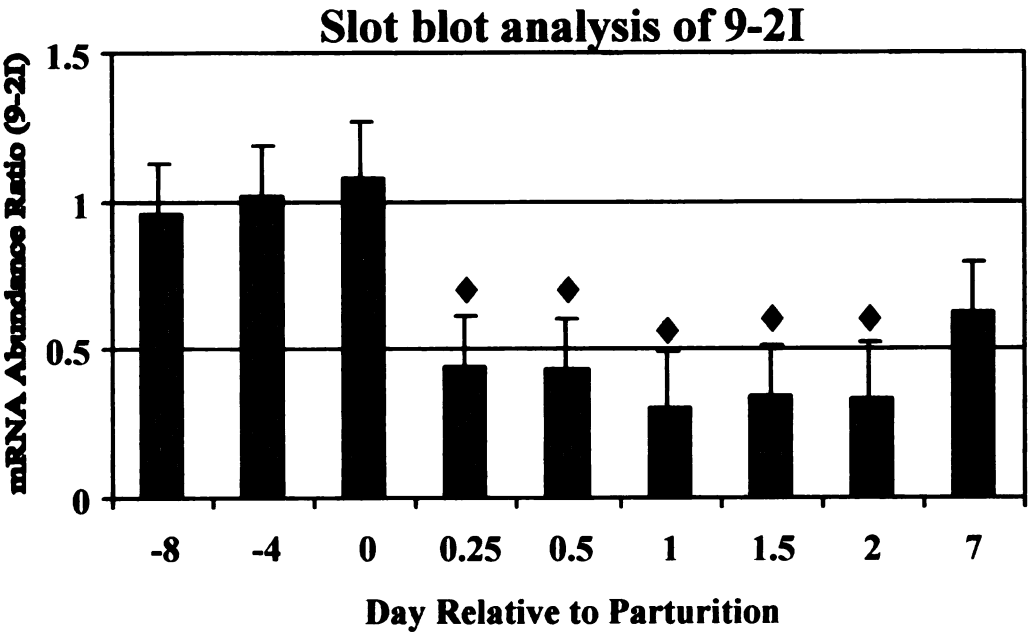
Figure 2.6 – mRNA abundance profiles of genes encoded by amplicons 3-10Ia and 9-2I in neutrophils from periparturient cows were obtained using slot blot hybridizations. Shown are the β-actin normalized neutrophil mRNA abundances (± SEM) from four cows at multiple parturient time points (relative to parturition on day 0). Parturition significantly repressed 3-10Ia mRNA (panel a, $P = 0.018$), with nadir being reached on day 2 postpartum. Neutrophil mRNA abundance of 9-2I was also significantly effected by parturition (panel b, $P = 0.003$), with lowest levels found on day 1 postpartum. For both genes, neutrophil mRNA abundance was significantly lower from day 0.25 through day 2 post partum ($P \leq 0.05$) compared to prepartum abundance (indicated by ◆).

Figure 2.6

a


Slot blot analysis of 3-10Ia
mRNA Abundance Ratio (3-10Ia)
5
4
3
2
1
0
-8
-4
0
0.25
0.5
1
1.5
2
7
Day Relative to Parturition

b


Slot blot analysis of 9-2I
mRNA Abundance Ratio (9-2I)
1.5
1
0.5
0
-8
-4
0
0.25
0.5
1
1.5
2
7
Day Relative to Parturition

E. Correlations between Neutrophil Gene Expression and Serum Steroid Profiles from Periparturient Cows

Three steroid hormones, cortisol, estradiol, and progesterone, are known to be critical to bovine parturition and may have effects on neutrophil functions (Guidry et al., 1976; Roth et al., 1982; Roth et al., 1983; Goff and Horst, 1997). Sera from the four animals used for slot blot analysis of neutrophil mRNA abundance were analyzed by RIA to characterize steroid concentration profiles throughout the peripartum period. Actual hormone profiles were reported in Figure 7 of Weber et al. (2002) and are found in **Figure 2.7** of this thesis. Briefly, cortisol increased significantly at parturition and continued to be significantly higher than basal levels for the first 24 hours post partum. Estradiol was significantly higher than basal levels at d -8, -4, 0, and 0.25 with a dramatic spike on d 0. Progesterone concentrations were high until d 0, at which time they plummeted to nearly undetectable levels (and remained so until d 7). Correlation analysis showed a clear association between neutrophil mRNA abundance of 3-10Ia and serum progesterone ($r = 0.44$, $P = 0.01$; **Table 2.1**). In addition, serum estradiol and progesterone concentrations both correlated positively ($r = 0.3595$, $P = 0.047$ and $r = 0.3495$, $P = 0.046$ respectively) with 9-2I mRNA abundance in neutrophils (**Table 2.1**). No significant correlations were detected between serum cortisol concentrations and neutrophil mRNA abundance of 3-10Ia and 9-2I (**Table 2.1**). Scatter plots and regression analysis of these relationships between neutrophil 3-10Ia mRNA abundance and hormones (**Figure A.5 panels a, b, c**) and neutrophil 9-2I mRNA abundance and hormones (**Figure A.5 panels d, e, f**) appear in **Appendix One**.

Figure 2.7 - Serum concentrations of three steroid hormones of bovine reproduction change significantly during parturition. In panel **(a)**, concentrations of serum cortisol rise sharply at parturition and return to basal by day 1.5 of lactation. In panel **(b)**, serum progesterone concentrations are high before parturition but plummet at parturition and stay low throughout day 7 of lactation. In panel **(c)**, serum estradiol concentrations begin to rise 8 days before parturition, peak at parturition, and rapidly return to basal concentration by day 0.5 of lactation. Data are presented as raw daily means ($\pm$ SEM). The solid black bar is the mean for mid-gestation control cows and the light gray bars are the daily means for parturient test cows. Bars without SEM had variation too low to be visualized on the graphs. Daily test cow means significantly different from the control cows mean are highlighted by asterisks (**$P \leq 0.01$; *$P \leq 0.05$). These data are presented in Weber et al., 2002 and are reprinted by permission from the editor of *Veterinary Immunology and Immunopathology* (**Appendix Two**).

Figure 2.7.

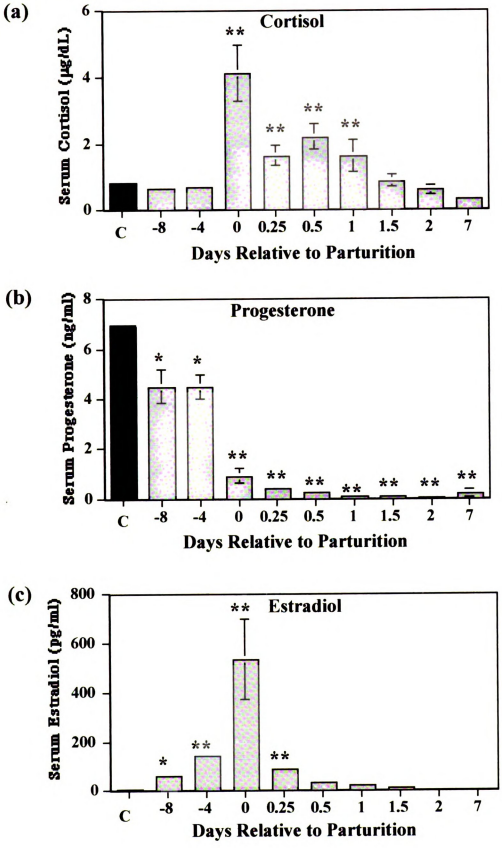

(a)
Cortisol
Serum Cortisol (µg/dL)
6
4
2
0
**
**
**
**
C
-8
-4
0
0.25
0.5
1
1.5
2
7
Days Relative to Parturition
(b)
Progesterone
Serum Progesterone (ng/ml)
8
6
4
2
0
*
*
**
**
**
**
**
**
**
C
-8
-4
0
0.25
0.5
1
1.5
2
7
Days Relative to Parturition
(c)
Estradiol
Serum Estradiol (pg/ml)
800
600
400
200
0
*
**
**
**
C
-8
-4
0
0.25
0.5
1
1.5
2
7
Days Relative to Parturition

Table 2.1 - Correlations[a] between serum steroid hormone concentrations and neutrophil mRNA abundance of 3-10Ia and 9-2I in periparturient dairy cows.

DD Product Amplicon	Cortisol	Estradiol	Progesterone
3-10Ia	-0.2614	0.2354	0.4430**
9-2I	-0.0389	0.3595*	0.3495*

[a] Pearson Correlation Coefficient

* $P \leq 0.05$; ** $P < 0.01$

F. Putative Identifications of Genes Differentially Expressed in Neutrophils of Periparturient Cows

Putative identities of 3-10Ia and 9-2I, as well as the other amplicons screened by cDNA dot blot hybridization, were obtained through DNA sequence and BLASTn analyses. Each amplicon contained approximately 225 bp of plasmid DNA, which was trimmed prior to BLASTn analysis. Significant homology was considered when an expectation value (E-value) was less than 10^{-4}. This value describes "the number of hits one can expect to see by chance when searching a database of a particular size" (from the National Center for Biotechnology web page BLAST Program Frequently Asked Questions http://www.ncbi.nlm.nih.gov/BLAST/blast_FAQs.html). The closer the E-value is to zero, the more significant the homology is to the sequence identified in the database. The 3-10Ia amplicon, which was approximately 375 bp in length after vector trimming, was homologous (E-value of 10^{-110}) to the bovine mitochondrial gene for cytochrome b (cytb) (**Figure 2.8**). The 9-2I amplicon displayed high homology (E-value of 10^{-52}) to the human insulinoma rig-analog mRNA encoding DNA-binding protein, which has been shown to encode ribosomal protein S15 (rig/RPS15) (**Figure 2.9**). The full-length cDNAs for cytb (1140bp) and rig/RPS15 (498 bp) are comparable in size to the mRNA species detected on Northern blots probed with 3-10Ia and 9-2I (**Figure 2.5**) respectively, further supporting that the identities of these genes were accurate. Putative identities of the remaining twelve amplicons shown by dot blot analysis to be differentially expressed in periparturient cow neutrophils (**Figure 2.4**) are listed in **Table 2.2**. Identities were not currently available in the public databases for all amplicons, but some appear to represent genes for DNA binding proteins or factors that have a role in

the citric acid cycle. Thus, the putative identities of amplicons obtained in this study demonstrate that we have identified cellular genes that are important to general life functions of neutrophils.

```
95  ctcagacgtagacaaaatcccattccacccctactataccactaaggacatcttaggggc 154
    ||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
636 ctcagacgtagacaaaatcccattccacccctactataccattaaggacatcttaggggc 695

155 cctcttactaattctagctctaatactactagtactattcgcacccgacctcctcggaga 214
    |||||||||||||||||||||||||||||||||||||||| || ||| ||||||||||||
696 cctcttactaattctagctctaatactactagtactatttgcgcccaacctcctcggaga 755

215 cccagataactacaccccagccaatccactcaacacacccccctcacatcaaacccgagtg 274
    |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
756 cccagataactacaccccagccaatccactcaacacacctcctcacatcaaacccgagtg 815

275 atacttcttatttgcatacgcaatcttacgatcaatccccaacaagctaggaggagtact 334
     |||||| |||||||||| |||||  |||||||||||||||| || |||||||||| ||
816 gtacttcctatttgcatatgcaattctacgatcaatccccaataaactaggaggagtcct 875

335 agccctagccttctctatcctaattcttgctctaatccccctactacacac 385
    |||||||| |||||| |||||||| |||  ||| || ||| ||||||||||
876 agccctagtcttctccatcctaatccttattctcattcccttactacacac 926
```

Figure 2.8 – The 3-10Ia amplicon was homologous (E-value 10^{-110}) to a region of the bovine gene for mitochondrial cytochrome b (See **Table 2.2** for Genbank Accession number). Shown are the aligned sequences for 3-10Ia (top line, highlighted in gray) and bovine mitochondrial cytochrome b (bottom line).

```
118 ttacttgaggggatgaaacgg-aggaatgggtggccccgataccgggccggccatgctt 176
    ||||||||| |||||||| ||| |||| |||||||||||||| ||||||||||||||||
467 ttacttgagagggatgaagcgggaggagtgggtggccccgatgccgggccggccatgctt 408

177 cacgggcttgttattgatggagaactcccctacgtagtggccgatcatcccacgcttgat 236
     |||||||||   |||||||||||||| || | ||||||||||||||| |  |||||||
407 tacgggcttgtaggtgatggagaactcgcccaggtagtggccgatcatctcgggcttgat 348

237 ttccacctggttgaaggtcttgccgttatanacgccaaccatgct 281
    |||||||||||||||||||||||||| || ||||| ||||||||
347 ctccacctggttgaaggtcttgccgttgtagacgcccaccatgct 303
```

Figure 2.9 – The 9-2I amplicon was homologous (E-value 10^{-52}) to a region of the human gene encoding rig/ribosomal protein S15 (See **Table 2.2** for Genbank Accession number). Shown are the aligned sequences for 9-2I (top line, highlighted in gray) and rig/ribosomal protein S15 (bottom line).

Table 2.2 - Summary of differentially expressed neutrophil genes.

Analysis	Amplicon	mRNA Abundance*	BLAST Results (DNA Sequence Homology)	E value	Accession Number§
Slot Blot	3-10Ia	Decreased	Bovine mitochondrial cytochrome b	10^{-110}	516661(Genbank)
	9-2I	Decreased	rig/Ribosomal Protein S15	10^{-52}	184553(Genbank)
Dot Blot	2-11I	Decreased	No significant homology	-	-
	2-14Ia	Decreased	No significant homology	-	-
	2-14Ib	Decreased	Human glutamate dehydrogenase I mRNA	10^{-22}	14745836(Genbank)
	3-10Ib	Decreased	Bovine mitochondrial cytochrome b	10^{-110}	516661 (Genbank)
	7-16I	Decreased	No significant homology	-	-
	7-20R	Decreased	EST	10^{-52}	482220 (TIGR)
	2-11R	Decreased	EST	10^{-15}	482220 (TIGR)
	3-10Ra	Decreased	DNA binding proteins^	10^{-68}	^
	3-10Rb	Decreased	DNA binding proteins^	10^{-113}	^
	3-11Ra	Decreased	GL004 protein, AD030 mRNA	10^{-44}	9910247, 12005891(Genbank)
	3-11Rb	Decreased	GL004 protein, AD030 mRNA	10^{-109}	9910247, 12005891(Genbank)
	9-7I	Increased	EST	10^{-1-}	664615 (TIGR)

* Change in mRNA abundance following parturition versus prepartum as tested by dot and/or slot blot hybridization.

§ Accession number from either Genbank or TIGR databases.

^ 3-10Ra and 3-10Rb demonstrated equally significant homology to the following: Homo sapiens, similar to PPAR binding protein mRNA (Genbank Accession # 13653337); Homo Sapiens, vitamin D receptor-interacting protein complex component DRIP205 mRNA (9789554); Homo sapiens, thyroid hormone receptor-associated protein component TRAP220 mRNA (3319289); and Homo Sapiens, mRNA for RB18A protein (2765321).

V. DISCUSSION

We have demonstrated in the current study that bovine blood neutrophils repress expression of multiple cellular genes in response to parturition (**Figures 2.4, 2.5, 2.6, and Table 2.2**), and that expression of two of these genes (9-2I and 3-10Ia) associates significantly with blood progesterone and estradiol concentrations (**Table 2.1 and Appendix One, Figure A.5**). Therefore, we accept our hypothesis that parturition induces changes in bovine blood neutrophil gene expression. The DNA sequence of 3-10Ia had high homology with the mRNA sequence for bovine mitochondrial cytochrome b (cytb). Similarly, 9-2I had high homology with the gene encoding the rig/ribosomal protein S15 (rig/RPS15). Both genes were down regulated by at least 45 % in blood neutrophils of periparturient cows, beginning approximately 6 hours post partum and continuing through to the second day of lactation (**Figure 2.6**).

Mitochondrial cytochrome b is a member of Complex III in the electron transport system that generates ATP (Becker et al., 1996). This energy-producing system is located on the inner mitochondrial membrane of cells. If the pronounced repression of cytb neutrophil mRNA levels after parturition is reflected by reduced protein expression, this could impact general energy metabolism in the cells leading to a crippling of the five main neutrophil functions (margination/migration, chemotaxis, phagocytosis, respiratory burst, phagosome-lysosome fusion). Each of these functions requires energy in the form of ATP. Although no work has been published on the cellular effects of inhibited cytb expression in neutrophils or other cells, naturally occurring mutations have been found in human cytb genes that result in truncated cytb or in disruption of the cytochrome bc_1 complex assembly and activity (reviewed in Fisher and Meunier, 2001). The negative

effects on cells that occur due to these mutations are related to respiratory metabolism and associate with the occurrence of diseases such as exercise intolerance, cardiomyopathy, myopathy, and Leber hereditary optic neuropathy. It is possible, therefore, that decreased cytb mRNA abundance in neutrophils could be connected to disease susceptibilities of periparturient dairy cows.

The rig/RPS15 gene encodes ribosomal protein S15, a key component of the small ribosomal subunit and one of the first proteins that binds with 16S rRNA during assembly of the ribosomal complex (Agalov et al., 2000; Nikulin et al., 2000). The consequences of the repressed rig/RPS15 gene expression we observed in blood neutrophils of our parturient dairy cows are unknown. However, proper formation of the small subunit of the ribosomal complex is required for the process of translation, so down regulated expression of this gene could have tremendous implications for the overall synthetic capacity of neutrophils around parturition. Further investigation of this neutrophil gene expression system in periparturient cows seems warranted. The DNA sequences of six additional genes shown in this study to be repressed in neutrophils around parturition (**Figure 2.4**) had significant homology to genes that encode several DNA binding proteins and enzymes of the citric acid cycle (**Table 2.2**). Like repressed cytb and rig/RPs15, down regulation of these types of cellular genes could be critically important to capacity of bovine neutrophils to function immunologically, but this is yet to be determined. The identities of six other differentially expressed neutrophil genes could not be determined at the time of writing this thesis, but several had high homology to ESTs available in the TIGR database (**Table 2.2**). It is only a matter of time before the identity of these genes will be known.

We tried to obtain a preliminary understanding of the potential factors that may have influenced gene expression in neutrophils of our periparturient cows by determining relationships between serum steroid concentrations and expression of 3-10Ia (cytb) and 9-21 (rig/RPS15). We started with the three main steroids of bovine parturition (cortisol, progesterone, and estradiol) because of the direct and pronounced effects on target gene expression in cells that express receptors for these hormones (Carson-Jurica et al., 1990; Beato et al., 1995). Our laboratory has demonstrated that bovine blood neutrophils express receptors for cortisol (Preilser et al., 2000a; Weber et al., 2002) and progesterone (Burton and Coussens, unpublished data), and human and rat leukocytes are known to express estradiol receptors (Ito et al., 1995; Stefano et al., 2000). Our correlation (**Table 2.1**) and regression analyses (**Appendix One, Figure A.5**) showed that blood progesterone and estradiol concentrations were significantly related to neutrophil expression of cytb and rig/RPS15. In another study we showed that blood cortisol concentration was significantly negatively correlated with neutrophil expression of L-selectin (Weber et al., 2002). Therefore, while very preliminary in nature, our data have pointed to the steroid hormones of bovine parturition as possible factors that disrupt the functional capacities of neutrophils through altered expression of key cellular and immune function genes. Studies in which these hormones are administered in controlled environments, including *in vitro*, will be required to discern their true effects neutrophil gene expression, and to ascertain if this means something to the functional capacities of the neutrophils. This will be continuing work of our group.

In closing, a brief comment on the use of DDRT-PCR to explore differential gene expression in leukocytes is offered. The randomness of the DDRT-PCR technique is well

known to cause a high rate of false positives (Kozian and Augustin, 1995; Zhang et al., 1998; Ace and Okulicz, 1999; Ali et al., 2001). We confirmed this in the current study by showing that not all gene expression changes identified in total leukocytes using DDRT-PCR could be confirmed in Northern blot analysis of total leukocyte RNA (data not shown). Also, the expression of several genes originally shown by DDRT-PCR to be induced in total leukocytes around parturition (e.g., 2-11I and 9-7I in **Figure 2.2**) were later shown to be repressed (2-11I) or unchanged (9-7I) in neutrophils using dot blot analysis (**Figure 2.4**). We recommend, therefore, that future studies use RNA from purified leukocyte populations when employing DDRT-PCR as a preliminary screening mechanism for differential gene expression, and that DDRT-PCR results be confirmed by dot and (or) Northern blot hybridizations.

VI. CONCLUSIONS

We conclude from the results of this study that bovine parturition affects the expression of multiple neutrophil genes. The identities of genes detected using high throughput dot blot analysis and confirmed by Northern and slot blot hybridization suggest that their repression following parturition may contribute to the numerous immune dysfunctions of bovine neutrophils collected from parturient dairy cows. Results of this study also point to the reproductive steroids as possible contributors to altered gene expressions in neutrophils of parturient dairy cows. Further studies are needed to substantiate these preliminary data and to determine if identified neutrophil genes are involved in immunity and disease susceptibility of periparturient dairy cows.

CHAPTER THREE

General Discussion and Conclusion

The periparturient period is a time when dairy cows undergo profound physical, metabolic, nutritional, and hormonal changes. Animal health is often compromised during this period in association with some of these changes. Numerous researchers have focused their investigations on blood neutrophils of periparturient cows due to the critical role of these leukocytes in innate immunity and host defense against intramammary infections. Collectively, this research has demonstrated that most neutrophil functions are impaired in periparturient dairy cows. For example, neutrophils demonstrate a decreased ability to migrate *in vitro* (Nagahata et al., 1988; Kehrli et al., 1989; Detilleux et al., 1994), reduced ability to produce ROS when stimulated *in vitro* (Kehrli and Goff, 1989; Detilleux et al., 1994), and possess lowered myeloperoxidase activity and oxidative capacity compared to neutrophils from cattle not undergoing parturition (Kehrli and Goff; 1989; Kehrli et al., 1989; Detilleux et al., 1994). Despite our knowledge that bovine neutrophils are dysfunctional around parturition, little research has been published describing why these phagocytes are so affected.

The research presented in this thesis demonstrates that mRNA abundances of multiple blood neutrophil genes are altered as a result of parturition. The abundance of mRNA encoded by two key cellular genes (mitochondrial cytochrome b and rig/ribosomal protein S15) showed clear reductions in neutrophils of four dairy cows following parturition (**Chapter Two, Figure 2.6**). In addition, the abundance of at least eleven other neutrophil transcripts were reduced at twelve hours after parturition

(**Chapter Two, Figure 2.4 and Table 2.2**). Therefore, we accept our hypothesis that parturition induces changes in blood neutrophil gene expression. Additional data presented in Chapter Two also demonstrates that modest but significant associations exist between abundance of altered transcripts in neutrophils and serum concentrations of progesterone and estradiol in periparturtient dairy cows (**Chapter Two, Table 2.1; Appendix One, Figure A.5**). We monitored these hormones in our study because of the well documented effects of steroids on gene expression in other cell systems (Evans, 1988; Carson-Jurica et al., 1990; Beato et al., 1995). Being lipophilic, steroid hormones easily cross the plasma membranes of target cells and bind to high affinity receptors present in the cells cytoplasm or nucleus (Becker et al., 1996). Such binding induces changes in the receptors, converting them into potent transcription factors that act on hormone-responsive genes (Carson-Jurica et al., 1990). Often receptor action on hormone responsive genes is direct and mediated by receptor binding to DNA motifs in the regulatory regions of the genes. However, hormone activated steroid receptors can also repress or induce target gene expression indirectly by interfering with or activating other inducing or repressing transcription factors that then act on the target gene (Beato et al., 1995). Whether direct or indirect, the actions of steroids on target gene expression are typically rapid and pronounced.

We were not surprised that the steroids involved in reproduction had significant associations with neutrophil gene expression in the current study. Previous researchers have demonstrated dramatic effects of glucocorticoids (Burton et al., 1995; Lee and Kehrli, 1998; Weber et al., 2002), estradiol, and progesterone (Roth et al., 1983; Miyagi et al., 1992) on bovine blood neutrophils. Weber et al. (2002) demonstrated that bovine

blood neutrophils express mRNA for the glucocorticoid receptor and Preisler et al. (2000a) showed that bovine neutrophils bind glucocorticoids and respond to such binding with altered trafficking *in vivo*. Unpublished work from our group has also shown that bovine leukocytes express mRNA for the progesterone receptor (Burton and Coussens, 2001), implying that these immune cells are sensitive to progesterone. In addition, estrogen and progesterone receptors are known to be expressed in human and murine neutrophils (Ito et al., 1995; Tibbetts et al., 1999; Stefano et al., 2000). Combined with the correlation data presented in Chapter Two of the current study, these published studies argue strongly for modulatory roles of cortisol, progesterone, and estradiol on neutrophil functions in periparturient dairy cows. Specifically, mitochondrial cytochrome b and rig/ribosomal protein S15 expression in blood neturophils appears to be highest when blood progesterone and (or) estradiol are highest. Of course, additional studies are needed to substantiate these relationships and to determine which pools of neutrophils may be affected by these parturient steroids (e.g. bone marrow, circulating, marginating, or tissue neutrophils).

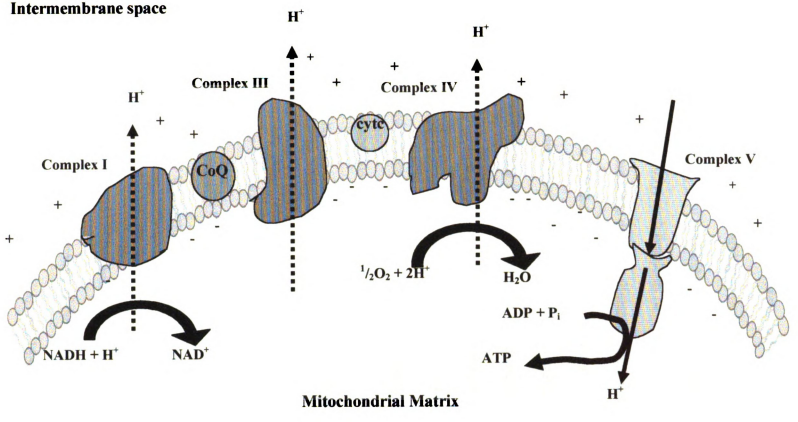
The pronounced decreases in mRNA abundance for mitochondrial cytochrome b (cytb) and rig/ribosomal protein S15 (rig/RPS15) observed following parturition in this study (**Chapter Two, Figure 2.6**) were of special interest because of the known functions of these genes in other cell systems. The protein encoded by the cytb gene is a key component of complex III in the electron transport system that generates mitochondrial ATP (**Figure 3.1**) (Becker et al., 1996). This system is located on the inner mitochondrial membrane of cells. Oxidation of NADH occurs in the mitochondrial matrix and provides an electron that is accepted by complex I of the electron transport

chain (**Figure 3.1, far left**). This electron is then transferred to coenzyme Q (**round molecule to the right of complex I in Figure 3.1**) prior to its acceptance by complex III (**Figure 3.1, left center**). Complex III passes the electron to cytochrome c (**Figure 3.1 center**) from which the electron is accepted by complex IV and passed to an oxygen resulting in the generation of water (**Figure 3.1, right center**). As the electron passes through complexes I, III, and IV, hydrogen ions are pumped out of the mitochondrial matrix to the intermembrane space creating a proton gradient (**Figure 3.1, dashed arrows**). The protons re-enter the mitochondrial matrix via passage through complex V, aiding in the initiation of ATP synthesis (**Figure 3.1, far right**). Much of the ATP utilized for general cellular functions is generated in the mitochondria in this manner (Becker et al., 1996).

Interestingly, DNA located within the mitochondria, not the nucleus, encodes cytb. Mitochondrial DNA is circular and more similar to that found in prokaryotes. The transcriptional regulation of this DNA is different from that which occurs in the nucleus. A few studies have demonstrated that mitochondrial RNA synthesis of several genes increases following administration of synthetic glucocorticoids (Van Itallie, 1990; Van Itallie, 1992). In light of the fact that progesterone, not cortisol, showed a positive relationship with cytb mRNA abundance in the current study, further studies on steroid regulation of this key gene seems warranted.

Figure 3.1 - Mitochondrial cytochrome b (cytb) is a key component of Complex III of the electron transport chain located in the inner mitochondrial membrane. Complex III accepts an electron from coenzyme Q and passes it to cytochrome c. This electron transport system results in the pumping of protons from the mitochondrial matrix to the intermembrane space creating a proton gradient. The gradient is removed when protons re-enter the mitochondrial matrix through complex V, providing the source of energy that drives ATP synthesis. Reduction in the amount and activity of mitochondrial cytb results in disruption of electron transport, proton gradient formation, and ATP synthesis by the cell. If the reduced cytb mRNA in neutrophils from parturient cows also reduces generation of ATP in the cell's mitochondria, profound negative effects on immune functions in these leukocytes are to be expected. This may begin to provide an explanation for the dramatically altered functionality of bovine neutrophils around parturition.

Figure 3.1


Intermembrane space
H+
H+
H+
Complex III
Complex IV
cyte
Complex I
CoQ
Complex V
1/2O2 + 2H+
H2O
ADP + Pi
NADH + H+
NAD+
ATP
H+
Mitochondrial Matrix

While still to be substantiated in future studies, reduced cytb mRNA abundance in parturient neutrophils may cause reduced expression of the cytb protein in mitochondria. If cytb proteins were absent or decreased, the ability of the cells to form complex III would be reduced resulting in disruption of the electron transport chain and deficient ATP production by the cells. Certainly, such problems have been recorded in numerous human diseases where cytb gene mutations disrupt cellular respiratory metabolism (reviewed by Fisher and Meunier, 2001). Recall from the literature review that only few mitochondria are present in mature blood neutrophils. If this integral portion of the electron transport chain were missing in these phagocytes, as may be the case when cytb mRNA abundance is reduced during parturition, general energy metabolism would be predicted to be in jeopardy. Because all immune functions of neutrophils require energy in the form of ATP, inhibited cytb expression could also be predicted to affect the surveillance, migration, phagocytosis, oxidative burst, and phagolysosome killing by bovine neutrophils. If true, the cytb results of the current study may begin to explain why neutrophils of periparturient cows are dysfunctional and thus why the animals are so susceptible to mastitis and other infectious diseases.

The down regulation of neutrophil rig/RPS15 mRNA observed in this study may also have negative consequences for the immune functions of these cells. Originally identified in a rat insulinoma cDNA library, the rig gene has been shown to encode for mammalian ribosomal protein S15 (RPS15) (Kitagawa et al., 1991). RPS15 was recently found to be a key component in the assembly of the small ribosomal subunit (**Figure 3.2**) and in prokaryotes is one of the first to bind with 16S rRNA during assembly of the small subunit (Agalarov et al., 2000; Nikulin et al., 2000). This protein

is required for recruitment of additional proteins to ensure that proper formation of the small subunit occurs. If the small subunit is not formed, the ability of the affected cells to translate mRNA into proteins may be generally inhibited. In this regard, it was interesting that three major proteins (CD62L, CD18, and G1) of bovine neutrophils are down regulated during periparturition (Lee and Kehrli, 1998; Kimura et al., 1999b; Weber et al., 2002; Burton and Weber, **Appendix One, Figure A.1**). Deficient expression of these and other proteins could signal neutrophils to become inactive or even apoptotic. Indeed, apoptosis could be an important mechanism for clearing aging neutrophils from the circulation, especially during times of neutrophilia (as occurs during parturition; Preisler et al., 2000a; Weber et al., 2002). Combined with repressed cytb expression in blood neutrophils of parturient cows, reduced expression of RPS15 could be a hallmark of aging (and dysfunctional) neutrophils. Clearly, these possibilities must be substantiated through additional research.

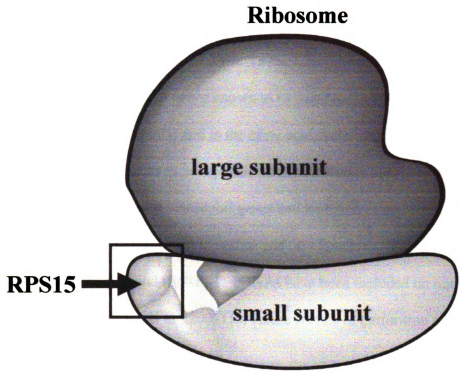


Figure 3.2 - Ribosomal protein S15 (RPS15) is an integral component of the small ribosomal subunit. Researchers have demonstrated in prokaryotes that RPS15 is one of the first ribosomal proteins to bind 16s rRNA, and induces binding of additional proteins for small subunit formation. When cells are deficient in RPS15, the small subunit may not form correctly and overall translational capacity of the cells may be inhibited. Preliminary evidence from our laboratory (Chapter Two; Weber et al., 2002; Burton and Weber, unpublished) and others (Lee and Kehrli, 1998; Kimura et al., 1999b) suggest that bovine blood neutrophils deficient in RPS15 mRNA also have deficient expression of key membrane proteins such as L-selectin, CD18, and G1 (examples of L-selectin and G1 in **Appendix One, Figure A.1**).

Finally, the putative identities of eleven additional genes observed in the current study as modestly to moderately down regulated in bovine blood neutrophils during parturition are noteworthy (**Chapter Two, Table 2.2**). The cDNA sequences of these genes had high homology with genes known to be involved in DNA binding (eg. by steroid and vitamin D receptors) and in the citric acid cycle. Again, therefore, we identified basic cellular genes as being affected in neutrophils during parturition. Several of our differentially expressed neutrophil genes had high homology with ESTs in the TIGR database, but with no known identities and (or) functions. These cDNAs, and ESTs along with the cytb and rig/RPS15 cDNAs have been included on our groups cDNA micorarrays (Burton et al., 2001) for future studies of parturition effects on gene expression in neutrophils.

In conclusion, the research presented in Chapter Two demonstrates clearly that parturition induces reductions in mRNA abundance of several key genes important for the basic cellular functions of neutrophils. Data presented in Chapter Two also implicates the reproductive steroid hormones as possible factors that influence neutrophil gene expression. Although the genes identified through this research are not considered classical immune response genes, their protein products are intimately involved in the basic functions of cells including respiratory metabolism, the citric acid cycle, transcription, and translation. Therefore, lowered mRNA abundance of these genes at parturition may help explain the general dysfunctions of blood neutrophils (surveillance, recruitment, phagocytosis, respiratory burst, and phagolysosome killing) that have been reported by bovine leukocyte biologists for several decades. In turn, these neutrophil dysfunctions are clearly associated with the heightened disease susceptibility of parturient

dairy cows. The work of this thesis has thus provided a launch point for future studies aimed at explaining the causes and consequences of gene repression in bovine neutrophils around parturition.

CHAPTER FOUR

Recommendations for Future Research

As mentioned in previous chapters, resting blood neutrophils actively express at least 750 genes. While this is a relatively small number of genes compared to expression in other cell systems, it is not surprising given that mature neutrophils are terminally differentiated cells with few mitochondria, Golgi apparatus, and excessive cytoplasmic granules packed full of proteins required for many of the cell's innate immune functions. Interestingly, researchers are beginning to publish data showing that exposure of neutrophils to various stimuli significantly alter global gene expression (Cowling and Birnboim, 2000; Newberger et al., 2000). Until our work, however, no one had demonstrated that bovine blood neutrophils respond to parturition with globally altered gene expression. We have now shown that the cortisol spike of parturition inhibits neutrophil expression of the L-selectin gene, preventing these leukocytes from marginating and causing neutrophilia (Weber et al., 2002). Furthermore, the work of the current study shows that mRNA abundances of at least eleven other neutrophil genes are repressed around parturition, two of which appear to associate with serum concentrations of progesterone and estradiol (cytb and rig/RPS15. However, much more work is required to fully confirm the identities and causes of the decreased mRNA levels of the neutrophil genes shown through this study. The tools are now in place to carry on such research. For example, the fourteen amplicons generated by DDRT-PCR and used on dot blots could now be used to screen a bovine leukocyte cDNA library to obtain full-length cDNA sequences. The full-length cDNAs would then be sequenced to confirm the

identities of cytb, rig/RPS15, and the other genes putatively identified. This may also help identify genes represented by those amplicons that demonstrated no significant homology to DNA sequences currently available in the public databases.

Neutrophils from periparturient cows accumulate in the blood resulting in pronounced neutrophilia (Nagahata et al., 1988; Kehrli et al,, 1989; Preisler et al., 2000a; Weber et al., 2002). Although many functional aspects of these phagocytes are altered, these cells' immediate fate is still unknown. The present study demonstrated mRNA of multiple neutrophil genes to be down regulated shortly after parturition when neutrophil numbers are at their highest in the blood. We have hypothesized that the decreased mRNA abundance is in response to fluctuating hormone levels but there is also the possibility that neutrophilia may be inducing other changes in the cell such as apoptosis. With apoptosis, cell death would result in an overall decrease in genes that could be expressed, mimicking a regulated decrease in mRNA abundance of numerous genes. Several methods can be utilized to determine if blood neutrophils from parturient cows are apoptotic. These include the use of fluorescent dyes, cell surface markers specific for apoptosis, and terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL), all methods that can be evaluated by flow cytometry which is readily available in our laboratory. In spring 2002, we plan to implement a few of these methods to determine the relative contributions of altered gene expression and apoptosis to the phenomena described in this thesis and our companion paper (Burton et al., 2001; Madsen et al., submitted). Both scenarios are unique and could potentially provide interesting explanations for the increased disease susceptibility of periparturient dairy cows.

In addition to confirmation of differential gene expression in neutrophils from periparturient cows, it would be of interest to observe if other situations result in similar changes in gene expression. This will be done by adding the 14 amplicons identified by DDRT-PCR to the bovine total leukocyte cDNA library created by the Center for Animal Functional Genomics at Michigan State University. Microarrays generated from this expanded cDNA library will be used for even broader studies of blood neutrophil gene expression in periparturient cows, including *in vitro* studies to observe individual effects of cortisol, estradiol, and progesterone on neutrophil gene expression. Future studies could also evaluate differences in bone marrow, blood, and tissue neutrophil gene expression under these scenarios. The results of these studies will help us gain a better understanding of gene expression in relation to the dysfunctional neutrophil biology observed during the periparturient period. They may also result in discovering which of these steroid hormones have the greatest affect on neutrophil function leading to increased disease susceptibility.

APPENDIX ONE

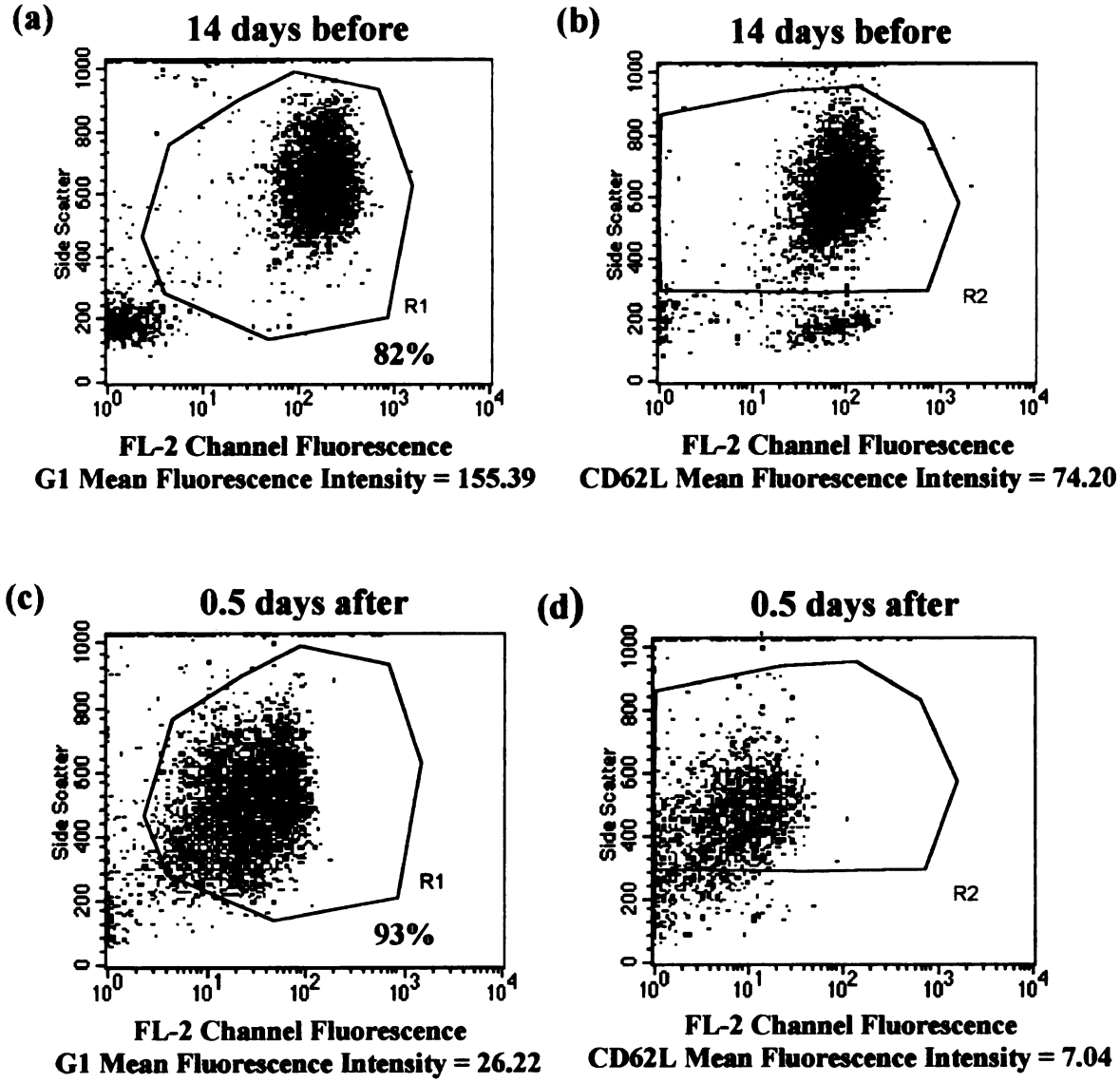


Figure A.1 – Purity of isolated neutrophils from d –14 and d 0.5 from which RNA was reverse transcribed and used in dot blot hybridization. An aliquot of purified neutrophils was obtained prior to the TRIzol step and fluorescently immuno-stained for the cell surface markers G1 and L-selectin (CD62L). G1 is neutrophil specific and indicates the purity of the cell population (panels a and c). Overall, our cell populations had a purity of 80% or greater. The decreased CD62L intensity (panels b and d) is well documented and characteristic of neutrophils from parturient dairy cows (Lee and Kehrli, 1998; Weber et al., 2002).

Representative DDRT-PCR Gels

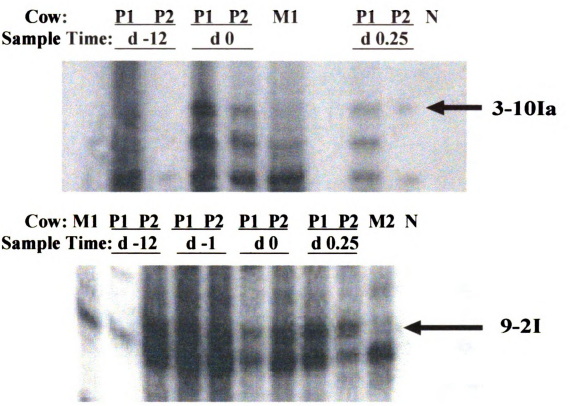


Figure A.2 – DDRT-PCR gel sections containing differentially expressed genes called 3-10Ia and 9-2I. Expression of 3-10Ia and 9-2I in neutrophils was followed through the entire flow of experiments outlined in **Chapter Two, Figure 2.1**. Letters above the lanes indicate the following samples and time points: mid-gestation control cows M1 and M2; pooled samples from d -175 to -130 prepartum; parturient test cows P1 and P2; sampled on d -12, -1, 0, 0.25 relative to parturition; N is the negative control (no RNA in reverse transcription reaction).

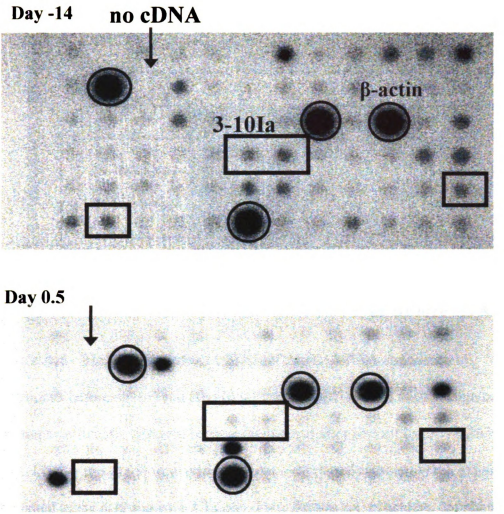


Figure A.3 – Autoradiographs of dot blots hybridized with radiolabeled cDNA created by reverse transcription of neutrophil RNA from pre- (day -14) and post partum (d 0.5) time points. All 14 amplicons were spotted in quadruplicate (example of 3-10Ia in boxes). β-actin was also spotted in quadruplicate as a control (dark circled spots) and four spots containing no DNA for negative control are indicated by the arrows (one replicate per blot).

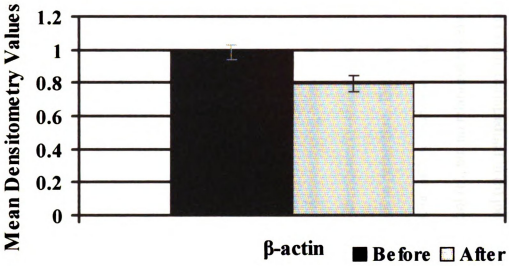
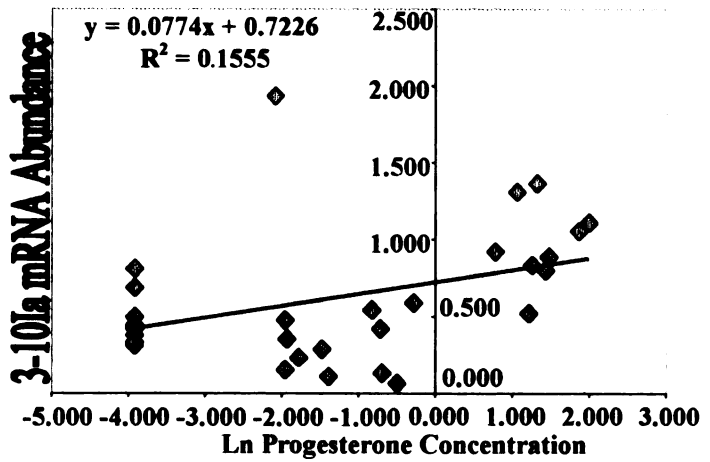
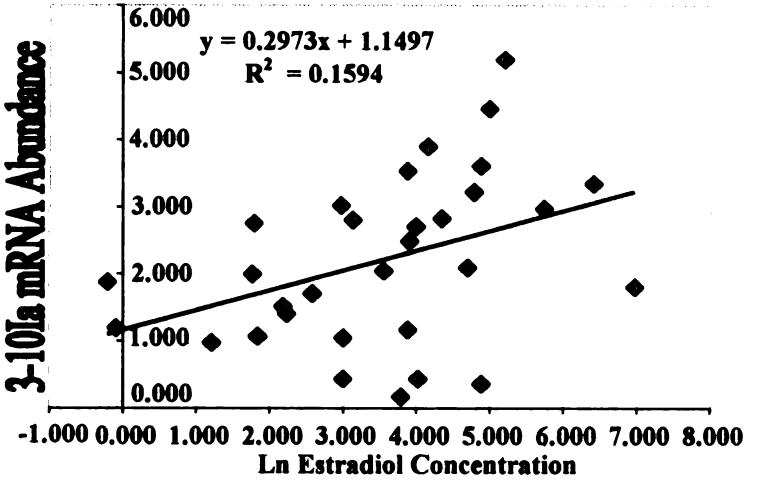


Figure A.4 – Mean dot densities (± SEM) of β-actin mRNA abundance in neutrophils before (d -14) and after (d 0.5) parturition. β-actin mRNA abundance was not significantly different between before and after parturition ($P > 0.10$ by t-test). Data in this graph were obtained from a shortened autoradiograph exposure compared to the data shown in **Chapter Two, Figure 2.4**, to achieve dot intensities that were measurable by the scanning densitometer.

(a) progesterone – 3-10Ia relationship



(b) estradiol – 3-10Ia relationship



(c) cortisol – 3-10 Ia relationship

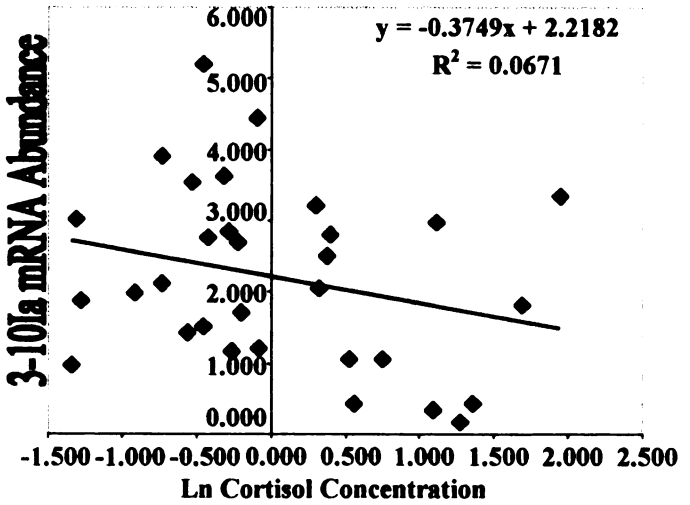
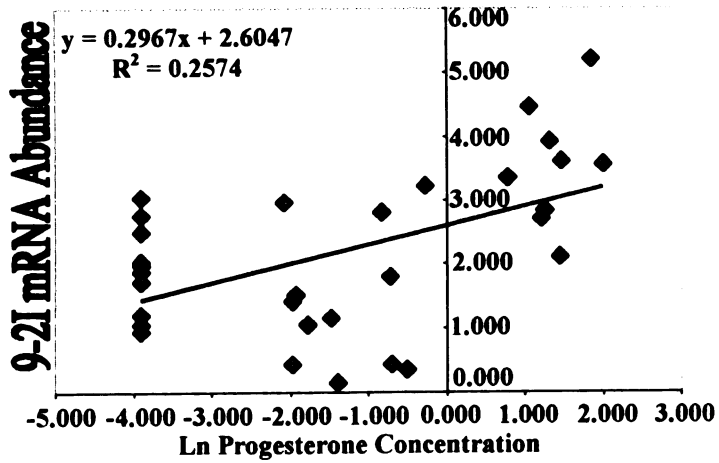
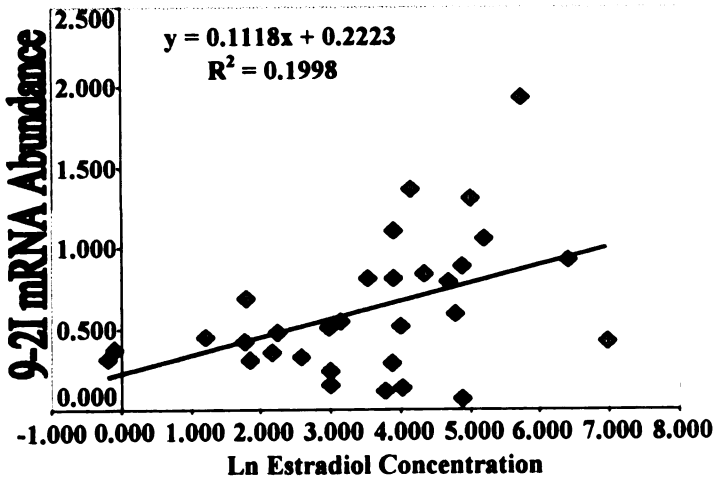


Figure A.5 - Scatter plots demonstrating relationships between neutrophil 3-10Ia mRNA abundance (y-axis) and steroid hormones (x-axis) involved in parturition. Data show positive relationships between 3-10Ia mRNA abundance and progesterone (a) or estradiol (b), but no relationship between 3-10Ia expression and cortisol (c) in periparturient dairy cows.

(d) progesterone – 9-2I relationship



(e) estradiol – 9-2I relationship



(f) cortisol – 9-2I relationship

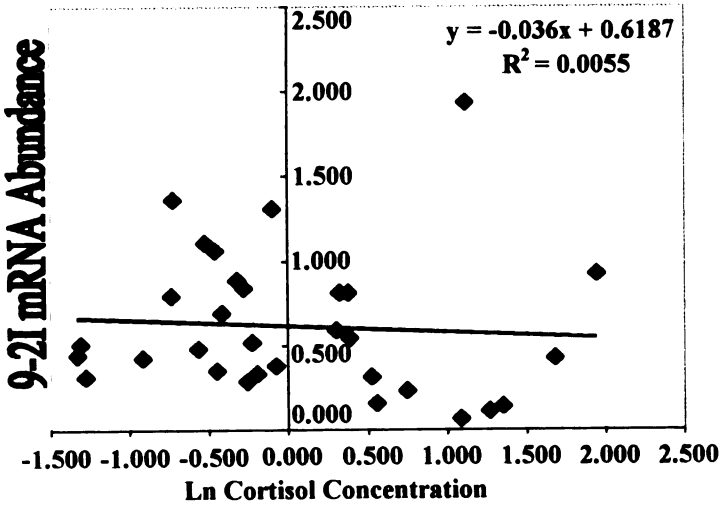


Figure A.5 continued... Scatter plots demonstrating relationships between neutrophil 9-2I mRNA abundance (y-axis) and steroid hormones (x-axis) involved in parturition. For 9-2I, mRNA abundance is positively related with serum progesterone (d) and estradiol (e), but not related with serum cortisol (f).

APPENDIX TWO

November 16, 2001

Dr. C.L. Baldwin
Editor-in-Chief for the Americas
Veterinary Immunology and Immunopathology
Department of Veterinary & Animal Sciences
Paige Laboratory
Holdsworth Way
University of Massachusetts
Amherst, MA 01003

Dear Dr. Baldwin:

I am writing to you in regards to the article "Pre-translational Regulation of Neutrophil L-selectin in Glucocorticoid-Challenged Cattle" by Weber et al. that has been accepted for publication in *Veterinary Immunology and Immunopathology* (revised manuscript VII #01-26). I am currently a Master's student at Michigan State University and am scheduled to defend my thesis on December 4, 2001. I request permission to include Figure 7 (Serum concentrations of three steroid hormones of bovine reproduction change significantly during parturition.) in Chapter Two of my thesis as this data was utilized in statistical analyses of my results.

Thank you for your prompt consideration of this manner!

Sincerely,

Sally A. Madsen

Sally A. Madsen

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Editors-in-Chief
Dr C.L. Baldwin
Dr. C.J. Howard
Dr. J. Naessens

Dr C.L. Baldwin
Department of Veterinary & Animal Science
Paige Laboratory
University of Massachusetts,
Amherst, MA 01003
Tel: 413-545-3167
Fax: 413-545-6326
E-mail: Cbaldwin@vasci.umass.edu

Ms. Sally A. Madsen
Dept animal Science
Anthony Hall
East Lansing, Michigan 48824-1225

Fax 517-353-1699

Dear Sally,

It is acceptable for you to include the figure in your thesis. Technically a thesis or dissertation is a publication and copyrighted by the university or individual since it is a publicly accessible document. However the practice of publishing your graduate degree research results that appear in those documents in peer-reviewed journals as well is not only long-standing but highly encouraged by the scientific community. This is because the data is usually more readily accessed through this medium and second it has been validated by the peer-review process.

Good luck with your defense.

Sincerely,

Cynthia L. Baldwin
Editor

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