USING ANTIBODY AND CELL-MEDIATED IMMUNE RESPONSE TO TEST ANTIGENS IN PERIPARTURIENT DAIRY COWS AS A MEASURE OF DISEASE RESISTANCE

By

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To my wife, the better half who inspires me to improve

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Abstract of Thesis Presented to the Graduate School
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Despite major advances in the dairy industry for sanitation, housing, milking strategies, and genetic trait selection; incidence of disease is still rising for Holstein dairy cows. This has sparked research aimed at identifying ways to incorporate genetic selection to improve broad-based immune responsiveness. For this to become a possibility, immune function must become a trait which can be quantified and correlated with risk of disease. For this study, both branches of adaptive immune function were considered due to the potential for an inverse relationship between the two. As a result, 774 cows were categorized based on their ability to mount an antibody-mediated immune response (AMIR), and 812 cows were categorized based on their ability to mount a cell-mediated immune response (CMIR). Immune response categorizations were high, medium, or low, such that the measured immune response for high > medium > low.

Categorization status for AMIR as well as CMIR was found to be significantly associated with mastitis occurrence. Medium immune responders were 1.76 and 2.14 times more likely to have an occurrence of moderate or severe mastitis than high immune responders for AMIR and CMIR respectively. Low AMIR cows were 2.90 times more likely to have an occurrence of ketosis than high responders. This association with ketosis followed a low > medium > high

pattern. For CMIR, low responders were 6.68 times more likely than high responders to have a retained fetal membrane (RFM). When only considering multiparous cows low responders for CMIR were 26.52 times more likely than high responders to have an occurrence of RFM. Although not statistically significant, medium CMIR status cows were 7.40 times more likely than high responders to have an occurrence of metritis.

When considering the performance traits of fertility and milk yield, high AMIR status was associated with reduced fertility and reduced milk yield. However, high CMIR cows produced significantly greater milk yields than medium and low responders.

Negative associations between higher levels of AMIR and reduced milk yield are likely attributed to neglecting immune function as a genetic selection trait. Associations between immune function and ketosis provide evidence for immune system involvement with energy-related metabolic conditions.

CHAPTER 1
INTRODUCTION

Disease Trend

The susceptibility of dairy cows to infectious disease is increasing. Genetic selection for increased milk yield without regard for disease resistance may be fueling this adverse effect (Harmon, 1994; Heringstad, 2000; Emanuelson, 1988). The rampant selection for increased milk production void of measures for resistance to mastitis has been found to result in a genetic increase of 0.02 cases of mastitis per cow per year (Strandberg and Shook, 1989). This translates into a genetic increase of 2 mastitis cases for every 100 dairy cows per year.

Immune Suppression

Immune suppression experienced around the time of calving has been well documented (Mallard et al., 1998; Lacetera et al., 2005; Kimura et al., 2006). This suppression is believed to be at least in part responsible for the increased risk of disease peripartum. The added stress associated with parturition and the abrupt change in 'lifestyle', work together to suppress immune function (Mallard et al., 1997; Van Kampen and Mallard, 1997). Different mediators of this immune depression include; endocrine hormones (Mallard et al., 1997), hypocalcemia (Kimura et al., 2006), and non-esterified fatty acids (NEFA) (Lacetera et al., 2004, 2005).

Selection for Disease Resistance

Since a national database for Holstein health disorders in the United States does not exist, it is impossible to genetically select directly against specific health disorders. Artificial insemination has provided some opportunity for producers to select for certain traits that vary in their degree of association with disease resistance. Selection to improve somatic cell score (SCS) (a logarithmic transformation of somatic cell count), productive life and structural udder traits (udder cleft, udder depth, rear udder height, rear udder width, and fore udder attachment) have

all been found to be significant predictors of susceptibility to clinical mastitis (Nash et al., 2000). Six studies estimating the genetic correlation between SCS and clinical mastitis all indicated a positive correlation averaging 0.71, and ranged from 0.37 to 0.98 (Nash et al., 2000). However, the relationship between SCC and intra-mammary infection is still unclear (Piccinini et al., 1999; Schukken et al., 1999). Also, with the exception of productive life, these traits are only associated with infections of the mammary gland.

The increasing incidence of disease associated with selection for increasing milk production may be partially explained by the association of increased severity and prevalence of immune suppression with elevated NEFA and deficiencies in calcium. These two postulated factors in immune suppression are also potentially correlated with increasing milk yield. However, previous work also indicates a strong genetic influence on immune responsiveness (Wagter et al., 2000; Mallard et al., 1998, Biozzi et al., 1979). Application of measures to place genetic selection pressure on immune responsiveness could potentially be used to overcome increasing infectious disease trends. Concerns for a negative association between milk yield and genetic potential for immune responsiveness are not substantiated (Wagter et al., 2003; Detilleux et al., 1995).

Breeding to improve immune function is a concept which has been examined and utilized in poultry (Soler et al., 2002; Cole, 1968), swine (Mallard et al., 1998), sheep (Woolaston and Baker, 1996), and mice (Biozzi et al., 1979). However, work aimed at categorizing general immune responsiveness in Holstein cattle is just recently gaining attention (Wagter et al., 2000, 2003; Hernandez et al., 2003). The obvious motive behind research aimed at identifying superior and inferior immune responsiveness is to serve as an indirect trait enabling selection for general broad-based disease resistance. This concept is appealing for several reasons. Since eradication

of environmental infection-causing organisms is impossible, the domestic bovine must rely primarily on its immune system to fight pathogens. Thus, cows are exposed to a wide variety of pathogens which are also proficient at altering their virulence mechanisms. As a result, selection should be for the cattle with the most robust repertoire of response against a variety of pathogens. Selection for increased immune responsiveness should reduce the strong dependency on vaccines and antibiotics. Increases in antibody titers to vaccinations should result in more efficient use of vaccine dosage and potentially a reduction in vaccine dosage (Wagter et al., 2000; Mallard et al. 1997). Selecting for increased immune responsiveness should also take steps toward addressing the concerns consumers have for excessive use of antibiotics and animal welfare issues. Finally, through a reduction in mastitis occurrence, SCC should also be reduced leading to increased cheese yield, dairy product quality and shelf life (National Mastitis Council, 1996).

Research by Wagter et al. (2000) has found a significant variation in a cow's ability to mount a humoral immune response. They also found that not all cows experience immune suppression around calving. This research used ovalbumin (OVA) as a novel antigen to elicit an antibody mediated immune response (AMIR) in 136 Holstein cows and heifers. They found the high responders had the lowest incidence of mastitis. They also found that antibody responsiveness to ovalbumin positively correlated with antibody response to the E. coli J5 vaccination (Rhône Mérieux, Lenexa, KS). Detilleux et al. (1995) found adequate genetic potential for immune responsiveness to exist among sires with top PTA for milk yield. An additional study found that selection for immune responsiveness does not predispose cows to reduced milk yield (Wagter et al., 2003).

Objectives

Using 136 cows the previous study by Wagter et al. (2000) had difficulty finding statistical significance between AMIR and disease risk. Also, this study did not include a measure of cell-mediated immune response (CMIR), which is the other branch of a cow's adaptive immunity.

In the present study we have further analyzed the association between immune responsiveness and disease through the use of a larger sample size. The objectives of this study were to categorize a cow's humoral response to ovalbumin as a measure of AMIR as well as delayed-type hypersensitivity (DTH) response to *Candida albicans* as a measure of CMIR (Hernandez et al., 2003, 2005). Associations between these immune categorizations and disease risk, namely mastitis, metritis, retained fetal membrane, ketosis, and displaced abomasum were tested. The effect of these immune categorizations on milk production, somatic cell count, and fertility were also tested.

CHAPTER 2
REVIEW OF LITERATURE

Immune System Basics

Introduction

In order to better understand the mechanisms involved in this research study, a basic review of the immune system is required. The immune system is comprised of several defense mechanisms. Most potential pathogens never elicit an immune response because the body's first line of defense, the epithelial surface, protects against the establishment of infection. The epithelial surface defense mechanisms can be divided into those mechanical, chemical, and microbiological. Mechanical is used to describe the defense provided through tight junctions between epithelial cells, or the movement of mucus by cilia in the respiratory tract. In the stomach, the low pH, enzymes (pepsin), antimicrobial fatty acids, and peptides found in the stomach provide a chemical barrier to infection (Risso, 2000). The normal flora found on the skin and in the gut provides a microbiological defense mechanism. The initial response is characterized as an innate nonspecific immune response while extended and/or repeat exposure to pathogen leads to an acquired immune response acting only on specific antigen.

Innate Immunity

When foreign microbes are able to pass the body's first line of defense, the immediate response is classified as innate immune response. Innate immune function is adept at distinguishing self from non-self by the use of cell-surface receptors which react with general features that are common among microbes called pathogen associated molecular patterns (PAMPs). Therefore this response is characterized as nonspecific effector recognition of pathogen which involves the phagocytic and inflammatory activity of the leukocytes, largely macrophages and neutrophils (Beutler and Rietschel, 2003). Macrophages and neutrophils

release various cytokines and chemokines which can initiate the inflammatory response. The inflammatory response works by recruiting additional effector molecules to the infection site, reducing the spread of infection by microvascular coagulation, and by promoting tissue repair (van der Poll, 2001).

Acquired Immunity

Infectious disease occurs when a microorganism succeeds in evading or overwhelming innate immune defenses. It is at this point where the acquired or adaptive immune response is required. Acquired immune function is characterized by specific recognition of antigen and has two main branches which provide means for the elimination of both extra and intracellular pathogens. Elimination of extracellular pathogen is performed by antibody-mediated immune response (AMIR) pathways. Eradication of intracellular pathogen is performed by cell-mediated immune response (CMIR) pathways.

Intracellular immunity

In certain instances, some forms of bacteria, parasites, and all viruses, replicate within cells and are not detected by extracellular immunity. On these occasions, the body employs methods to combat intracellular pathogens which largely involve the function of T-lymphocytes (T-cells). T-cells only respond to antigen that is accompanied by a major histocompatibility complex (MHC), forming an MHC:antigen complex. This reaction requires a specific T-cell receptor for a response to take place (Jensen, 2007).

There are two classes of MHC molecules: MHC class I molecules are expressed by nearly every nucleated cell in vertebrates. MHC class II molecules are only expressed by professional antigen presenting cells (APC), which are a special group of phagocytic cells. Dendritic cells are the most common APC, but B-lymphocytes and macrophages can also function as APCs' (Jensen, 2007). These APCs' specialize in displaying a small portion of processed antigen bound

to an MHC class II molecule on the APC's surface (Savina and Amigorena, 2007). Antigen processing for presentation with an MHC class II molecule involves the endocytic pathway. This pathway begins when exogenous antigen has been endocytosed by an APC.

There are two main classes of T-cells; CD4+ T-cells (T_H), and CD8+ T-cells (T_C). The T_C cell is responsible for cytotoxic activity or cell killing. These cells initiate their cytotoxic activity on cells displaying antigen bound to an MHC class I molecule which is expressed by nearly every nucleated cell in vertebrates. The T_H cell is probably the most important of the T-cells. T_H cells only respond to antigen bound to an MHC class II molecule, meaning they only respond to antigen presented by dendritic cells, macrophages or B-lymphocytes, also called APCs'. There are two subsets of T_H cell; T_H1 subset is responsible for cell-mediated functions such as delayed-type hypersensitivity (DTH) and activation of cytotoxic T-cells; T_H2 subset is largely responsible for B-cell activation. The functions of the two subsets of T_H cell serve to activate cells in both branches of the adaptive immune response, which is why T_H cells are of special importance.

Naïve T-cell activation requires specific antigen presentation by an APC to a T_H cell. The activation of a T_H cell initiates the release of cytokines which can: activate the cytotoxic activity of T_C cells, stimulate the chemotaxis of leukocytes, activate B-lymphocytes, and also cause differentiation into memory T-cells. Activated T_H and T_C cells have a relatively short life-span while the memory T-cells being long-lived can last for the duration of the cow's life.

Extracellular immunity

B-cells use immunoglobulins for antigen recognition and are concerned with the elimination of extracellular pathogens. Non-membranous immunoglobulins, called antibodies, function by binding to the antigen which elicited the response. This binding or coating (opsonization) can neutralize the pathogen, and also flags the pathogen for phagocytosis or complement activity. For a B-cell to differentiate into effector cells (activation), they require

accessory signals from an activated T_H2 T-cell. B-cell activation occurs when they bind antigen with its membranous immunoglobulin. The antigen is then internalized and degraded. A peptide fragment from the antigen is later displayed on the cells surface with an MHC class II molecule. A specific interaction between the MHC: antigen peptide complex and an armed T_H2 T-cell send activation signals to the B-cell. This allows the differentiation into antibody secreting plasma cells and memory B-cells (Parker, 1993).

Immunologic memory

Adaptive immunity is also associated with immunologic memory which results in a more rapid and effective immune response to pathogens that have been previously encountered. An antibody response profile indicates features which are common to every antibody response (Figure 2-1). The extent of these features or kinetics are different for a primary immune response (first antigen encounter) and secondary immune response (>1 antigen encounter). After antigen exposure, every antibody response begins with an initial lag phase to allow for somatic hypermutation and clonal differentiation into effector cells. This phase is followed by an increase in antibody concentration until a peak concentration is reached. After a peak response is reached, it is followed by a steady decline in antibody concentration. In a primary response the humoral response results from the activation of naïve lymphocytes, whereas in a secondary immune response it is memory lymphocytes which are activated. These memory lymphocytes have greater affinity for their specific antigen, which facilitates greater immune response upon repeated exposure. Memory lymphocytes are also long-lived, and can provide life-long immunity to their specific pathogen. A secondary immune response is characterized by a shorter lag phase, greater magnitude, longer duration and greater antibody affinity to antigen when compared to a primary immune response. A primary antibody response consists primarily of IgM isotype antibody, while a secondary response consists largely of IgG isotype. There is also substantial

variation in the kinetics of an antibody response due to antigen type, administration route, presence of adjuvant, and species exposed to antigen.

Relationship Between Intracellular and Extracellular Immunity

Several studies have cited inverse relationships between intracellular immunity and extracellular immunity (de Vries, 1995; Biozzi et al., 1979; Rupp et al., 2007). Although the three referenced studies support the claim of an inverse relationship, they are all concerned with different aspects of the immune system.

The work performed by Biozzi et al (1979) supported an inverse relationship between CMIR and AMIR on the basis of intracellular catabolism of antigen. Breeding mice for high antibody mediated immune responsiveness (AMIR) was associated with slower intracellular catabolism of antigen. The explanation for this finding was that slowed antigen processing also related to prolonged antigen presentation which results in greater stimulation for the production of antibodies. The mice selected for high AMIR were more resistant to extracellular pathogens, however; the support for an inverse relationship enters when these high AMIR mice were more susceptible to intracellular pathogens. The slowed intracellular catabolism which was favorable for AMIR is believed to be unfavorably associated with relevant measures of cell-mediated immune function (CMIR).

Research conducted by Rupp et al., (2007) found support for an inverse relationship on the basis of MHC alleles at the DRB3.2 locus. As previously discussed, MHC molecules are responsible for antigen presentation to T lymphocytes. This experiment found significant relationships between available alleles at the MHC locus DRB3.2 and measures of AMIR and CMIR. However, these relationships were inversely related. Alleles which confer high measures for CMIR associated with low measures of AMIR, and vise versa.

Another paper by de Vries (1995) outlined a mediator for an inverse relationship on the basis of cytokine expression by T_H2 cells. As previously described, T_H2 lymphocytes are involved with activation of an AMIR while T_H1 lymphocytes are involved with activation of a CMIR. T_H1 lymphocytes secrete the cytokine interleukin-10 (IL-10). This release of IL-10 works to block the function of T_H2 lymphocytes (Figure 2-2).

Delayed-Type Hypersensitivity

Hypersensitive immune responses are often termed, inappropriate immune responses to antigen. There are four types of hypersensitive reactions, three of which are mediated by antibodies (Type I-III), and one is mediated by T-lymphocytes (Type IV). A Type IV hypersensitivity response is called a delayed-type hypersensitivity reaction (DTH) due to a characteristic delayed response by comparison to the acute phase reactions of immediate hypersensitivity (Type I). A DTH reaction generally peaks around 24-72 hrs post secondary exposure. The other three hypersensitive reactions are termed immediate hypersensitivity because reaction peaks occur within minutes or hours of secondary exposure.

A DTH immune response requires the stimulation of T_H1 cells to form memory T_H1 cells. Therefore a DTH reaction requires previous exposure to the specific antigen. Upon secondary antigen exposure, antigen presentation cells (APC) take up the antigen and display it in conjunction with MHC class II molecule to the previously formed memory T_H1 cells. When memory T_H1 cells bind to APC's, cytokines are released and chemotaxis of predominantly macrophages and neutrophils occur at the exposure site resulting in a granuloma. Clinically, a palpable lump occurs which is largely composed of these macrophages and neutrophils and to a far less degree, T-cells.

DTH reactions are frequently used to detect exposure to large intracellular antigens. The most common of these antigens would be *Mycobacterium tuberculosis*. DTH reactions have also

been previously used as a means in which to quantify cell-mediated immune function (Mallard et al., 1998; Hernandez et al., 2003, 2005). There are no other known *in vivo* methods for quantifying CMIR. In order to mount a DTH immune response the subject must have been previously exposed to the antigen at least two weeks prior. This is required to provide an adequate period for T-cell clonal expansion and differentiation into memory T-cells. The antigen is then injected into the subject intradermally and after 24-72 hours, detection of this responseis by examination for a palpable lump at the injection site.

Indirect Enzyme-Linked Immunosorbent Assay (ELISA)

The indirect ELISA is a method for detection and quantification of antibody that reacts against a specific antigen. These methods for indirect ELISA have been used in the past as a means to quantify an individual cow's antibody mediated immune responsiveness to a novel antigen (Burton et al., 1989; Mallard et al., 1997; Wagter et al., 2000). For the indirect ELISA, antigen is coated to the surface of a microtiter well and after sufficient time, excess free antigen is washed away. Nonspecific reactions are blocked by a non-reactive protein. Serum (or some other fluid) which potentially contains the anti-antigen primary antibody of interest, is added to the microtiter well. This allows the primary antibody to bind the antigen which is attached to the side of the well. After sufficient time has elapsed, the free unbound antibody is washed away. An enzyme-conjugated secondary antibody is added which binds to the constant region of the primary antibody which is adhered to the side of the well. After sufficient time, free secondary antibody-enzyme conjugate is washed away and a substrate for the remaining enzyme is added. The enzyme-substrate reaction is a color producing reaction which is quantified in terms of optical density using a specialized spectrophotometer. This application can be used to detect previous exposure to a particular infectious disease (Wild, 2001).

Periparturient Immune Suppression

The periparturient period for a dairy cow is accompanied by many abrupt events and changes in management which provide stress during this time frame. Various management strategies are employed during the transition period in an effort to ease this major adjustment. However, the act of parturition, lactation, changes in ration and entry into the milking herd have various implications which provide mediators for immune suppression. This immune suppression has been detected in several studies (Lacetera et al., 2005; Saad et al., 1989; Detilleux et al., 1994; Park et al., 1992). This is believed to be at least partially responsible for the increased incidence of disease found soon after calving. Several studies have been conducted to identify the mediators responsible for immune suppression (Mallard et al., 1997; Lacetera et al., 2005; Kimura et al., 2006).

Neuroendocrine Effect

Neuroendocrine-immune modulation has been researched due to the identification of neuroendocrine receptors on the surface of lymphocytes as well as their ability to release neurotransmitters and hormones such as growth hormone and insulin-like growth factor-1 (Badolato et al., 1994; Blalock, 1994). Findings such as these indicate that metabolic changes induced by neuroendocrine mediators also have implications on the immune system (Besedovsky and Del Ray, 1996; Dardenne and Savino, 1996). To a certain degree immune cells resemble small pituitary glands (Von Ruecker and Schmidt-Wolf, 2000). These discoveries have sparked research aimed at determining the effect stress hormones have on lymphocyte function in periparturient dairy cattle.

Circulating levels of growth hormone (GH) has been found to have a positive correlation with antibody responsiveness to ovalbumin (OVA) measured in blood serum ($r^2 = 0.29$, $p \leq 0.001$) or milk whey ($r^2 = 0.31$, $p \leq 0.0005$) (Mallard et al., 1997). Antibody produced in

response to an *E. coli* J5 vaccination (Rhône Mérieux *E. coli* J5, Rhône Mérieux, Lenexa, KS) was also significantly correlated with GH ($r^2 = 0.18$, $p \leq 0.04$) (Mallard et al., 1997).

Insulin-like growth factor-1 (IGF-1) has been found to be negatively associated with antibody responsiveness measured in blood serum ($r^2 = -0.19$, $p \leq 0.04$) as well as milk whey ($r^2 = -0.22$ $p \leq 0.01$) (Mallard et al., 1997). Also, antibody responsiveness was significantly influenced by an interaction between week relative to calving and IGF-1 concentration ($p \leq 0.005$) (Mallard et al., 1997).

The correlation between cortisol concentration and antibody responsiveness has also been studied. Cortisol levels were found to be positively associated with antibody responsiveness ($r^2 = 0.17$, $p \leq 0.06$) (Mallard et al., 1997).

The relationship these classical hormones have with each other was also examined. The relationship between GH and cortisol have shown to have a direct relationship with both having maximum concentrations at calving. On the other hand, during this time frame, IGF-1 concentrations were at a minimum, yielding a negative correlation. GH and cortisol levels decreased in the weeks following calving, while IGF-1 concentrations increased until peak lactation (Mallard et al., 1997).

Effect of Negative Energy Balance

Nonesterified fatty acids

Periparturient dairy cows at or near the onset of lactation, frequently undergo negative energy balance, which simply means, the energy requirements for milk production and final stages of calf development prepartum, exceed energy intake (Adewuyi et al., 2005). Dairy cows tend to amplify this condition by frequently exhibiting a reduction in dry matter intake beginning in the days prior to calving. To compensate for the deficit in energy, adipose tissue lipolysis occurs which produces free fatty acids in the blood called non esterified fatty acids (NEFA)

(Adewuyi et al., 2005). Research has discovered that overconditioned cows not only lost significantly more body condition when compared to medium or thin conditioned cows, but also had significantly higher NEFA concentrations (Figure 2-3) (Lacetera et al., 2005). Periparturient dairy cow body condition and blood NEFA concentration is negatively correlated with lymphocyte function as measured by reductions in peripheral blood mononuclear cell (PBMC); DNA synthesis, immunoglobulin M (IgM) secretion (Figure 2-4), and interferon-gamma (IFN-γ) secretion (Figure 2-5) (Lacetera et al., 2005, 2004). In this study body condition was a binary trait with overconditioned cows in one group while medium and thin conditioned cows in the other. The significance of NEFA and/or body condition with lymphocyte function is generally only found in overconditioned cows. At the time of this particular work it was speculated that for periparturient dairy cows, alterations in lymphocyte function may proportionally relate to loss in body condition as assessed by changes in BCS.

Effect of hyperketonemia

The common state of negative energy balance for periparturient dairy cows also is a predisposing factor for development of hyperketonemia, a condition whereby levels of ketone bodies are elevated in the blood. The production of milk for today's dairy cow requires large demands for glucose. To meet this demand during a time of suppressed dry matter intake, dairy cows undergo intense gluconeogenesis. It is during this time where a large portion of serum NEFA is directed to the liver which is then synthesized into ketone bodies. The serum ketones found in cattle are acetone, acetoacetate, and β-hydroxybutyrate (Baird, 1982). Several studies have reported suppressed immune responsiveness associated with the presence of elevated ketone bodies (Franklin et al., 1991; Hoeben et al., 1997). However, other studies do not replicate this antagonistic relationship with the immune system (Nonnecke et al., 1992).

Effect of Hypocalcemia

Calcium plays a critical role in the activation of immune cells. Immune cell activation involves signal transduction pathways which involve inositol 1,4,5-trisphosphate binding to receptors on the endoplasmic reticulum (ER) which in turn stimulate the release of calcium ions (Ca^{2+}) into the cells cytoplasm (Grafton and Thwaite, 2001; Lewis, 2001). The level of the resulting rise in intracellular Ca^{2+} has been used as a measure of immune cell responsiveness (Partiseti et al., 1994; Baus et al., 1996). Also, an in vivo study in rats showed that extracellular fluid calcium level is a primary indicator for intracellular calcium status (Mailhot et al., 2000).

It is known that for a dairy cow in the periparturient period, the demands for calcium and risk of hypocalcemia greatly increase as production of colostrum and milk initiates. Because calcium is critical to immune cell activation, it has been hypothesized that the increased demands for calcium may unfavorably affect intracellular calcium levels, which in turn could affect immune cell activation potential. Also, periparturient cows who have undergone mastectomy, do not develop hypocalcemia, and more importantly, do not encounter the same degree of immune suppression as lactating periparturient cows (Goff and Kimura, 2002; Nonnecke et al., 2003).

Researchers have discovered that calcium levels in the blood as well as calcium levels stored in the ER decline in the days up to calving. They also found serum calcium levels were significantly correlated with the intracellular Ca^{2+} response as well as Ca^{2+} stored in the ER (Kimura et al., 2006). Due to the tremendous production of milk and its demand for calcium, these research findings substantiate the claim that cows can be at least partially immune suppressed during the periparturient period due to deficiencies in calcium.

Lactogenesis Effect

It has been hypothesized that immune suppression around calving is partly due to the sequestering of available systemic immunoglobulins (Ig) into the mammary gland for colostrum

and milk. This has also been theorized to explain the differences between high and low antibody responders to ovalbumin, where the low responders are low due to increased sequestering of Ig into the mammary gland. This theory was challenged when Wagter et al. (2000) compared the antibody response to OVA in serum to that in whey. For the theories to be supported by a correlation analysis, a negative or inverse relationship should be revealed, however, correlation analysis indicated a positive significant relationship within each test herd (Herd 1, $r = 0.45$, $p < 0.0001$; Herd 2, $r = 0.28$, $p < 0.001$; Herd 3, $r = 0.44$, $p < 0.001$).

Dexamethasone

Dexamethasone is a synthetic glucocorticoid which is commonly used to initiate parturition in cows within the last 30 days of gestation. This prepartum use provides consistent highly effective results; however, it has also been consistently associated with increased risk of retained fetal membranes (Beardsley et al., 1976; Peters and Poole, 1992). Also, several studies have identified a direct immunosuppressive activity to measures of innate as well as adaptive immunity which are associated with the use of this glucocorticoid (Burton et al., 1995; Burton and Kehrli, 1995, 1996).

Disease Trend

Associated with breeding for increased milk production without regard for immune responsiveness, a concomitant rise in infectious disease occurred (Emanuelson et al., 1988; Harmon, 1994; Wagter et al., 2003). For mastitis, Nordic data reveal that genetic correlation with milk production ranges between 0.24 and 0.55 with an average of 0.43 when large field data sets are analyzed (Heringstad et al., 2000). After assuming a conservative genetic correlation between mastitis and milk production of 0.30; Strandberg and Shook (1989) state that under traditional progeny testing programs without selection for mastitis, a genetic increase of 0.02 cases of

mastitis per cow per year is the result. So for every 100 cows, there would be a genetic increase of 2 cases of mastitis per year.

The mediators behind the correlation between production and mastitis have not been fully determined. Detilleux et al. (1995) used 137 periparturient cows when they found selection for high milk yield did not produce genetic lines with unfavorable measures for innate and adaptive immune function. However, this study may not have been able to adequately reflect mediators for immune suppression which may bridge the gap between clinical mastitis and milk yield. The effect of NEFA (Lacetera et al., 2005), hyperketonemia (Suriyasathaporn et al., 2000) and calcium (Kimura et al., 2006) are three previously described mediators for immune suppression that can also correlate with milk production. As increased milk production per cow is achieved, demands for energy and calcium also increase. This increase in demand for both energy and calcium could make the potential for a deficiency in both energy and calcium an ever increasing risk. Since a deficiency in energy can lead to the production of both NEFA and ketone bodies, and both can suppress the immune system. Also, a deficiency in calcium inhibits immune cell activation which also suppresses immune function (Kimura et al., 2006); it can be hypothesized that selection for increased milk production without regard for immune responsiveness contribute to the mediators of immune suppression. The resulting increase in occurrence of immune suppression can be at least partly responsible for the increased risk of disease.

Selection for Disease Resistance

The concept of breeding animals for disease resistance is not new. Selections against specific genes responsible for disease have provided means to prevent or reduce the risk of a given condition and have emphasized the effect genetics plays in disease resistance. Selection for disease resistance is most effective when the given condition is associated with a single or small number of genes. In poultry, selections against specific genes responsible for Marek's disease

have made great strides (Cole, 1968). In dairy cattle, identification of a genetic defect responsible for bovine leukocyte adhesion deficiency (BLAD) has drastically reduced the risk of this condition (Kehrli et al., 1990). In sheep, the association between a certain genotype and natural scrapie risk has been studied (Hunter et al., 1997). In swine, certain genes associated with risk of salmonella and E. coli diarrhea has been studied (Edfors-Lilia et al., 2000).

Several different methods have been developed to select for disease resistance. Some of these methods are concerned with reducing the incidence of a specific condition (Nash et al., 2000) or pathogen, while others are more broad-based in their approach and work to reduce the incidence of many related conditions (Wagter et al., 2000). Generally speaking, more broad-based approaches tend to have slower genetic progress for a specific condition, but during that time frame the genetic progression is favorable for a larger spectrum of conditions.

Direct Versus Indirect Selection

In certain instances, selection for disease resistance is accomplished by directly selecting against the disease itself. Using this approach, one study compared mastitis frequencies of progeny from the best bulls for mastitis resistance to the progeny of the worst bulls (Steine, 1996). In this research, they found the three worst bulls for a mastitis resistance index had daughters with twice the mastitis frequency of daughters of the three bulls with the best index values. This type of selection requires an extensive database with records of disease occurrence in the pedigree of a given animal.

Other methods of genetically selecting for disease resistance in dairy cows have involved the use of indirect traits (Nash et al., 2000; Heringstad et al., 2006; Wagter et al., 2000). Because there is not a national database for Holstein disease occurrence in the United States, it becomes impossible to directly select against particular infectious diseases. So identification of an

effective indirect trait is required. For this to be useful, it must be correlated with the phenotype of interest (disease resistance), it must also be easy to measure and heritable (Kelm et al., 2001).

Disease Resistance Through Artificial Insemination

Somatic cell count

In dairy cows, the widespread use of artificial insemination has enabled producers the ability to select sires based on predicted transmitting abilities (PTA) for phenotypic traits associated with disease resistance. Somatic cell score (SCS) is a selection trait available for dairy producers using artificial insemination and is simply a logarithmic transformation of somatic cell count (SCC). Elevations in SCC are seen as a response to microbial infestation in the mammary gland and are reported as the number of leukocytes present in 1 mL of milk. Thus, it can not only be used to identify the presence of microbes in the mammary gland but it can also to a certain degree be a potential measure of innate immune response to infection. Selection for reduced SCS is considered because of the correlation between SCC and clinical mastitis. The association between SCC and clinical mastitis has been extensively researched (Nash et al., 2000; Rogers et al., 1998; Heringstad et al., 2006). Several studies cite strong correlations between SCC and clinical mastitis (Heringstad et al., 2006; Nash et al., 2000). Nash et al (2000) cites an average genetic correlation of 0.71 over six studies between SCC and clinical mastitis. Heringstad et al. (2004) cites a range in genetic correlation dependent on phase of lactation ranging from 0.37 to 0.73.

The exact genetic relationship between selection for lower SCS and clinical mastitis is not completely understood. Because SCC reflects the amount of leukocytes in milk, and this can also be an indicator for innate immune responsiveness to microbial infestation; there is a belief that genetically selecting for reduced SCS in ‘healthy’ cows may also be concurrently selecting for reduced immune responsiveness. Previous research found that milk SCC prior to experimental

challenge with *S. aureus*, was actually higher in cows that resisted infection compared to those who became infected (Piccinini et al., 1999; Schukken et al., 1999). However, there is substantial disagreement with this philosophy. Two studies found that high immune responders, determined by antibody responsiveness to ovalbumin, had significantly lower LS means for SCC than low responders (Wagter et al., 2000; Mallard et al., 1997). Additionally, Kelm et al. (1997) found a tendency for greater functional ability of neutrophils from cows with lower estimated breeding values (EBV) for SCS during the periparturient period.

In light of the concerns posed by Piccinini et al. (1999) and Schukken et al. (1999), recent work has concluded that SCS should be considered a heterogeneous trait with SCC of healthy cows separate from SCC of mastitic cows (Heringstad et al., 2006). This study found the heritability of SCS in healthy cows to be 0.08, while the heritability in mastitic cows equals 0.03.

Structural traits of the udder and productive life

Udder conformational traits have also been used to help select for resistance to clinical mastitis infection. Udders with good attachment and cleft provide greater distance between the teat canal and the ground, or other potential fomites. Also, proper conformation can be associated with proper function and use of milking machines, which can also provide an avenue for microbial intramammary infiltration. PTAs' for traits such as; udder cleft, udder depth, rear udder height, rear udder width, and fore udder attachment have been found to be statistically significant predictors for clinical mastitis risk (Nash et al., 2000).

The use of PTAs' for SCC and udder conformational traits has provided some means to genetically select for disease resistance. However, these traits only associate with infections of the mammary gland, and for the case of selection for reduced SCS, we are still unsure if we are also unintentionally selecting for an unfavorable reduction in immune responsiveness.

PTAs' for productive life have also shown to be significant predictors of clinical mastitis infection. Productive life is a measure of the "stay ability" or the ability of the daughters for a particular sire to resist being culled. Selection for this trait has a very crude application to disease resistance especially when you consider the array of potential reasons for being culled. However, productive life has been found to be significantly associated with clinical mastitis (Nash et al., 2000).

Disease Resistance Through Specific Attributes of the Immune System

Lymphocyte subsets

Alterations in populations of T lymphocyte subsets have led to speculation about a potential role in predicting disease resistance. One study found a special correlation between features of T lymphocyte populations and the periparturient period. During this period of typical immune suppression, mammary gland secretions contained fewer numbers of T-lymphocytes and the ratio for subsets CD4:CD8+ was less than 1 (Park et al., 1992). Special attention was then placed on the ratio of T-lymphocyte subsets and their ability to predict disease. Additional studies have found that the CD4:CD8 ratio in mammary gland secretions was lowest in cows with *Staphylococcus aureus* mastitis (Park et al., 1993; Sordillo et al., 1991). Park et al. (1993) found that responsiveness to antigen by CD4+ T lymphocytes was incrementally reduced with increasing presence of CD8+ T lymphocytes. Park et al. (2004) found that mastitis susceptible cows had CD4:CD8 ratios of less than 1 in mammary gland secretions as well as peripheral blood.

Major histocompatability complex

Another arena for exploration involves the identification of specific MHC haplotypes or gene alleles which are associated with favorable measures of immune function. This could potentially serve as a genetic marker for disease resistance. The biological relevance stems from

the previously discussed role of MHC in antigen presentation to T lymphocytes. The capacity to adequately present antigen is fundamental to mounting an effective immune response. Bovine MHC is also referred to as the bovine leukocyte antigen (BoLA) and is encoded by highly polymorphic genes. Several studies have found significant differences in immune responsiveness and resistance to disease with alterations in class I and II MHC haplotypes and gene alleles (Rupp et al., 2007; Park et al., 2004; Aaerestrup et al., 1995; Rupp and Didier, 2003; Kelm et al., 1997). A recent study by Rupp et al. (2007) looked at the alleles for the DRB3.2 locus. This is the location encoding the MHC class II antigen binding site, making this region highly polymorphic. This study found several associations between different alleles and measures of immune function, disease resistance, and performance.

Broad-Based Immune Responsiveness

Selection for improved immune responsiveness against disease has been studied in poultry (Soler et al., 2002; Heller et al., 1992; Kean et al., 1994), swine (Mallard et al., 1998), sheep (Woolaston and Baker, 1996), mice (Biozzi et al., 1979) and Holstein cattle (Mallard et al., 1997; Wagter et al., 2000; Hernandez et al., 2003). Selecting for increased immune responsiveness is to provide an indirect trait that potentially correlates with broad-based disease resistance. Along with the previously mentioned mediators for immune suppression which affect immune responsiveness, there appears to be a significant genetic effect which determines the magnitude of a particular cow's immune responsiveness. Significant variation exists in the ability of periparturient dairy cows to mount an immune response indicating that not all cattle experience the same degree of immune suppression around calving (Mallard et al., 1997; Wagter et al., 2000).

The concept of selecting for broad-based disease resistance as opposed to resistance to particular pathogens/diseases is appealing. It is true that selection against specific conditions will

generally provide the quickest genetic progress for that particular condition. However, this often results in little or no genetic progress for other conditions. Also, due to the mechanics of the immune system, simply selecting for resistance to one condition without regard for other diseases, may introduce susceptibility to other conditions (Biozzi et al. 1979; de Vries, 1995; Rupp et al., 2007).

The principles behind the decision to select for broad-based disease resistance are as follows: The cow relies on the immune system as the principle means to which pathogens are fought. Eradication of environmental infection-causing organisms is impossible, so cows are going to be exposed to a wide variety of pathogens. Through mutations, these organisms are capable of altering their virulence and defense mechanisms including resistance to antibiotics. Selection for increased immune responsiveness should reduce the dependency on vaccines and antibiotics. Increases in antibody titers to vaccinations should result in more efficient use of vaccine dosage and potentially a reduction in vaccine dosage (Wagter et al., 2000; Mallard et al. 1997). Selecting for increased immune responsiveness should take steps toward addressing the concerns consumers have for excessive use of antibiotics and animal welfare issues. Through increased immune responsiveness and a reduction in mastitis occurrence, SCC should also concurrently reduce (Mallard et al., 1997; Wagter et al., 2000) which is associated with increased cheese yield, shelf-life, and dairy product quality (National Mastitis Council, 1996).

Differences between high and low immune responders

Biozzi (1979) made some interesting discoveries while studying the differences between mice bred for high and low antibody responsiveness. This study found that there was no difference in the amount of antibody released from individual plasma cells of the high and low responders. However, they found the high responders did multiply and differentiate at a significantly faster rate than low antibody responders. They found that antigen was catabolized at

a significantly faster rate in the low responders when compared to high responders. The rationale explaining this finding is that slower catabolism of antigen is associated with slower antigen processing and prolonged antigen presentation. This extended antigen presentation is associated with greater lymphocyte stimulation and activation.

Correlation with infectious disease risk

Several studies have researched the correlation between immune responsiveness and disease incidence. These studies can also be used to further understand the individual aspects of the immune system and their particular role in the prevention of a given condition.

While breeding mice for high and low antibody responsiveness, Biozzi et al (1979) naturally found that his high antibody responder line was more resistant to extracellular pathogens. However, the high antibody responders were more susceptible to intracellular pathogen. This is believed to be due to the slowed intracellular catabolism of antigen associated with the high antibody responders. When concerned with intracellular immunity, it is the speed at which the cell is able to break down the antigen which positively reflects the potential of intracellular immunity.

In swine, high immune responders as assessed by measures for AMIR, CMIR, and innate immune function, were found to have considerably less peritonitis and pleuritis following a *Mycoplasma hyorhinis* infection (Mallard et al., 1998). However, it was also noted the high immune responders as assessed by estimated breeding values (EBV) for AMIR, CMIR, and innate immune function had more arthritis than the low responders. This is believed to be the result of selection for increased cell-mediated responsiveness and its association with the inflammatory response.

Mastitis occurrence within antibody response categorization has been studied in Holstein dairy cows. Although not statistically significant, mastitis incidence for the high responders was

lowest in 2 of the 3 study herds. In these two herds the high responders did not have an incidence of mastitis. There was also reason to question the validity of the herd having more mastitis in the high responders. In this herd, all cases of mastitis were in first parity heifers. The incidence of mastitis is generally higher for multiparous cows compared to primiparous. Relating to mastitis, two studies have also looked at the correlation between antibody responsiveness to ovalbumin and antibody response to the *E. coli* J5 vaccination (Rhône Mérieux, Lenexa, KS) (Mallard et al., 1997; Wagter et al., 2000). In both instances they found that the correlation between antibody titers to the *E. coli* J5 vaccine and response to ovalbumin were positive and significant. Wagter et al. (2000) reported a general correlation of $r = 0.56$ ($p < 0.0001$). The *E. coli* J5 vaccination has been proven to be associated with reduced SCC, reduced time for clearance of *E. coli* in milk, and less milk production loss following intramammary challenge (Wilson et al., 2007). Because of this efficacy there is true biological relevance in selecting for increased immune responsiveness to ovalbumin.

Mallard et al. (1997) also compared the incidence of disease between high and low AMIR cows. In this study their main focus was the effect of cortisol, GH, and IGF-1 on antibody response profiles. However, they also looked at disease occurrence over the 3 categorizations for antibody response. They found that disease incidence was smallest for high responders (group 1). This finding also followed the pattern of high <medium (group2) < low (group3) for incidence of disease.

Correlation with energy-related metabolic disease

Several studies have cited links between risk of infectious disease and risk of metabolic disorders (Curtis et al., 1985). Often times in these studies, causality is not established. For the case of one condition (metritis) causing the other condition (ketosis), one might use the example of a postpartum cow with metritis who as a result reduces feed intake. This results in negative

energy balance and the development of ketosis-fatty liver complex. Another explanation for this association is provided through Lacetera et al (2005) and Suriyasasathaporn et al. (2000). Because the presence of NEFA and ketone bodies is closely linked with negative energy balance and energy-related metabolic conditions (ketosis); it is possible that the presence of NEFA and ketone bodies serve to suppress immune function which increases the risk for infectious disease (Figure. 2-2).

The discovery of elevated NEFA concentrations in response to inflammatory agents (Steiger et al, 1999; Kushibiki et al., 2003) has sparked research to determine if early lactation mastitis can cause ketosis-fatty liver complex in dairy cows (Waldron et al, 2006). However, this work concluded that the results do not indicate mastitis to be causal for energy-related metabolic disorders. Instead, they did suggest the possibility for a potential protective effect by the immune system on metabolism during early mammary infection.

In some instances, research studying immune responsiveness reveals a relationship with a disease previously not understood to be correlated with the immune system. Schukken et al. (1988) studied the relationship between an infectious disease (mastitis) and retained fetal membranes. This study revealed that cows having a retained fetal membrane were more likely to have a case of mastitis shortly after calving. Retained fetal membrane is a condition which is now understood to be the result of a faulty immune response. The body must be able to identify the placenta as foreign and mount an appropriate immune response against the placenta soon after parturition. Research has demonstrated that neutrophil chemotaxis as well as killing ability is impaired in both pre and postpartum cows that will/had a retained placenta (Kimura et al., 2002). Because of this, it is possible that the prepartum use of dexamethasone and its association with increased risk of retained fetal membranes might be partially explained by the

immunosuppressive action of dexamethasone (Burton et al., 1995; Burton and Kehrli, 1995, 1996).

We do not exactly know the precise magnitude or the role the immune system components have on many metabolic activities. The complexity of the immune system *in vivo* often times provide unpredicted results during research study. The possibility of the immune system serving a substantial role in body metabolism is considered plausible.

Correlation with milk production

Selection for improved immune responsiveness should yield a trend toward reduced incidence of disease. Although this strategy provides many benefits for animal welfare, the extent to which this philosophy is adopted will be strongly influenced by economics. A decrease in disease occurrence will provide obvious financial benefits through a reduction in treatment costs and also a reduction in milk loss from disease or treatment of disease. However, there is considerable speculation that any economic benefits observed may be counteracted by decreased performance. In other species, the energy and nutritive demands of a superior immune system have been shown to reduce performance. The energy and nutrients required for maintenance and activation of a responsive immune system could otherwise be used for other phenotypic traits (Klasing et al., 1987, 1998; Soler et al., 2003). Concerning dairy cows, the positive correlation between milk production and clinical mastitis (Emanuelson et al., 1988; Harmon, 1994) provides some evidence that financial benefits may be off-set by a reduction in milk yield.

Along with the previously cited avian study, additional research has been conducted to study the ramifications of superior immune responsiveness with performance. In swine, Mallard et al. (1998) found that growth performance was consistently significantly greater for high immune responders when compared to both the low immune responders and the control group. In this research, pigs were selectively mated for high and low immune responsiveness over the

course of eight generations using measures for innate and adaptive immunity (AMIR & CMIR). The trend of improved rate of gain for the high immune responder line compared to the rest was first identified in generation 0 and continued through generation 7.

In Holstein cows, Wagter et al. (2003) looked at milk production within high, medium, and low AMIR categorizations. Because parity significantly contributed to variation in 305 day milk yield, they conducted their analysis within parity. For first parity cows the low responders produced significantly more milk than the medium and high responders. However, for second parity cows, there was no statistical difference between the high and low as well as high and medium response groups. For third parity cows, the high responders produced significantly more milk than the low and medium response groups (Wagter et al., 2003). Another study showed that dairy cows genetically selected for high milk yield over seven generations did not produce unfavorable measures for innate and adaptive immune function when compared to cows selected for average milk production (Detilleux et al., 1995).

The findings of these studies indicate that although there is an association between selection for increased milk yield and increased risk of clinical mastitis, selection for improved immune responsiveness should not predispose cows to reduced milk yield. It also indicates adequate variation in immune responsiveness among sires with high PTA for milk yield to support selection for both increased milk yield as well as increased immune responsiveness

Heritability of measures for immune responsiveness

The level of heritability expected during selection for measures of immune responsiveness indicate the rate in which genetic improvement can be made. If the proposed measure for immune responsiveness has low levels of heritability, the effectiveness of an indirect trait would be very limited.

The heritability of measures for AMIR and CMIR has been tabulated in pigs (Mallard et al., 1998). These calculations were configured on over 1200 observations through 8 generations of selection for high and low immune responsiveness. Heritability of AMIR was estimated to be 0.268 while CMIR heritability estimates equaled 0.163. The heritability of AMIR as measured by antibody responsiveness to ovalbumin has also been tabulated for Holstein periparturient cows (Wagter et al., 2000). These estimates of heritability ranged 0.32 to 0.64 dependent on week relative to calving. The lower value for this range (0.32) coincided with the heritability of antibody response to OVA measured at calving. Having the lowest estimate occur around calving may be explained by the various stress factors and mediators for immune suppression which are occurring during this period.

Categorizing AMIR and CMIR

Introduction

Studies which associate immune responsiveness with disease risk usually involve correlative studies associating an iddices of immunity with risk for disease. The method chosen to stimulate the immune system by which to measure immune response is of primary importance. The technique used should represent a subject's overall potential to resist disease. Methods used to categorize immune responsiveness for the purpose of selection for disease resistance should have a very general approach which gives consideration to all aspects of immune function. When considering the separate roles in the immune system for AMIR and CMIR as well as the previous work which identifies a potential inverse relationship between the two (Biozzi et al., 1979; de Vries, 1995, Rupp et al. 2007), inclusion of both branches becomes essential. Due to the potential for an inverse relationship, a failure to consider both branches upon genetic selection may result in increased susceptibility in the branch not considered.

Antibody-mediated immune response

There are two different techniques that have previously been reported to categorize AMIR in Holstein cows. In both instances, cows were categorized during the periparturient period with ovalbumin as the test antigen to elicit the humoral response. The study cows were injected with the antigen at week -8, week -3, and week 0 relative to calving. Blood samples to measure the ensuing antibody response were collected on week -8, week -3, week 0, week +3, and week +6. Antibody response was detected by ELISA and the resulting OD values were used to categorize AMIR function (Wagter et al., 2000; Mallard et al., 1997). However, these studies differ in how AMIR categorizations were extrapolated from the OD values.

Mallard et al. (1997) found that the sample size of 33 cows and heifers partitioned into three groups. All cows responded well to the initial antigen exposure at week -8, however it was the responses to the subsequent antigen exposures which determined their categorization. High responders had an above average response to all three antigen administrations. The medium responders responded well to both of the prepartum antigen administrations, yet responded poorly postpartum to the week 0 injection. The low responders mounted poor pre and postpartum responses to the week -3 and week 0 administrations of antigen.

Wagter et al., (2000) categorized AMIR in 136 cows and heifers. In this study an index was generated which used the change in OD value over the intervals between the antigen injection/blood collection periods. This resultant value was then used to categorize cows as high, med, or low antibody responders. The formula for this index is as follows (Eq. 2-1):

$$y_{total} = I_1 + \beta_1 I_2 + \beta_2 I_3 + I_4 \qquad (2\text{-}1)$$

Where:

- y_{total} = total antibody
- I_1 = change in optical density (OD) between week -8 and week -3

- I_2 = change in OD between week -3 and week 0
- I_3 = change in OD between week 0 and week +3
- I_4 = change in OD between week +3 and week +6
- β_1 & β_2 = either 1.0 or 1.5

The coefficient β_1 takes on a value of 1.0 if I_2 is positive, representing a positive antibody response around parturition. If I_2 is negative, representing a lack of response around calving, β_1 takes on a value of 1.5 which serves to magnify or inflate the negative response. This rationale was also applied to β_2 concerning I_3.

To categorize immune responders, the mean and standard deviation for all the generated y_{total} values was configured. Cows were classified as high responders if they had an y_{total} value greater than the mean plus one standard deviation. A medium responder had an y_{total} value within the mean plus one standard deviation and the mean minus one standard deviation. A low responder had an y_{total} value below the mean minus one standard deviation.

Cell-mediated immune response

Methods employed to categorize cell mediated immune function have all utilized delayed-type hypersensitivity (Hernandez et al., 2005, 2003; Mallard et al., 1998). Because this reaction is mediated by T_H1 cells it is an indicator of intracellular immunity. This is the only in vivo method known which enables categorization of CMIR.

Quantification and therefore categorization of the CMIR comes from measurements taken at the antigen injection site. As previously stated, DTH reactions require previous exposure to the antigen intended to elicit the DTH response. To initiate the DTH response, the antigen is injected intradermally and double skin-fold measurements are taken to serve as a baseline. All measurements should be taken with three repetitions while using the average of the three for analysis. After 24 to 48 hours, measurements of the injection site are once again taken which

should reflect the palpable lump indicative of a DTH response. The degree to which the measurements increased can be used as an indicator of cell mediated immune responsiveness.

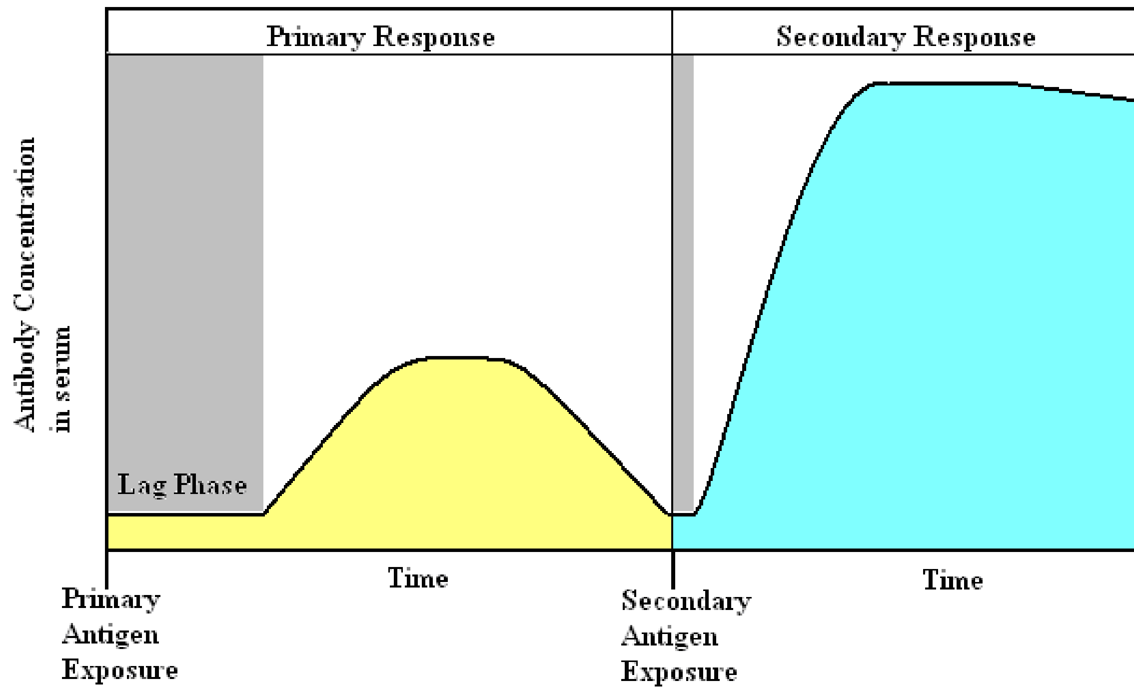

Figure 2-1. General depiction of a primary and secondary response to antigen "x". There is substantial variation in the kinetics of an antibody response due to antigen type, administration route, presence of adjuvant, and species exposed to antigen.

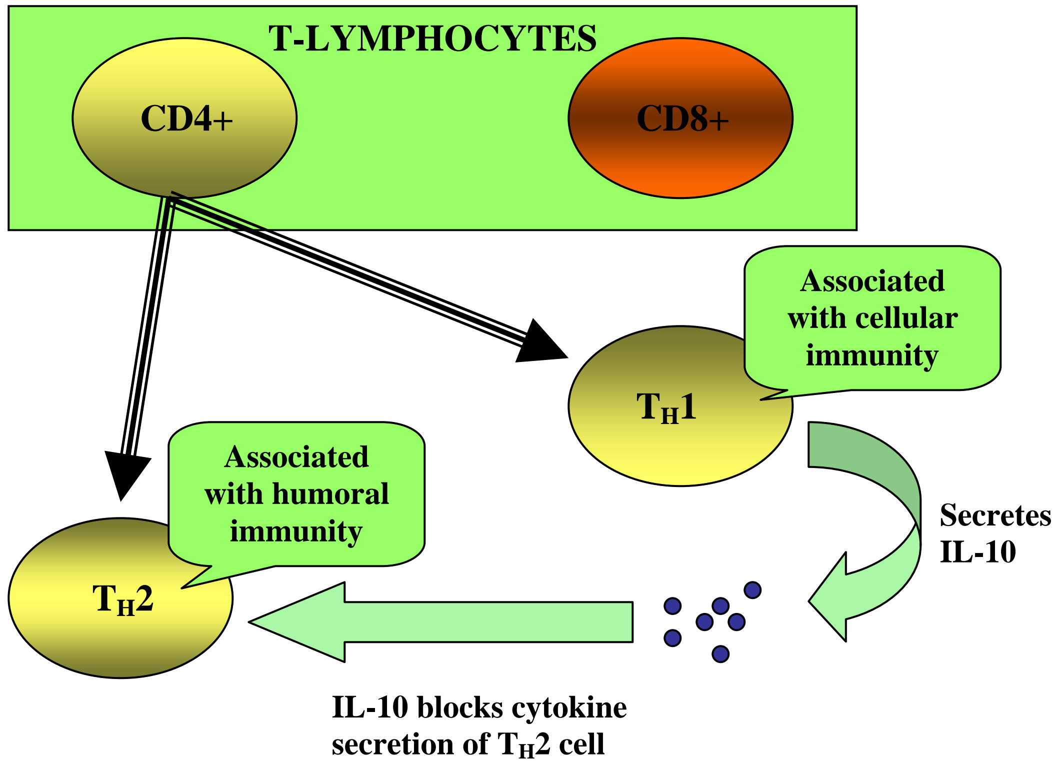

Figure 2-2. Depiction of the inverse relationship between AMIR and CMIR as a result of IL-10.

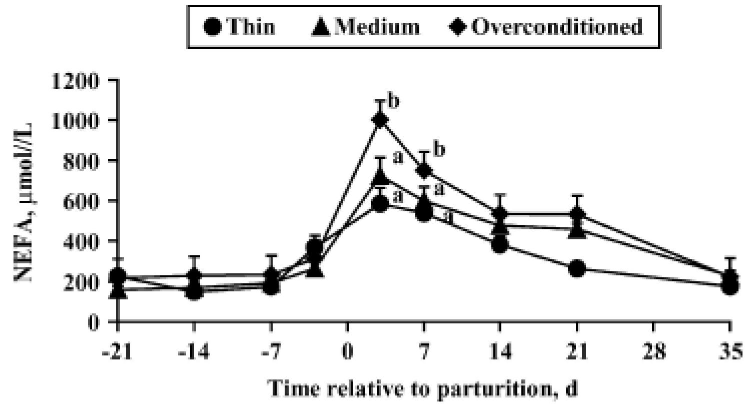

Figure 2-3. Plasma NEFA in thin, medium and overconditioned cows during the peripartum period. Values with different letters differ significantly ($P < 0.01$). Values reported are LS means ± SEM. Reprinted with permission from: Lacetera, N., D. Scalia, U. Bernabucci, B. Ronchi, D. Pirazzi, and A. Nardone. 2005. Lymphocyte Functions in Overconditioned Cows Around Parturition. J. Dairy Sci. 88:2010-2016. Figure 2, page 2012.

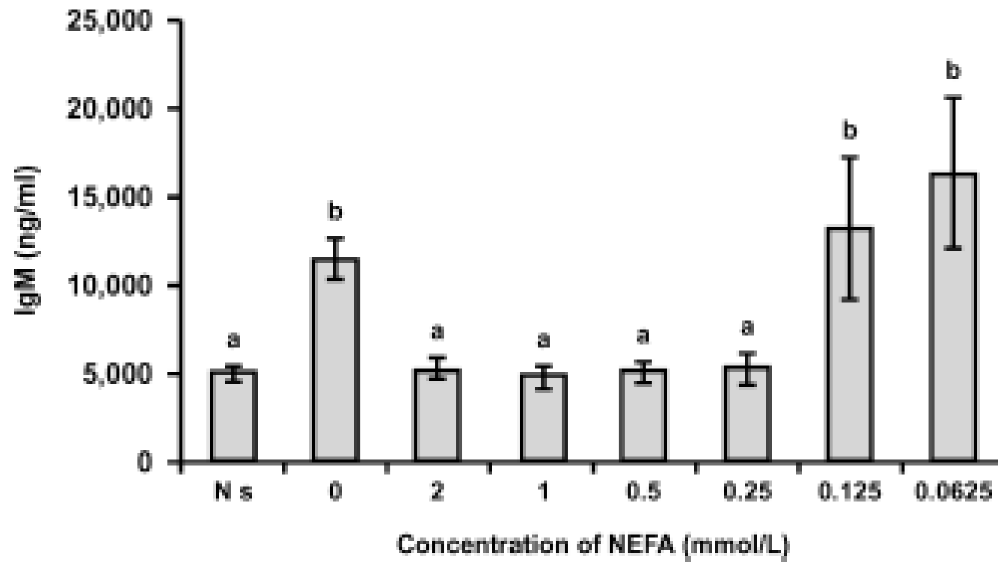

Figure 2-4. Effects of NEFA on IgM secretion in peripheral blood mononuclear cells stimulated with pokeweed mitogen. Values reported are mean ± SEM. Columns with different letters differ significantly ($P < 0.05$). Ns = Not stimulated. Reprinted with permission from: Lacetera, N., D. Scalia, O. Franci, U. Bernabucci, B. Ronchi, and A. Nardone. 2004. Short Comm: Effects of Nonesterified Fatty Acids on Lymphocyte Function in Dairy Heifers. J. Dairy Sci. 87:1012-1014. Figure 2, page 1014.

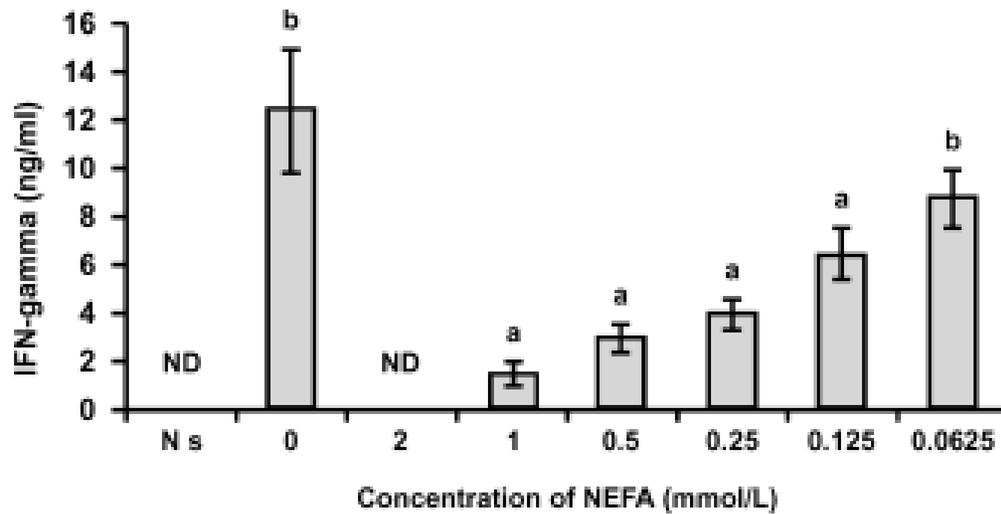

Figure 2-5. Effects of NEFA on interferon-γ secretion in peripheral blood mononuclear cells stimulated with concanavalin A. Values reported are mean ± SEM. Columns with different letters differ significantly ($P < 0.05$). Ns: not stimulated; ND: not detectable. Reprinted with permission from: Lacetera, N., D. Scalia, O. Franci, U. Bernabucci, B. Ronchi, and A. Nardone. 2004. Short Comm: Effects of Nonesterified Fatty Acids on Lymphocyte Function in Dairy Heifers. J. Dairy Sci. 87:1012-1014. Figure 3, page 1014.

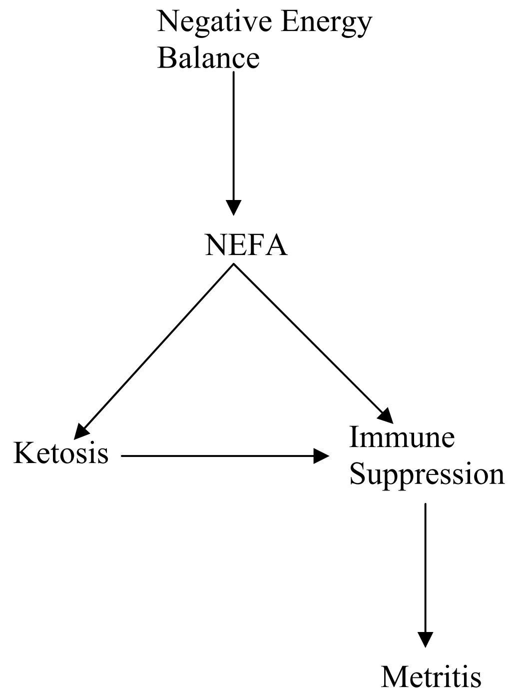

Figure 2-6. Flow chart describing potential relationship between energy-related metabolic condition (ketosis) and infectious condition (metritis).

CHAPTER 3
METHODS UTILIZED FOR THE CATEGORIZATION OF AMIR AFTER GENERATING ELISA OPTICAL DENSITY VALUES

Introduction

Enzyme linked immunosorbent assay (ELISA) is commonly used to detect the presence of a particular antigen, or specific antibody in body fluids or tissues (Wild, 2001). Previous work used the generated optical density (OD) values from an indirect ELISA as a means to quantify antibody mediated immune responsiveness (AMIR) for individual dairy cows (Mallard et al., 1997; Wagter et al., 2000; Hernandez et al., 2003). In our research we utilized these previously described methods. However, after examination of the results, it was decided that further adjustments to the OD values were required. We also found that not all cows were naïve to the test antigen. This chapter discusses the identification of cows previously exposed to the test antigen, and describes the reasoning behind the adjustments made to the OD values, and the methodology employed to make these adjustments. This chapter also discusses the alternate methods attempted to categorize AMIR and the justification for the method chosen.

Study Population

In total, 875 Holstein cows/heifers were enrolled into the study population at approximately 8 weeks (wk-8) prior to expected calving. In cows, this was the initiation of the dry period. Animals were enrolled if the expected dry period length was less than 90 days, if reconfirmed pregnant at enrollment and also if found in good health with no obvious signs of disease. All test animals were from a single herd in north central Florida which maintains exceptional record keeping. All cows and heifers were enrolled between September 9th and December 31st, 2004, calved between October 25th, 2004 and March 12th, 2005, and exited between November 9th, 2004 and March 28th, 2005. All cows and heifers received a routine dry off, prefresh, and fresh cow protocol.

Exclusion Criteria

A reduction in the study population was necessary to maintain the integrity of the study. Of the 875 cows and heifers, 13 were removed due to missing samples at one of the blood collection periods. An additional 88 were removed either because they were not naïve to test antigen or because they did not meet the interval or dry period length exclusionary criteria which will be discussed later in the chapter. A total sample size of 774 with 433 cows and 341 heifers upon enrollment were analyzed. Because measurement of AMIR concluded 2 weeks after calving, heifers will be referred to as primiparous cows and the cows at enrollment will be referred to as multiparous cows (primiparous = first lactation; or multiparous = second or greater lactation).

Removal of Cows Previously Exposed to Antigen

Ovalbumin (OVA) was chosen as the antigen to stimulate AMIR. The rationale behind this decision came from the ability of OVA to stimulate a humoral response, the low likelihood of prior exposure, and the previously successful use of this antigen as a tool to categorize AMIR in dairy cows (Mallard et al., 1997; Wagter et al., 2000; Hernandez et al., 2003). For this trial, the periods of antigen exposure and blood collection occurred at enrollment (wk-8), entry into springer pen (wk-3), and calving (wk0). An additional blood sample was collected 2 weeks after calving (wk+2) (Figure 3-1). Blood samples were collected to determine antibody response to antigen.

The OD value for wk-8 (OD_{-8}) was to serve as a covariate for the antibody response to OVA. Equal treatment for the measurement of AMIR requires that all animals are naïve to test antigen at this point. This is critically important due to the differences between primary and secondary antibody responses (Figure 2-1). If a portion of cows mount a secondary response due to previous exposure and are being compared with cows mounting a primary response, this introduces unequal treatment into your study population.

Methodology and Results

After review of the OD_{-8} values it was apparent some cows were most likely not naïve or had high nonspecific reactivity to the test antigen, so a natural cut-off point of an $OD_{-8} = 0.455$ was used. This point corresponded with 1 standard deviation above the mean for all OD_{-8} values. As a result of this analysis, 38 animals (23 primiparous and 15 multiparous) were termed "not naïve to test antigen" and were removed from the study.

Effect of Parity on Antibody Response to Ovalbumin

A repeated measures analysis using OD_{-8} as a covariate with the mixed procedure of SAS revealed that multiparous cows responded significantly higher than primiparous cows at every antibody response measurement week ($p<0.0001$). Due to this effect, adjustments to OD values as well as AMIR categorizations were all made with respect to parity (Figure 3-2). Explanations for this finding may include higher levels of stress and therefore immune suppression for younger cows experiencing lactation and parturition for the first time compared to multiparous cows. Another explanation involves the presence of a more extensive antibody repertoire in older cows. This could simply be due to the effect of time, allowing greater exposure to a broader array of pathogens. Another explanation

Interval Variation Adjustment

Defining Intervals: Antigen was injected and blood was collected at specified time frames (Figure 3-1). Variation in the number of days between these points of blood collection/antigen injection has a strong influence on the measured antibody response. These interval lengths reflect the duration between previous antigen exposure and measurement of antibody response; which is relevant due to the kinetics of an antibody response profile (Figure 2-1). In every antibody response there is a lag phase followed by a period of increasing antibody concentration up to a peak response which is followed by a steady decline in antibody concentration. Introducing

variation in the number of days between points of blood collection/antigen injection generates inconsistency in the phase of antibody response profile where antibody response was measured.

For this research, interval 1 (Int_1) was defined as the duration in days between sampling periods identified as wk-8 and wk-3. Interval 2 (Int_2) was defined as the duration in days between sampling periods, wk-3 and wk0. Interval 3 (Int_3) was defined as the duration in days between sampling periods, wk0 and wk+2.

Strictly for the purpose of monitoring the change in body condition score (BCS), additional intervals were identified. Interval 4 (Int_4) was defined as period between wk-3 and wk0 which is an indication of the change in BCS over the transition period. Interval 5 (Int_5) was defined as period between wk-8 and wk0. Also, interval 6 (Int_6) was defined as period between wk-8 and wk+2.

Interval Exclusionary Criteria: One exclusion criteria at study assignment was based on dry period length (Int_1 plus Int_2). If this period was greater than 90 days, they were removed from the study. Although this restriction consists of Int_1 plus Int_2 it does not adequately put restrictions on individual lengths for either Int_1 or Int_2. However, it can eliminate cows with various metabolic problems from skewing results.

A minimum interval length of 12 days was set for both Int_1 and Int_2. This does work to restrict specific interval lengths to a minimum, but it still leaves room for substantial variation to occur. Applying these exclusionary criteria an additional 50 animals were eliminated yielding a study population of 774.

Due to the resources available and nature of a large-scale dairy farm, considerable variation in the lengths for Int_1 and Int_2 did occur. To correct the OD values for this effect, adjustments were made to OD values based on the duration of the interval leading up to the

respective OD value. For example, adjustments to the wk-3 OD (OD_{-3}) were made based on the length of Int_1 for a respective cow.

With Int_3 concluding at a sampling period occurring after calving (wk+2) with animals managed in the milking herd, this duration was under our control which greatly reduced the variation in Int_3 lengths. No adjustments were required for the OD_{-8} values because this was the initial point of exposure.

Methods for OD Adjustment

Statistical analysis to determine the effect of interval length on OD value was performed using the GLM procedure of SAS. The OD response to be adjusted was the outcome variable while potential explanatory variables for each cow included:

- Int_Y = interval length in days for interval "y"
- BCS_X = categorization of body condition score for sampling week "x"
- ΔBCS_Y = change in body condition score over interval "y"
- OD_X = optical density value at sampling week "x"
- Dex = binary effect, whether cow received dexamethasone prior to calving
- Sick = binary effect, incidence of either medium or severe case of mastitis, metritis, ketosis, or displaced abomasum within 16 DIM. Only used for analysis to correct OD_{+2}, for reasons to be discussed later.

Explanatory variables remained in the model if the effect showed a tendency ($P < 0.10$) to predict the desired OD value. With $\alpha = 0.05$, if statistical significance was found with the main effect (Int_y), the resultant parameter estimate was applied to Equation 3-1 to correct the corresponding OD values. If a given OD value was adjusted based on interval length, the model predicting the OD value for the subsequent sampling period would have the adjusted OD from the previous sampling week.

$$AOD_{ZPX} = OD_{ZPX} + PE_{YP} * (Mint_{YP} - Int_{ZPY}) \qquad (3\text{-}1)$$

Where:

- AOD_{ZPX} = the adjusted OD value for cow "z" in parity "p" for week "x"
- OD_{ZPX} = OD value for cow "z" in parity "p" for week "x"
- PE_{YP} = parameter estimate for the effect interval "y" has on OD_x values for parity "p". Also, interval "y" must always immediately precede week "x"
- $Mint_{YP}$ = median number of days for interval "y" for parity "p"
- Int_{ZPY} = the actual interval length for cow "z" in parity "p" for interval "y"

Results OD_{-3}

The length in days for Int_1 was under considerable variation ranging from 12-55 days in primiparous cows (Figure 3-3).View of a scatter plot depicting the relationship between Int_1 and OD_{-3} values do not reveal any obvious pattern. Model effects were: Int_1, OD_{-8}, BCS_{-8}, and ΔBCS_1. For primiparous cows, Int_1 was not found to be a significant predictor of OD_{-3} values ($p = 0.34$, $\beta = -0.0015$). Because of this, no adjustments were made to primiparous cow OD_{-3} values.

The Int_1 variation for multiparous cows ranged from 21-71 days (Figure 3-4). Inspection of the scatter plot depicting this relationship revealed an obvious association. The model effects were Int_1, and OD_{-8}. There was a significant linear effect between Int_1 and OD_{-3} values ($p < 0.0001$, $\beta = -0.0081$). This parameter estimate represents the slope of the fitted line for the linear model. The median Int_1 in multiparous cows equaled 36. This parameter estimate and median was then used to generate the adjusted OD_{-3} values for each respective multiparous cow (Eq. 3-1).

Results OD_0

The range in days for Int_2 also had considerable variation. For primiparous cows, this range extended from 12 to 45 days (Figure 3-5). Model effects were Int_2, OD_{-3} ΔBCS_2, and Dex.

The variation in length of Int_2 had a significant effect on OD_0 values ($p < 0.0001$, $\beta = -0.0246$).The median Int_2 duration of days was 21. This parameter estimate and median was then used on primiparous cows to adjust the OD_0 values (Eq. 3-1).

For multiparous cows, the length of Int_2 ranged from 12-44 days (Figure 3-6). The scatter plot depiction of the relationship clearly indicates an association. This plot also clearly represents a characteristic secondary immune response with a longer duration of peak response. Model effects were Int_2, and OD_{-3}. The effect of Int_2 length significantly effected OD_0 values ($p < 0.0001$, $\beta = -0.0211$). The calculated median was 22 days. This parameter estimate and median was used to adjust OD_0 values for multiparous cows (Eq. 3-1).

Results OD_{+2}

For primiparous cows the variation in Int_3 ranged 12-21 days (Figure 3-7). For this analysis the model effects included Int_3, OD_0, and ΔBCS_4. The length of Int_3 was a significant predictor for OD_{+2} in primiparous cows ($p < 0.0032$, $\beta = -0.0298$). The calculated median number of days was 16. The parameter estimate and median was used to adjust OD_{+2} values in primiparous cows (Eq. 3-1).

For multiparous cows the range for Int_3 was 12-20 days (Figure 3-8). This model included Dex,, OD_0, and Int_3. However, Int_3.was not a significant predictor for OD_{+2} in multiparous cows ($p = 0.21$, $\beta = -0.0104$). No adjustments were made to OD_{+2} in multiparous cows.

An additional GLM model was run after the discovery that parity was not a significant predictor for OD_{+2} as determined by a linear regression model (Eq. 3-2) which is later discussed. This new model analyzed associations with OD_{+2} irrespective of parity. The remaining model effects were Sick, OD_0, and, Int_3. In this instance the model revealed Int_3 is a significant predictor for OD_{+2} ($p < 0.0044$, $\beta = -0.0182$). After consideration, this will not be used to adjust OD_{+2} values due to the added power of a repeated measure analysis in this application.

Analysis of Classification Methods

As previously discussed in chapter 2, there are two different techniques that have been previously reported to categorize AMIR in periparturient Holstein cows. In both instances, cows were categorized with ovalbumin as the test antigen to elicit the humoral response. The study cows were injected with the antigen at wk-8, wk-3, and wk0 relative to calving. The ensuing antibody response was detected by ELISA and the resulting OD values were used to categorize AMIR function (Wagter et al., 2000; Mallard et al., 1997). The response to antigen introduced at wk0 was detected on wk+3 and wk+6 postpartum.

Use of interval changes in OD: The method for the actual AMIR categorization in the publication by Wagter et al. (2000) used an index based on the change in OD response over the intervals between blood sampling/ antigen injection periods (Eq. 2-1). As previously discussed in chapter 2, this index weights those intervals (β_1 & β_2) around calving if they show a decline in OD value, which represents a decline in antibody concentration.

$$y_{total} = I_1 + \beta_1 I_2 + \beta_2 I_3 + I_4 \qquad (2\text{-}1)$$

Where:

- y_{total} = total antibody
- I_1 = change in optical density (OD) between week -8 and week -3
- I_2 = change in OD between week -3 and week 0
- I_3 = change in OD between week 0 and week +3
- I_4 = change in OD between week +3 and week +6
- β_1 & β_2 = either 1.0 or 1.5

In this study, inspection of the OD values for the respective weeks revealed a point where antibody response was at an apparent maximum. If there was an improvement after subsequent antigen injection, these maximal points in antibody concentration only slightly improved. If this peak response was achieved early in the study (OD_{-3}), which would indicate a high prepartum responder; there would be little if any room for an added response. As a result an index

concerned with weighting the change in OD during the intervals adjacent to calving (Eq. 1-1), may negatively impact the categorization of high immune responders who reached this peak response early because there was little or no room to further respond. Speculation for causation of this finding most likely involves the natural function of feedback inhibition.

Early postpartum measures of immune function: The period immediately following parturition is a common occasion for increased incidence of disease. As a result it may be hypothesized that substantial sickness could contribute to immune suppression. This effect of substantial sickness could be a confounding variable for antibody responsiveness to OVA detected early postpartum. This would make it difficult to study the effect measures of immune responsiveness have on disease risk if the association could also be in the opposing direction.

Objectives: The 2 objectives were as follows: 1) To analyze the potential for a maximal antibody response and its possible effect on antibody response categorization. 2) To study the potential for an effect of sickness on early postpartum measures of antibody responsiveness. This is performed by analyzing the relationship between OD_{+2} values and substantial sickness.

Methods

Maximal response: For this analysis, the relationship between OD value and the subsequent interval change in OD (I) was studied. This was performed for the relationship between OD_{-3} and I_2, and also between OD_0 and I_3.

The total sample size for this analysis was 774 cows. Cows were arranged according to their OD_{-3} value and grouped into one of 14 groups with 55 cows per group except for group 14 which had 59 cows. The top 55 cows for OD_{-3} became group 1 while the bottom 59 cows for OD_{-3} became group 14. This same process was also performed based on OD_0 values.

A one-tailed two sample t-test was used to determine if the OD value group had a significant effect on the subsequent interval change in OD. For this analysis, I_2 and I_3 had a

normal distribution, however in both cases, only group 1 cows could be compared to group 2 cows due to statistical differences in variance between group 1 and the rest of the groups. The null hypothesis is: H_o: Group 1 I_2 – Group 2 $I_2 \geq 0$. Rejecting the null with statistical significance means that Group 1 I_2 is significantly smaller than Group 2 I_2. A linear regression model using the REG procedure of SAS was also used to see if OD_{-3} had a significant effect on I_2, or if OD_0 had a significant effect on I_3.

Effect of sickness on OD_{+2}: For the statistical analysis a linear regression model with the REG procedure of SAS. OD_{+2} served as the outcome variable while potential explanatory variables included:

- BCS_x = categorization of body condition score for sampling week "x"
- ΔBCS_y = change in body condition score over interval "y"
- OD_0 = optical density value at wk0
- Dex = binary effect, whether cow received dexamethasone prior to calving
- Sick = binary effect, incidence of either medium or severe case of mastitis, metritis, ketosis, or displaced abomasum within 16 DIM.
- Parity = binary effect, either primiparous or multiparous

Explanatory variables remained in the model if the effect showed a tendency ($P < 0.10$) to predict the dependant OD value. For statistical significance, $\alpha = 0.05$. The Corr procedure of SAS was also used to test the correlation between OD_0 and OD_{+2} in healthy cows as well as those classified as sick within 16 DIM.

Results and Discussion

Maximal response: After sorting the OD_{-3} values from greatest to least, it was discovered that of the top 20 cows for OD_{-3} value, 15 had a smaller OD_0 value (75%), yielding a negative interval 2 change in OD. In these instances, 15 of the top 20 OD_{-3} responders would have an

amplified ($\beta = 1.5$) negative I_2 value applied to their AMIR index if using an index which weights interval changes in OD.

After grouping the OD_{-3} values from 1 to 14, with group 1 being the top 55 OD_{-3} values and group 14 the bottom 59 OD_{-3} values; this revealed a significantly smaller change in OD over interval 2 for group 1 cows compared to group 2 cows ($p < 0.0001$) (Figure 3-9). This value actually averaged below 0 (-0.05) for these top 55 OD_{-3} responders. Linear regression also revealed OD_{-3} is a significant predictor for I_2 ($p < 0.0001$; $\beta = -0.28$). A negative β indicates that as OD_{-3} values increase, I_2 values decrease.

Repeating the same process by ranking cows based on OD_0 values in order to compare interval 3 change in OD, revealed similar results. Due to missing wk+2 blood samples the sample size for this analysis was 754. As a result group 14 had 39 cows while group 1 – 13 had 55. Of the top 20 cows for OD_0 values, 15 had negative interval 3 changes in OD (75%). Also, group 1 cows based on OD_0 values had a significantly smaller interval 3 change in OD compared to group 2 cows ($p < 0.0001$) (Figure 3-10). Linear regression also revealed OD_0 is a significant predictor for I_3 ($p < 0.0001$; $\beta = -0.50$). A negative β indicates that as OD_0 values increase, I_3 values decrease.

In these instances of high OD values followed by a subsequent negative interval, the interval is not negative due to a poor subsequent OD value. Of the top 20 cows for OD_{-3}, 18 still had OD_0 values above the third quartile for the population and the other 2 were still above the median for the population. For the top 20 cows for OD_0, all 20 still had OD_{+2} values above the third quartile for the population. The negative interval was simply the result of an inability to respond further. As a result, additional indexes were generated and analyzed based on their correlation with disease incidence. This finding is likely due to feedback inhibition due to the

difficulty in boosting a subject that already has a high antibody concentration. Although OD adjustments were made to accommodate for interval length variation; a cow which experiences a secondary exposure during peak response from a previous exposure to the specific antigen will not be boosted to the degree a cow is that received the antigen after peak response due to feedback inhibition.

Effect of sickness on OD+2: For this analysis, there were a total of 754 cows, 40 of these cows were classified as “Sick” as defined within 16 DIM. Parity was not a significant predictor of OD_{+2} ($p = 0.54$); as a result, it was not included in the model and all cows were considered together (Eq. 3-2).

$$OD_{+2} = Sick + Dex + OD_0, \quad (3\text{-}2)$$

The analysis revealed that sickness, as previously defined, was a significant predictor of OD_{+2} ($p = 0.0156$). The difficulty in this analysis is proving the direction of the association. Did the occurrence of sickness within 16 DIM cause a suppression in immune responsiveness; or did inferior immune responsiveness cause sickness within 16 DIM. Because OD_0 occurs prior to the incidence of disease, and this was a significant predictor of OD_{+2} ($p < 0.0001$), you can be fairly certain the incidence of sickness had an effect on immune responsiveness.

Correlation analysis was employed to study the relationship between OD_0 and OD_{+2} at fixed levels of sickness. Among cows considered healthy within 16 DIM, correlation analysis revealed OD_0 is positively correlated with OD_{+2} ($r^2 = 0.64$, $p < 0.0001$). Furthermore, within sick cows, the correlation between OD_0 and OD_{+2} has an even greater significantly positive correlation ($r^2 = 0.73$, $p < 0.0001$).

For further understanding, an additional linear regression model was constructed. The rationale behind this analysis comes from OD_0 and OD_{+2} being positively correlated in sick as

well as healthy cows. If it was the level of immune response that caused susceptibility to sickness, the strong positive correlation should allow OD_0 to be able to serve as the outcome variable. It is known that OD_0 reflects an immune response occurring prior to the high risk period for sickness. For this analysis, parity was significant ($p < 0.0001$). As a result, this analysis was performed at fixed levels of parity.

For the 333 primiparous cows the independent variables remaining in the model were, Sick, and OD_{+2}. This analysis reveals sickness is not a predictor for OD_0 ($p < 0.387$). For the 422 multiparous cows the independent variables remaining in the model were Sick, and OD_{+2}. This analysis also reveals sickness is not a predictor for OD_0 ($p < 0.592$). For further evidence, it will be discussed later how models which do not include the +2wk antibody response data tend to have a stronger association with disease risk.

Alternate Index Methods for AMIR Categorization

Although only similar to the AMIR index (Eq. 1-1) in Wagter et al. (2000), a comparable index was generated (Eq. 3-3). This was chosen based on the utility of the previous index. In similar fashion, another index (Eq. 3-4) was derived due to the previously described effect of early postpartum sickness on immune responsiveness.

$$y_{total} = I_1 + \beta_1 I_2 + \beta_2 I_3 \qquad (3\text{-}3)$$

$$y_{total} = I_1 + \beta_1 I_2 \qquad (3\text{-}4)$$

Where:

- y_{total} = total antibody
- I_1 = change in optical density (OD) between week -8 and week -3
- I_2 = change in OD between week -3 and week 0
- I_3 = change in OD between week 0 and week +2
- β_1 & β_2 = either 1.0 or 1.5

The rationale behind two additional indexes is based on the assumption that a favorable AMIR will have greater correlation with the actual magnitude of the antibody concentration rather than changes in antibody concentration over intervals. In the case of a maximal antibody response, an index should reflect a cow's ability to maintain a high concentration of specific antibody. This should all be accomplished while also using measures which give special attention to antibody responses occurring peripartum.

The first index (Eq. 3-5) includes the postpartum OD_{+2}, while the second (Eq. 3-6) does not. In these indexes, the direct magnitudes of the OD values are considered. However, in the case of OD_0 and OD_{+2} they are still weighted, but this can be positively or negatively and only in proportion to the level of increase or decrease for I_2 and I_3. If I_2 is slightly negative, then OD_0 is multiplied by a number slightly under 1 yielding a smaller value. If I_2 is slightly positive, then OD_0 is multiplied by a number slightly over 1, yielding a larger value.

$$y_{total} = OD_{-3} + OD_0 *(1 + I_2) + OD_{+2} * (1 + I_3) \quad (3\text{-}5)$$

$$y_{total} = OD_{-3} + OD_0 *(1 + I_2) \quad (3\text{-}6)$$

Where:

- y_{total} = total antibody
- OD_{-3} = optical density value at wk-3
- OD_0 = optical density value at wk0
- I_2 = change in OD between week -3 and week 0
- I_3 = change in OD between week 0 and week +3

For each index (Eq. 3-3, Eq. 3-4, Eq. 3-5, Eq. 3-6), 2 different approaches were taken to extrapolate AMIR categorizations from the generated y_{total} values. In each approach the AMIR categorization was determined within parity due to the effect of parity on antibody responsiveness to OVA (Figure 3-2). The first approach involved calculating the mean and

standard deviation of all y_{total} values (Figure 3-11) (Wagter et al., 2000, Hernandez et al. 2003). This was performed separate for multiparous and primiparous cows. High responders were those cows with y_{total} values greater than the mean plus one standard deviation. Low responders were those with y_{total} values less than the mean minus one standard deviation. Medium responders had y_{total} values within ± one standard deviation. If dealing with a normal distribution of data, roughly 68% of the data will fall within ± 1 standard deviation of the mean. This leaves roughly 16% for high AMIR responders and 16% for low AMIR responders.

Because this method only categorizes the extreme 32 % of the population into high or low immune responders, calculations of quartiles were used. This method allowed us to set the top 25% of data as high AMIR, while the bottom 25% as low AMIR. The resulting middle 50% was classified as medium responders. In this case, the extreme 50% of the population is categorized as high or low responders. The configuration of the quartiles was also calculated within parity.

With 4 different possible equations (Eq. 3-3, 3-4, 3-5, 3-6) to generate antibody y_{total} values and 2 different methods to extrapolate AMIR classifications from each equation, an analysis of each possibility is required to determine which method is chosen. For this determination a raw association between each method and incidence of disease was used (Table 3-1).

Results

The worst association with disease is clearly Equation 3-5 using the SD classification method (Table 3-1). It appears Equation 3-3 has a closer association with resistance to mastitis and retained fetal membrane. However, Equation 3-4 and Equation 3-6 are more closely associated with resistance to metabolic conditions. These two equations do not include OD_{+2} data from early postpartum. Use of Equation 3-3, Equation 3-4, and Equation 3-6 appear to be the best options. Use of standard deviation versus use of quartiles appears negligible.

Using incidence of mastitis for an entire lactation as a binary outcome variable, logistic regression using the LOGISTIC Procedure of SAS was used to identify the equation which can be best used to predict susceptibility to mastitis. Independent variables remained in the model if they showed a tendency ($p < 0.10$) to predict mastitis incidence. The potential model effects were:

- BCS_x = categorization of body condition score for sampling week "x"
- ΔBCS_y = change in body condition score over interval "y"
- Dex = binary effect, whether cow received dexamethasone prior to calving
- Parity = binary effect, either primiparous or multiparous
- SCCavg = the average SCC collected monthly for the first 10 months
- ARC = antibody response categorization using either the standard deviation or quartile method for Equation 3-3, Equation 3-4, or Equation 3-6.

The effects which remained in the model were: SCCavg, ΔBCS_5, Parity, and ARC. Equation 3-3 was not a significant predictor for mastitis using either the standard devation ($p = 0.726$) or the quartile method ($p = 0.701$). Equation 3-4 showed a tendency to predict mastitis incidence using the standard deviation ($p = 0.116$) and quartile method ($p = 0.089$). Equation 3-5 was not a significant predictor for mastitis using either method ($p = 0.816$, $p = 0.893$). Using Equation 3-6, the standard deviation method was not a significant predictor ($p = 0.3646$), however, using the quartile method, Equation 3-6 was a significant predictor for mastitis incidence ($p= 0.028$).

The two equations revealing any ability to predict mastitis incidence did not include early postpartum (wk+2) measurements for antibody response. These methods appear to have a closer association with susceptibility to mastitis. Equation 3-6 was the only equation with a significant ability to predict mastitis. This is also the only equation which alleviates concerns about antibody

saturation and the effect of early postpartum sickness. In light of these findings, in an effort to streamline the method in which AMIR categorization is accomplished, Equation 3-6 using the quartile method will be used from here on out.

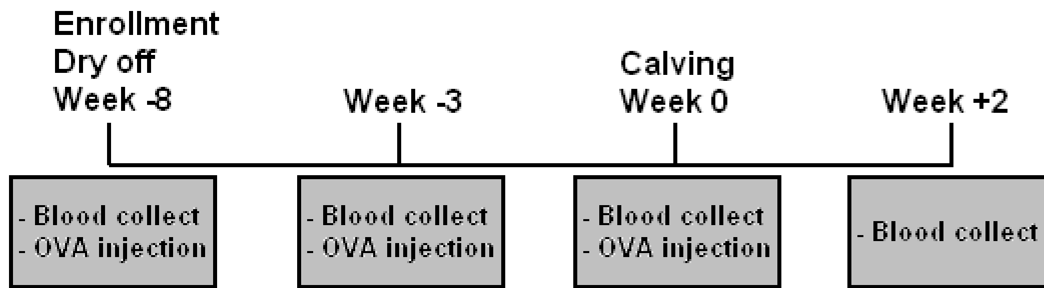

Figure 3-1. Basic outline for treatment/ sampling for antibody mediated immune responsiveness.

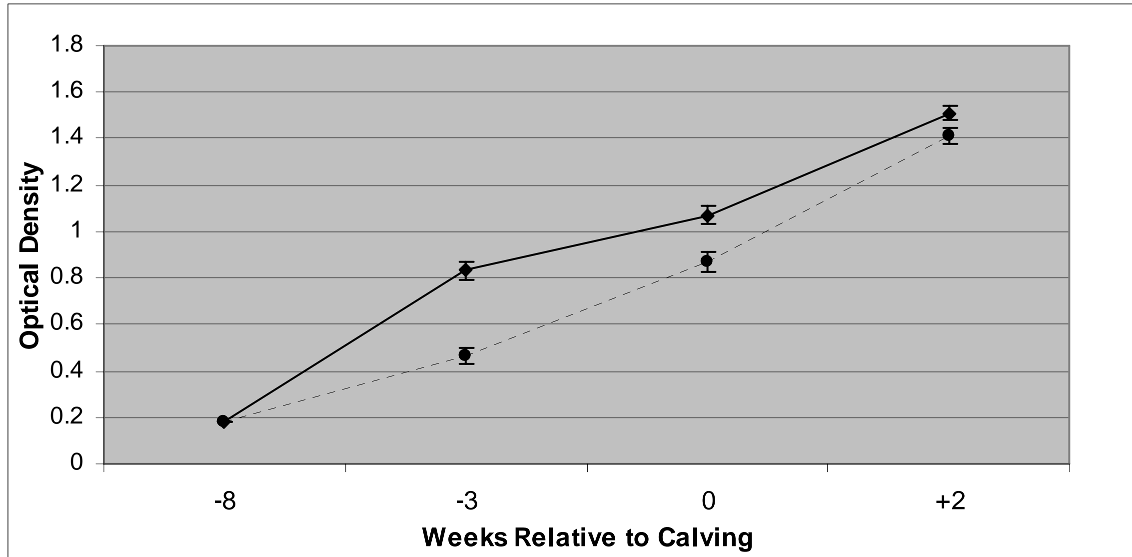

Figure 3-2. Optical density values reflecting antibody response to OVA by sampling period separated by parity. A) Solid line is for multiparous cows. B) Dotted line is for primiparous cows. Bars indicate 95% confidence intervals.

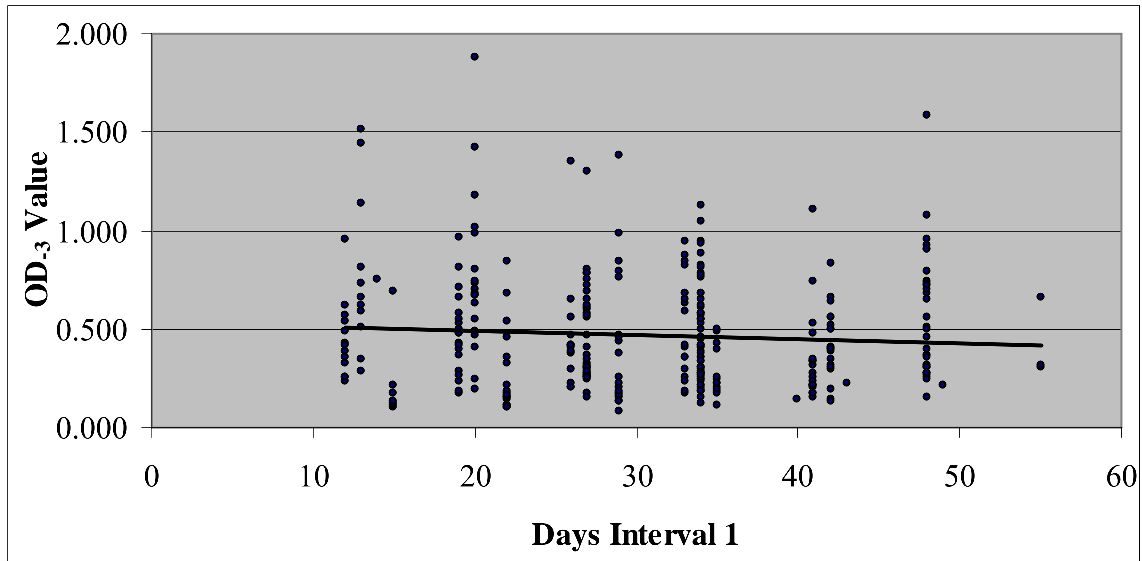

Figure 3-3. Scatter plot of OD_{-3} values by length of interval 1 for primiparous cows. Fitted line not significant ($p = 0.34$, $\beta = -0.0015$).

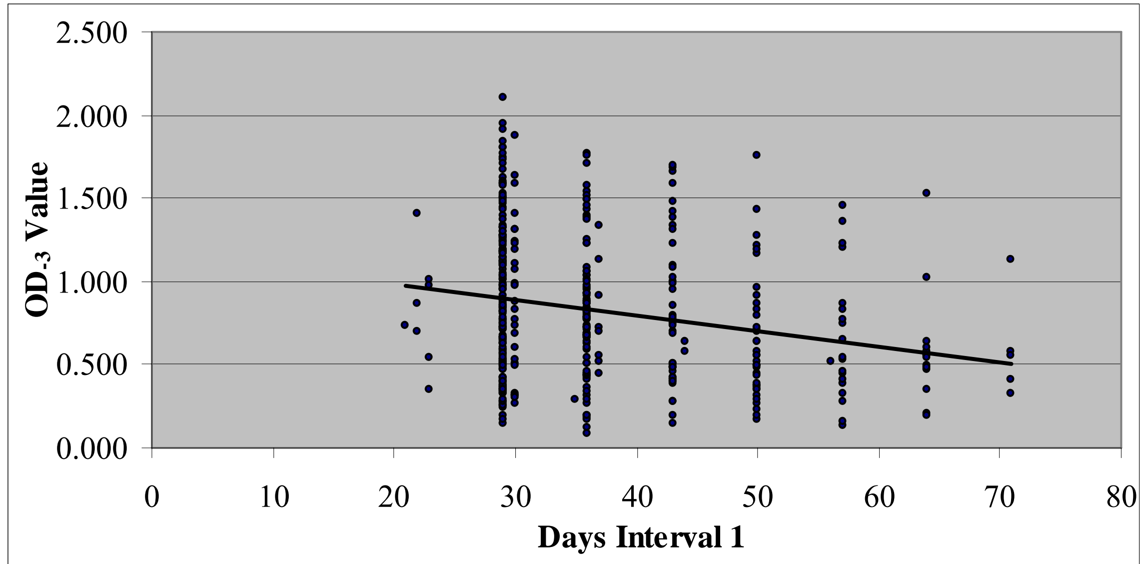

Figure 3-4. Scatter plot of OD_{-3} values by length of interval 1 for multiparous cows. Fitted line is significant ($p < 0.0001$, $\beta = -0.0081$).

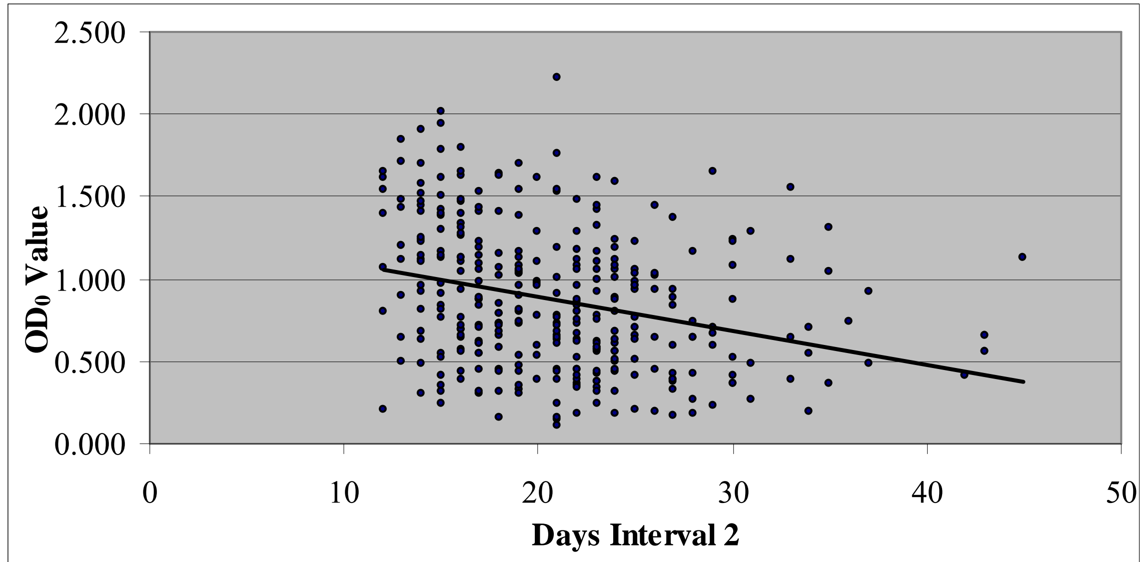

Figure 3-5. Scatter plot of OD_0 values by length of interval 2 for primiparous cows. Fitted line is significant ($p < 0.0001$, $\beta = -0.0246$).

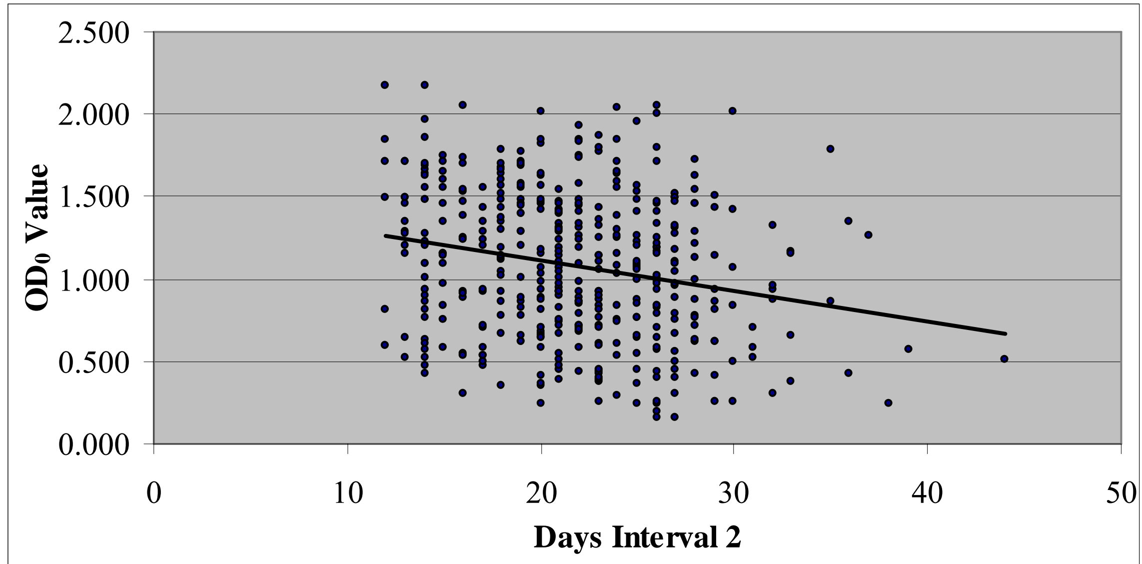

Figure 3-6. Scatter plot of OD_0 values by length of interval 2 for multiparous cows. Fitted line is significant ($p < 0.0001$, $\beta = -0.0211$).

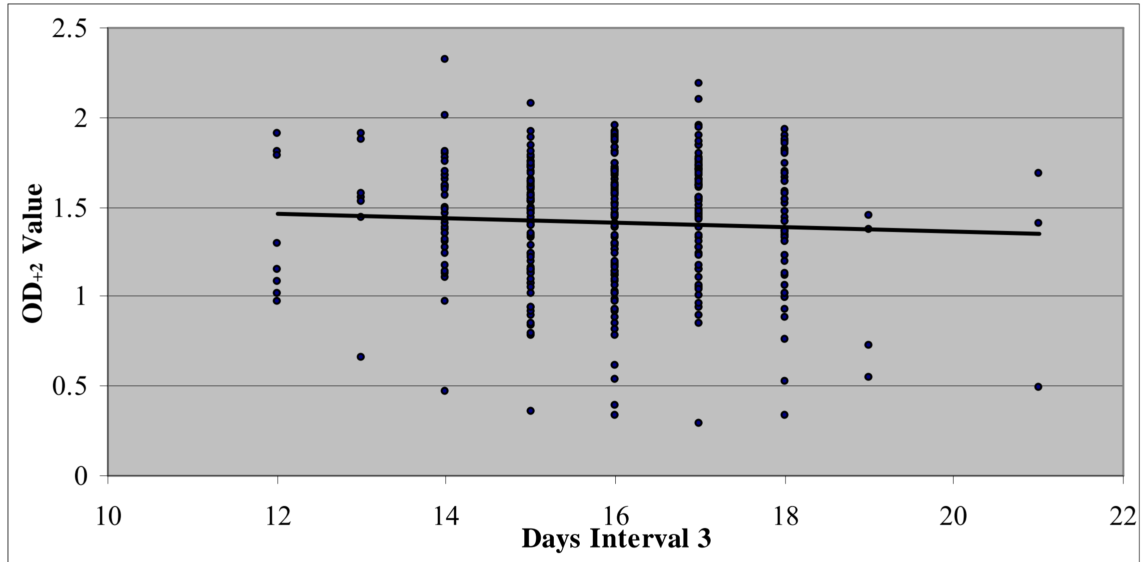

Figure 3-7. Scatter plot of OD_{+2} values by length of interval 3 for primiparous cows. Fitted line is significant ($p < 0.0032$, $\beta = -0.0298$).

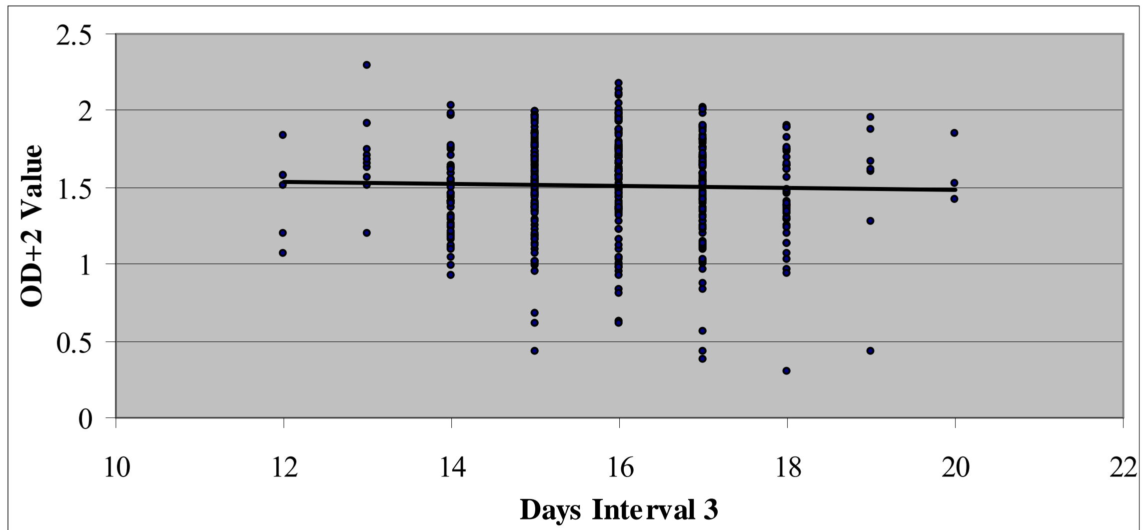

Figure 3-8. Scatter plot of OD_{+2} values by length of interval 3 for multiparous cows. Fitted line is not significant ($p = 0.21$, $\beta = -0.0104$).

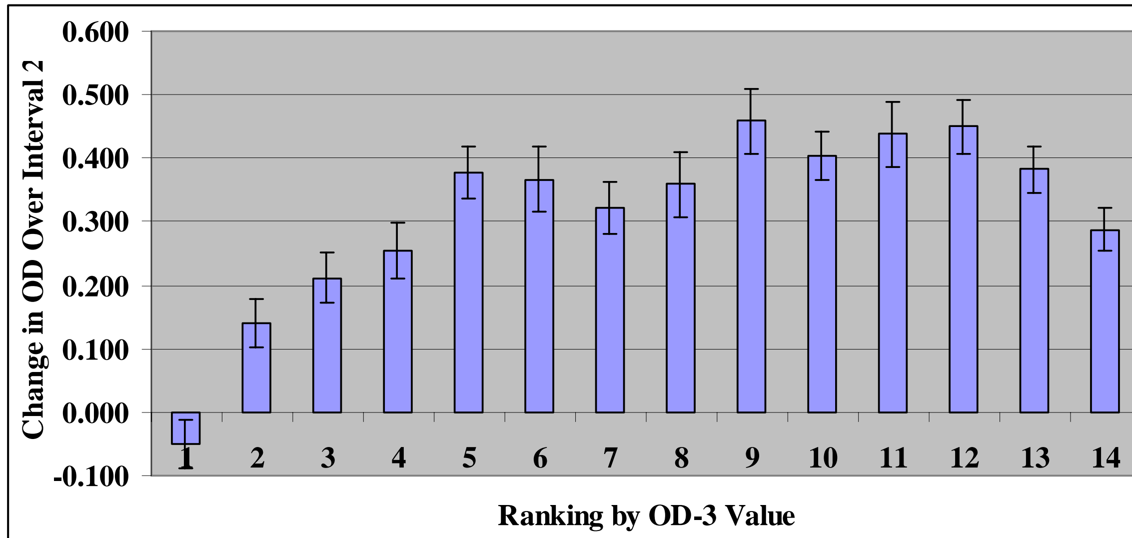

Figure 3-9. Graph reflecting the effect of maximal antibody response. Cows were ranked based on their OD_{-3} value (x-axis) with 55 cows per group ranking except group 14 which has 59 cows. Cows in group 1 have the top 55 OD_{-3} values. Cows in group 14 have the bottom 59 OD_{-3} values. The "y" axis represents OD_0 - OD_{-3}. Data points indicate the average change in OD over interval 2 for each group. Bars reflect SEM

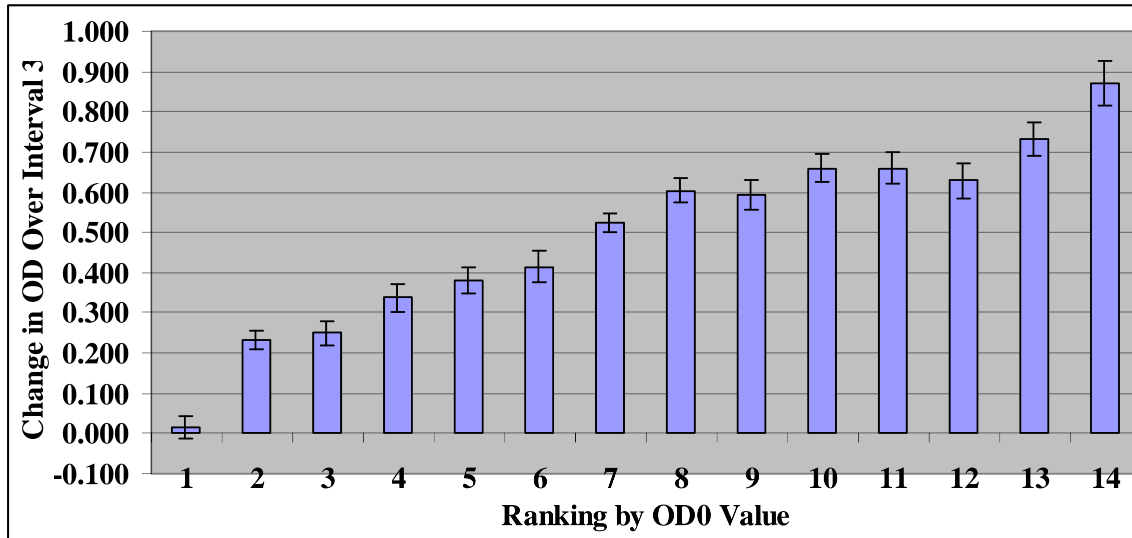

Figure 3-10. Graph reflecting the effect of maximal antibody response. Cows were ranked based on their OD_0 value (x-axis) with 55 cows per group ranking except group 14 which has 39 cows. Cows in group 1 have the top 55 OD_0 values. Cows in group 14 have the bottom 39 OD_0 values. The “y” axis represents the change in OD over Interval 3 (OD_{+2} - OD_0). Data points indicate the average change in OD over interval 3 for each group. Bars reflect SEM.

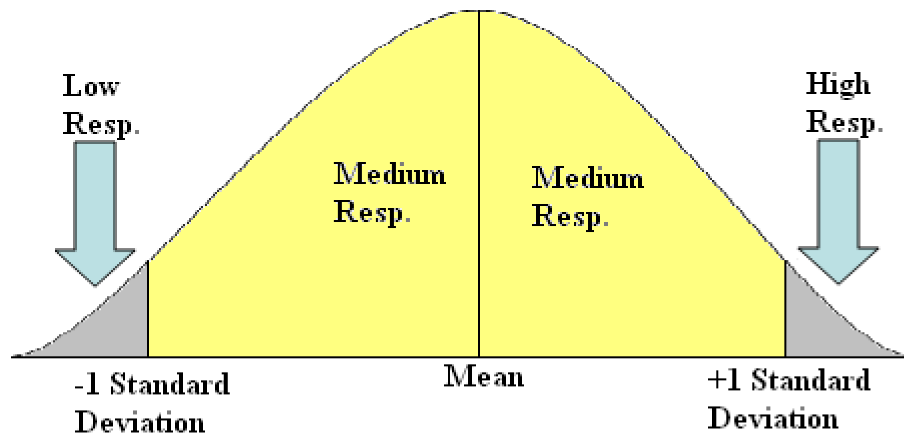

Figure 3-11. Depiction of a method used to categorize antibody mediated immune responsiveness. Mean and standard deviation are calculated based on y_{total}[1] values for the population. With a normal distribution roughly 16% of the population becomes high responders, 16% become low responders, and roughly 68% are medium responders.[1]y_{total}, total antibody response value generated through the use of an immune response index (Equation 3-3, Equation 3-4, Equation 3-5, Equation 3-6).

Table 3-1. Incidence of disease for each antibody response categorization method.

Mastitis

	Equation 3-3[1]		Equation 3-4[2]		Equation 3-5[3]		Equation 3-6[4]	
Categorization	St.Dev[5]	Quart[6].	St.Dev	Quart.	St.Dev	Quart.	St.Dev	Quart.
Low	27.45	27.98	23.73	24.12	25.69	27.71	24.35	23.35
Medium	26.93	27.86	28.05	29.33	26.64	26.30	27.39	30.14
High	22.83	21.95	23.01	22.84	26.42	25.47	24.77	21.74

Metritis

	Equation 3-3		Equation 3-4		Equation 3-5		Equation 3-6	
Categorization	St.Dev	Quart.	St.Dev	Quart.	St.Dev	Quart.	St.Dev	Quart.
Low	4.50	5.32	7.69	7.45	6.56	5.32	7.69	6.38
Medium	5.08	4.79	4.08	3.46	4.17	4.26	4.06	3.72
High	7.41	6.42	7.63	6.95	8.80	7.49	7.81	7.49

Metritis in cows having retained fetal membrane.

	Equation 3-3		Equation 3-4		Equation 3-5		Equation 3-6	
Categorization	St.Dev	Quart.	St.Dev	Quart.	St.Dev	Quart.	St.Dev	Quart.
Low	22.22	37.50	43.75	42.11	45.45	33.33	43.75	36.84
Medium	32.50	21.43	20.69	19.05	22.86	20.83	21.43	20.00
High	0.00	27.27	20.00	20.00	22.22	30.77	18.18	25.00

Ketosis

	Equation 3-3		Equation 3-4		Equation 3-5		Equation 3-6	
Categorization	St.Dev	Quart.	St.Dev	Quart.	St.Dev	Quart.	St.Dev	Quart.
Low	4.50	4.79	6.92	7.45	4.92	5.85	9.23	7.45
Medium	4.68	5.03	4.67	4.50	4.74	4.76	3.84	4.23
High	2.75	2.66	0.76	1.06	2.38	2.13	1.55	1.60

Displaced Abomassum

	Equation 3-3		Equation 3-4		Equation 3-5		Equation 3-6	
Categorization	St.Dev	Quart.	St.Dev	Quart.	St.Dev	Quart.	St.Dev	Quart.
Low	4.50	4.26	3.08	3.72	4.92	4.26	3.85	3.19
Medium	2.06	1.85	2.24	2.12	1.78	1.85	2.02	2.38
High	3.67	2.66	3.79	2.66	3.97	2.66	3.88	2.66

Retained fetal membrane

	Equation 3-3		Equation 3-4		Equation 3-5		Equation 3-6	
Categorization	St.Dev	Quart.	St.Dev	Quart.	St.Dev	Quart.	St.Dev	Quart.
Low	8.11	8.51	12.31	10.11	9.02	9.57	12.31	10.11
Medium	7.49	7.41	5.89	5.56	6.92	6.35	5.66	5.29
High	5.50	5.85	7.58	7.98	7.14	6.91	8.53	8.51

[1]Equation 3-3, refer to text. [2]Equation 3-4, refer to text. [3]Equation 3-5, refer to text. [4]Equation 3-6, refer to text. [5]St Dev.,standard deviation, refers to a method used to extrapolate antibody response categorizations (Figure 3-11). [6]Quart, quartiles, refers to a method used to extrapolate antibody response categorizations

CHAPTER 4
CATEGORIZATION OF PERIPARTURIENT ANTIBODY RESPONSE TO OVALBUMIN AND ITS RELATIONSHIP WITH COMMON DISEASES AND PERFORMANCE MEASURES OF HOLSTEIN DAIRY CATTLE

Introduction

The abrupt transition from a pregnant non-lactating cow to a non-pregnant lactating cow has a deleterious effect on immune function around the time of calving. This periparturient immune suppression is well documented (Lacetera et al., 2005; Saad et al., 1989; Detilleux et al., 1994; Park et al., 1992) and believed to be at least partially responsible for the increased risk of disease during this period. This effect appears to have several contributing mediators such as, increased stress and hormones associated with stress (Van Kampen and Mallard , 1997; Mallard et al., 1997), hypocalcemia (Kimura et al., 2006), negative energy balance with the resultant effect of nonesterified fatty acids (Lacetera et al., 2005) and hyperketonemia (Franklin et al., 1991; Hoeben et al., 1997).

Although these mediators have a significant effect on peripartum immune responsiveness, there also appears to be a gradual decline in immune competence associated with genetic selection focused on production traits which is made evident by the increasing risk of disease (Harmon, 1994; Heringstad, 2000; Emanuelson, 1988). This trend is occurring despite the efforts and major advances in sanitation and housing. This effect is believed to be fueled by placing the priority for genetic selection toward increased milk yield with little selection pressure for measures of disease resistance. Selection for increased milk production void of measures for resistance to mastitis has been found to result in a genetic increase of 0.02 cases of mastitis per cow per year (Strandberg and Shook, 1989). This translates into a genetic increase of 2 mastitis cases for every 100 dairy cows per year.

To overcome this unfavorable trend, selection pressure is being applied to traits associated with disease resistance. Selection for decreased SCC, productive life, and structural traits of the udder have been studied (Nash et al., 2000; Heringstad et al., 2006) and further implemented. However, associations between these traits and resistance to disease are rather crude and do not directly focus on the immune system which is primarily responsible for host defense. As a result, measures of the immune function have been evaluated as indicators of health (Wagter et al., 2000; Rupp et al., 2007; Park et al., 2004).

Significant associations with mastitis risk between differing populations of T lymphocyte subsets have been reported (Park et al., 1993, 2004). Several studies have found significant differences in immune responsiveness and resistance to disease with alterations in class I and II MHC haplotypes and alleles (Rupp et al., 2007; Park et al., 2004; Rupp and Didier, 2003). Other studies have compared incidence of disease among cows categorized based on their antibody-mediated immune responsiveness (AMIR) to test antigen (Wagter et al., 2000; Mallard et al., 1997; Hernandez et al., 2003).

Research by Wagter et. al. (2000) reported substantial variation among individual cow's ability to mount an antibody response around calving. In fact, not all cows experience peripartum immune suppression and cows could be categorized based on AMIR to ovalbumin (OVA). The high responders for AMIR had the lowest incidence of mastitis in two of the three study herds. Individual cow's antibody responsiveness to OVA also had a positive significant correlation with antibody titers to *E. coli* J5 vaccination. This response to OVA was highly heritable, ranging from 0.32 to 0.62 depending on week relative to calving (Wagter et al., 2000).

Although Wagter et al. (2000) did find favorable associations between this measure of AMIR and disease risk; with 136 cows and heifers spread over three herds, it was difficult to find

statistical significance. In the current trial this problem was addressed by incorporating a much larger sample size in order to more adequately study the relationship between AMIR and disease risk. The hypothesis being that high AMIR will be associated with lower disease risk. The objectives were to categorize cows based on AMIR to OVA and then test for associations between AMIR and disease incidence, namely; mastitis, metritis, retained fetal membrane, ketosis, and displaced abomasum. Additionally, possible associations between AMIR categorization and reproductive efficiency, milk yield and somatic cell score were examined.

Materials and Methods

Research Sample Cows

In total, 875 Holstein cows/heifers were enrolled into the study population at 8 weeks (wk-8) prior to calving. In cows, this was the initiation of the dry period. Animals were enrolled if the expected dry period was less than 90 days, if reconfirmed pregnant and if found in good health with no obvious signs of disease. All test animals were from a single herd in north central Florida which maintains exceptional record keeping. All cows and heifers were: enrolled between September 9th and December 31st, 2004; calved between October 25th, 2004 and March 12th, 2005; and sampling ended between November 9th, 2004 and March 28th, 2005. All cows and heifers received a routine dry off, prefresh, and fresh cow protocol.

Animal Removal and Interval Criteria

Of the originally enrolled 875 cows and heifers, 13 were removed due to missing data, yielding 862. Interval 1 (Int_1) was defined as time from enrollment (wk-8) to entry into springer pen (wk-3). Interval 2 (Int_2) was defined as time from entry into springer pen (wk-3) to calving (wk0) (Figure 4-1). Animals were removed from the study if the period from wk-8 to wk0 (dry period length for cows) was more than 90 days. Additional animals were excluded if Int_1 or Int_2 was less than 12 days in length. These time frames are relevant because they coincided with

antigen exposure and blood sampling to measure antibody response to antigen. Interval 3 (Int_3) was defined as the period between calving (wk0) and end of the sampling period (wk+2). No exclusionary criterion was needed for Int_3 because this interval was under investigator control. Of the 862 cows, 50 were removed because they did not meet one of these interval restrictions.

A preliminary analysis was used to remove an additional 38 animals found not naïve to test antigen. This resulted in a sample size of 774 with 433 cows and 341 heifers. The heifers upon enrollment will be referred to as primiparous cows while cows upon enrollment will be referred to as multiparous cows. Test animals were sired by 237 different sires, which should provide adequate variation in the gene pool for a sire effect on immune responsiveness.

Body Condition Scoring

Body condition was scored in ¼ point increments using the 5-point scale by Ferguson et al. (1994). Body condition scoring (BCS) was grouped into high, medium, and low categories. For cows during wk-8, wk-3, and wk0, a BCS between 3.0 – 3.75 was coded medium, those below 3.0 were coded low and those above 3.75 were coded high. Heifers at wk-8, wk-3, and wk0 were coded medium if BCS was between 3.0 – 3.5. A BCS above 3.5 was considered high, and a BCS below 3.0 was considered low. At wk+2, all animals were coded normal if BCS fell in the range of 2.75 – 3.5.

The loss of body condition, not just BCS alone was reported to be responsible for alterations in lymphocyte function (Lacetera et al., 2005; Wentink et al., 1997; Kaneene et al., 1997). To account for this factor the interval change in BCS for various intervals was calculated. Interval 4 was defined as the period between wk-3 and wk+2, which serves as an indicator of BCS lost over the transition period. Interval 5 was defined as the period between wk-8 and wk0. Interval 6 was defined as the period between wk-8 and wk+2.

Immunization

Ovalbumin was chosen as the test antigen due to its inert properties, its ability to stimulate antibody, a cow's reduced likelihood of previous exposure and its previous success as a tool to categorize AMIR (Wagter et al. 2000; Mallard et al., 1997; Hernandez et al., 2003). Animals received 1 mg ovalbumin (OVA; chicken albumin, Type VII, Sigma-Aldrich, St. Louis, MO, USA) at three times; week -8 (wk-8), week -3 (wk-3), and week 0 (wk 0) relative to calving (Figure 4-1). The wk-8 and wk-3 OVA immunizations were dissolved in *Escherichia coli* J5 vaccine (J5 bacterin, Pfizer Inc., Kalamazoo, MI, USA) with the manufacturer's adjuvant which coincided with the farm's routine vaccine protocol. At wk0, the 1 mg OVA was dissolved in 1 ml phosphate buffer saline (PBS, pH 7.4). All suspensions were then mixed with a type 1(*C. albicans* raw whole cell material, Greer Laboratories, Lenoir, NC, USA) antigen to stimulate a cell-mediated immune response (CMIR) and vortexed for at least one minute (Refer to chapter 5 for CMIR analysis).

Blood Collection and Processing

To determine antibody response to OVA, blood was collected by caudal venipuncture at; wk-8, wk-3, wk0, and wk+2 relative to calving (Figure 4-1). At calving (wk0), the blood sample was collected within 12 hours of parturition. Samples were collected into sterile 10 ml evacuated blood collection tubes with no additive, then put on ice during transport back to lab. Serum was collected after centrifugation at 4,000 rpm and stored at -70º C.

Enzyme-Linked Immunosorbent Assay

A cow's specific antibody response to OVA was detected using an indirect enzyme-linked immunosorbent assay (ELISA) method as previously described (Burton et al., 1989; Wagter et al., 2000; Mallard et al., 1997). For positive control sample, 10 lactating cows received 1 mg OVA and 0.5 mg Quil A adjuvant suspended in 1 ml PBS on day 0 and 14. On day 21, serum

from these cows was collected and pooled for positive control sample. Negative control samples consisted of a pool of serum from cows at wk-8.

Polystyrene 96-well plates (Immulon II, Fisher Scientific Co. Ltd., Pittsburgh, PA, USA) (Figure 4-2) were stored for 48 h at 4º C after being coated with 100 µL/ well of 1.4 mg OVA dissolved in 1 mL carbonate-bicarbonate coating buffer (pH 9.6). Plates were washed four times in a plate washer (ELX50 plate washer, Biotek Instruments, Inc., Winooski, VT, USA) with wash buffer solution containing PBS and 0.05 % Tween 20 (Sigma-Aldrich) (washing buffer, pH 7.4). Blocking solution (PBS pH 7.4, 3% Tween 20, 1% bovine serum albumin) was added (200 µL / well) and plates incubated at room temperature for 1 h. Plates were washed 4 times before applying 100 µL / well of control and test sera. All samples were diluted to 1/50 and 1/200 dilutions using wash buffer solution. Positive and negative controls (1/50 and 1/200) were run in quadruplicates while test sera (1/50 and 1/200) were run in duplicates placed in separate diagonal plate quadrants (Figure 4-3). Plates were then incubated for 2 h at room temperature. After being washed 4 times, 100 µL / well of alkaline phosphatase-conjugated rabbit anti-bovine IgG (whole molecule; Sigma Chemical Co., St. Louis, MO) dissolved in Tris buffer solution (TBS, pH 7.4 and 0.05% Tween) at a 1/38000 dilution was added, and plates incubated for 1 h at room temperature. Plates were washed 4 times before 80 µL / well of p-nitrophenyl phosphate disodium were added. Plates were incubated for 30 min at room temperature out of direct light. Optical density (OD) values were then determined for each sample with an ELISA plate reader (MRX Revelation, Dynes Technologies VA, USA) set at an absorbance of 405 ηm and 630 ηm (Revelation software, Dynes Technologies VA, USA).

Coefficient of variation was calculated for the 1/50 and 1/200 positive controls to determine whether plates were accepted or rejected. The coefficient of variation for the 1/50

dilution was calculated by dividing the standard deviation by the mean of the 1/50 positive control values. This was also performed for the 1/200 dilution. The maximum allowable variation for each plate was 20% for either the 1/50 or 1/200 positive control dilution.

In order to correct for variation among plates a correction factor was determined for each plate. The correction factor was determined by comparing the positive control OD values of each plate to the mean of every plate. The mean of each plates 1/50 positive control dilution was summed with the mean of the 1/200 positive control dilution. This additive positive control value was then divided into the mean of every plate's additive value. The resultant value functioned as the correction factor. Since each test sample was run in duplicate, each test samples mean 1/50 OD value was summed with the mean 1/200 OD value. This value was then multiplied by the plate's correction factor to determine the samples corrected OD value to be utilized in statistical analysis.

Preliminary Analysis

Removal of non-naive

Due to the differences in the kinetics of a primary and secondary antibody response (Figure 2-1), it was important that all cows receive equal treatment which requires all cows to be naïve to OVA at enrollment. This should result in low OD values for wk-8 (OD_{-8}) due to the lack of antibody with affinity for OVA. Inspection of the OD_{-8} values revealed that some cows were not naïve. Sorting the cows based on their OD_{-8} values revealed a natural cut-off where the values began increasing rapidly. This point also coincided with one standard deviation above the mean for the OD_{-8} values. As a result 38 cows were excluded due to not being naïve to test antigen. For further discussion refer to chapter 3.

Parity effect

A repeated measures analysis using OD_{-8} as a covariate with the mixed procedure of SAS revealed that multiparous cows responded significantly higher than primiparous cows at every antibody response measurement week ($p<0.0001$). Due to this effect, adjustments to OD values as well as AMIR categorizations were all made with respect to parity (Figure 3-2). Explanations for this finding may include the presence of a more extensive antibody repertoire in older cows. This could simply be due to the effect of time, allowing greater exposure to a broader array of pathogens.

Interval analysis and optical density value adjustment

The length of the interval between antigen administration and blood sampling has strong relevance due to the different phases of an antibody response (Figure 2-1). As a result, an analysis was performed to determine this effect and subsequently adjust the OD values for interval length (Figure 3-3, 3-4, 3-5, 3-6, 3-7, 3-8). A generalized linear model was constructed using the GLM procedure of SAS to determine if the duration in days of Int_1, Int_2, or Int_3 significantly influenced OD_{-3}, OD_0, or OD_{+2} respectively. For this analysis, OD response served as the outcome variable while possible explanatory variables included:

- Int_Y = interval length in days for interval "y"
- BCS_X = categorization of body condition score for sampling week "x"
- ΔBCS_Y = change in body condition score over interval "y"
- OD_X = optical density at previous sampling week "x"
- Dex = binary effect, whether cow received dexamethasone prior to calving
- Sick = binary effect, incidence of either medium or severe case of mastitis, metritis, ketosis, or displaced abomasum within 16 DIM. Only used for analysis to correct OD_{+2}, for reasons discussed in chapter 3.

Explanatory variables remained in the model if the effect showed a tendency ($P < 0.10$) to predict the desired OD value. With $\alpha = 0.05$, if statistical significance was found with the main effect (Int_y), the resultant parameter estimate was applied to Equation 3-1 to correct the corresponding OD values. If a given OD value was adjusted based on interval length, the model predicting the OD value for the subsequent sampling period would have the adjusted OD from the previous sampling period.

$$AOD_{ZPX} = OD_{ZPX} + PE_{YP} * (Mint_{YP} - Int_{ZPY}) \quad (3\text{-}1)$$

Where:

- AOD_{ZPX} = the adjusted OD value for cow "z" in parity "p" for week "x"
- OD_{ZPX} = OD value for cow "z" in parity "p" for week "x"
- PE_{YP} = parameter estimate for the effect interval "y" has on ODx values for parity "p". Also, interval "y" must always immediately precede week "x".
- $Mint_{YP}$ = median number of days for interval "y" for parity "p"
- Int_{ZPY} = the actual interval length for cow "z" in parity "p" for interval "y"

As a result the OD_{-3} values were adjusted for multiparous cows only. The OD_0 values were adjusted for both multiparous and primiparous cows. The OD_{+2} values were adjusted for heifers only. For further reference refer to chapter 3.

Classification analysis

Maximal response: Previous work devised an index using the interval change in OD value to generate total antibody values used to extrapolate AMIR classifications (Wagter et al. 2000). This index weighted those values around calving if they showed a decline in antibody response. In this study, inspection of the OD values for the respective weeks revealed what appeared to be a maximal antibody response. If there was an improvement after subsequent antigen injection, these maximal points in antibody concentration only slightly improved. If this peak response was

achieved early in the study trial (OD_{-3}), which would indicate a high prepartum responder; there would be little if any room for an added response. As a result an index concerned with weighting the change in OD during the intervals adjacent to calving (Eq. 1-1), may negatively impact the categorization of great immune responders who reached this saturation point early because there was little or no room to further respond.

After sorting the OD_{-3} values from greatest to least, it was discovered that of the top 20 cows for OD_{-3} value, 15 had a smaller OD_0 value (75%), yielding a negative interval 2 change in OD. In these instances, 15 of the top 20 OD_{-3} responders would have an amplified ($\beta = 1.5$) negative I_2 value applied to their AMIR index if using an index which weights interval changes in OD.

After grouping the OD_{-3} values from 1 to 14, with group 1 being the top 55 OD_{-3} values and group 14 the bottom 59 OD_{-3} values, a one-tailed two sample T-test was used to determine if the OD value level grouping had a significant effect on the subsequent interval change in OD. This revealed a significantly smaller change in OD over interval 2 for group 1 cows compared to group 2 cows ($p < 0.0001$) (Figure 3-9). This value actually averaged below 0 (-0.05) for these top 55 OD_{-3} responders. Linear regression also revealed OD_{-3} is a significant predictor for I_2 ($p < 0.0001$; $\beta = -0.28$). A negative β indicates that as OD_{-3} values increase, I_2 values decrease.

Repeating the same process by ranking cows based on OD_0 values in order to compare interval 3 change in OD, revealed similar results. Due to missing wk+2 blood samples the sample size for this analysis was 754. As a result group 14 was comprised of 39 cows while group 1 – 13 comprised of 55. Of the top 20 cows for OD_0 values, 15 had negative interval 3 changes in OD (75%). Also, group 1 cows based on OD_0 values had a significantly smaller interval 3 change in OD compared to group 2 cows ($p < 0.0001$) (Figure 3-10). Linear regression

also revealed OD_0 is a significant predictor for I_3 ($p < 0.0001$; $\beta = -0.50$). A negative β indicates that as OD_0 values increase, I_3 values decrease.

In these instances of high OD values followed by a subsequent negative interval, the interval is not negative due to a poor subsequent OD value. Of the top 20 cows for OD_{-3}, 18 still had OD_0 values above the third quartile for the population and the other 2 were still above the median for the population. For the top 20 cows for OD_0, all 20 still had OD_{+2} values above the third quartile for the population. The negative interval was simply the result of an inability to respond further. As a result, additional indexes were generated and analyzed based on their correlation with disease incidence.

Early postpartum measures of immune function: Previous work utilized the antibody response measured early postpartum as a tool to categorize AMIR (Wagter et al., 2000; Mallard et al., 1997).This period immediately following parturition is a common occasion for increased incidence of disease. This effect of substantial sickness could be a confounding variable for antibody responsiveness to OVA detected early postpartum. As a result it may be hypothesized that substantial sickness could contribute to immune suppression. This would make it difficult to study the effect measures of immune responsiveness have on disease risk if the association could also be in the opposing direction.

A linear regression model was used with the REG procedure of SAS. OD_{+2} served as the outcome variable while potential explanatory variables included:

- BCS_x = categorization of body condition score for sampling week “x”.
- ΔBCS_y = change in body condition score over interval “y”.
- OD_0 = optical density value at wk0.
- Dex = binary effect, whether cow received dexamethasone prior to calving.

- Sick = binary effect, incidence of either medium or severe case of mastitis, metritis, ketosis, or displaced abomasum within 16 DIM.
- Parity = binary effect, either primiparous or multiparous.

Explanatory variables remained in the model if the effect showed a tendency ($P < 0.10$) to predict the dependant OD value. For statistical significance, $\alpha = 0.05$. The resulting model was as follows (Eq. 3-2):

$$OD_{+2} = Sick + Dex + OD_0, \qquad (3\text{-}2)$$

The analysis revealed that sickness, as previously defined, was a significant predictor of OD_{+2} ($p = 0.0156$). The difficulty in this analysis is determination of causality, did the occurrence of sickness within 16 DIM cause a suppression in immune responsiveness; or did inferior immune responsiveness cause sickness within 16 DIM. Because OD_0 occurs prior to the incidence of disease, and this was a significant predictor of OD_{+2} ($p < 0.0001$), it appeared that the incidence of sickness had an effect on immune responsiveness.

Correlation analysis using the Corr procedure of SAS was employed to study the relationship between OD_0 and OD_{+2} at fixed levels of sickness. Among cows considered healthy within 16 days in milk, correlation analysis revealed OD_0 is positively correlated with OD_{+2} ($r^2 = 0.64$, $p < 0.0001$). Furthermore, within sick cows, the correlation between OD_0 and OD_{+2} has an even greater significantly positive correlation ($r^2 = 0.73$, $p < 0.0001$). For further reference see chapter 3.

Antibody-Mediated Immune Response Classification

To alleviate the concerns about the effect of antibody saturation and early postpartum sickness, a new index was generated for the categorization of AMIR (Eq. 3-6). The rationale behind this index is that favorable AMIR will have greater correlation with the actual magnitude of the antibody concentration rather than changes in antibody concentration over intervals. In the

case of antibody saturation, an index should reflect a cow's ability to maintain a high concentration of specific antibody. This should all be accomplished while also using measures which give special attention to antibody responses occurring peripartum.

$$y_{total} = OD_{-3} + OD_0 *(1 + I_2) \quad (3\text{-}6)$$

Where:

- y_{total} = total antibody
- OD_{-3} = optical density value at wk-3
- OD_0 = optical density value at wk0
- I_2 = change in OD between week -3 and week 0

In this index, the direct magnitudes of the OD values are considered. However, in the case of OD_0 it is still weighted, but this can be positively or negatively and only in proportion to the level of increase or decrease for I_2 and I_3.

Extrapolation of AMIR categorizations occurs by use of the y_{total} values for individual cows and then configuring the quartiles respective of parity (primiparous vs. multiparous). Cows within the bottom 25% for a respective parity are categorized as low AMIR responders, while cows in the top 25% for a respective parity are termed high AMIR responders. The remaining middle 50% are categorized as medium AMIR responders.

Diseases

Identification of disease was performed by farm personnel who were blinded to immune response categorizations. The diseases of interest for this project were; mastitis, metritis, retained fetal membranes, ketosis, and displaced abomasum. All diseases were recorded as yes/no binary responses for the trial period of the current lactation.

Mastitis was recorded through 365 DIM as light, medium, or severe. Severity was recorded as light if there were no systemic signs and milk was slightly watery with minimal clots (gargot).

Severity was medium if there were no systemic signs and a substantial amount of clots were observed in the milk. Severity was severe if there were systemic signs, watery milk, and a substantial amount of clots in the milk.

Metritis was recorded through 30 DIM as light, medium or severe. Severity was light if there was an abnormal vaginal discharge and a palpable uterine lumen. Severity was medium if there was a purulent vaginal discharge, with an enlarged, not-flaccid uterus. Severity was severe if there was a purulent foul-smelling vaginal discharge, with an enlarged flaccid uterus.

Association between AMIR and energy related metabolic conditions including ketosis and DA were also analyzed. Ketosis was recorded through 30 DIM as light, medium, or severe. Ketosis was coded as light if the urine contained 15 mg/dL of ketone bodies. Severity was medium if the urine contained 40 mg/dL of ketone bodies. The severity was severe if the urine contained > 80 mg/dL of ketone bodies. Displaced abomasum was recorded through 50 DIM.

Because retained fetal membranes are now understood to result from an inadequate immune response (Kimura et al., 2002), the relationship between AMIR and RFM was also considered. Retained fetal membranes were identified if there was placental retention 24 h postpartum.

Milk Yield, Somatic Cell Score, and Reproductive Efficiency

Milk yield was gathered from the Dairy Herd Information Association (DHIA) records for the current lactation using ME305, which is an estimate of the milk yield for 305 DIM. For categorical data, low milk producers were identified if there 305 day milk was in the bottom 25% of the study group. High milk producers were those in the top 25% of the study group. The remaining middle 50% were classified as medium milk producers.

Somatic cell score (SCS) was gathered from DHIA records. The average SCS was determined for the first 3 test days, the first 6 test days, and for the first 10 test days, which are an indication of the average SCS for the first 90, 180, and 300 DIM respectively.

To analyze for associations between AMIR categorization and reproductive efficiency, a binary pregnancy term was used which simply indicated if a given cow was pregnant by 150 DIM. Addition quantitative variable measures of fertility included; number of days that a cow is not pregnant (days open), and number of times bred.

Statistics

To analyze the associations between risk of disease and reproductive efficiency with AMIR categorization, a logistic regression model was developed using the LOGISTIC procedure of SAS. Other than the main effect (AMIR categorization), possible explanatory variables include:

- BCS_x = categorical effect of body condition score for sampling week "x".
- ΔBCS_y = quantitative variable, change in body condition score over interval "y".
- Dex = binary effect, whether cow received dexamethasone prior to calving.
- Parity = binary effect, either primiparous or multiparous.
- RFM = binary effect, incidence of retained fetal membrane.
- CDiff = binary effect, if reported difficult calving.
- Ketosis = binary effect, incidence of ketosis.
- SCSavg = somatic cell score average over 10 monthly test days.

For analyzing the relationship between AMIR categorization and the quantitative variables for SCS, 305 day milk yield, and reproductive efficiency, linear regression was used with the REG procedure of SAS. All categorical variables will be analyzed using logistic regression using the LOGISTIC procedure of SAS.

All relevant effects were put in the model. A backwords elimination procedure was used to determine the final model. Explanatory variables remained in the model if they showed a

tendency ($p < 0.10$) to predict the outcome variable. Statistical significance was determined by setting $\alpha = 0.05$.

Results

Mastitis and Metritis

No significant association was found between AMIR status and mastitis within 100 DIM. When considering the incidence within 365 days; there were 169 cases that were either medium or severe (22%). Forty (40) cases were recorded in primiparous cows and 129 in multiparous cows. Effects remaining in the model were: SCS average, ΔBCS_6, Parity. Antibody response categorization was a significant predictor of moderate and severe mastitis risk ($p = 0.0082$) (Table 4-1). Although the low responders were not statistically different than medium and high responders collectively ($p = 0.12$), the medium responders were 1.76 (CI = 1.08 - 2.89) times more likely to have an occurrence of moderate or severe mastitis than high responders.

The recorded incidence for light medium or severe metritis during the first 30 DIM was remarkably low with only 41 cases; 27 cases in primiparous cows and 14 cases in older cows (5.3% overall). Remaining model effects were: Parity, BCS_0, Dex, and RFM. Antibody response categorization was not significantly associated with occurrence of metritis in this analysis ($p = 0.29$) (Table 4-1).

Ketosis Displaced Abomasum and Retained Fetal Membrane

Only 45 cases of ketosis occurred within 30 days of calving (5.8%). Primiparous cows accounted for 24 cases while multiparous accounted for 21 cases. The model included ΔBCS_6 and the main effect (AMIR category). The low responders were 2.90 (CI = 1.10 – 7.62) times more likely to develop ketosis than high responders (Table 4-1) (Figure 4-4).

Displacement of the abomasum occurred in 21 cows (2.8%), with 12 from primiparous cows and 9 cases in multiparous cows. Remaining model effects were ΔBCS_4, and ketosis. In this analysis, AMIR was not a significant predictor of DA incidence (Table 4-1).

There were 57 cases of RFM for the study population (7.4%). Primiparous cows accounted for 18 cases while multiparous cows accounted for 39 cases. Remaining model effects were: ΔBCS_5, and BCS_0. The AMIR categorization was not a significant predictor of RFM incidence ($p = 0.17$) (Table 4-1).

Milk Yield and Somatic Cell Score

For the analysis of the effect of AMIR categorization on milk yield, the contributing model effects were parity, number of days open, and the binary trait of mastitis. In this analysis, AMIR categorization had a tendency to predict milk yield ($p = 0.06$, $\beta = -347.34$) (Figure 4-5). The analysis of the effect of AMIR on SCS included ΔBCS_6, milk categorization, and the binary mastitis variable. The effect of AMIR on SCS was not a significant predictor of SCS ($p = 0.40$).

Reproductive Efficiency

The model analyzing the effect of AMIR category on pregnancy at 150 DIM included the explanatory variables; ΔBCS_4, and milk categorization. The effect of AMIR was a significant predictor for pregnancy by 150 DIM ($p = 0.003$). The low antibody-mediated responders were 2.32 (CI = 1.44 – 3.75) times more likely than high responders to become pregnant by 150 DIM. Also, the low antibody-mediated responders were 1.57 (CI = 1.20 – 2.05) times more likely to become pregnant by 150 DIM than medium and low responders collectively (Figure 4-6).

Discussion

The significantly higher odds of mastitis for medium antibody-mediated responders compared to high responders indicate the validity of using antibody response to OVA as a measure of AMIR. These results coincide with the findings in Wagter et al. (2000) where low

responders had the highest occurrence of mastitis, and antibody response to OVA was significantly correlated with antibody response to *E. coli* J5 vaccination. The lack of additional statistical findings for mastitis and metritis are likely due to low recorded disease frequency.

Mechanisms linking energy-related metabolic disorders and measures of immune response are not completely understood. Associations between ketosis and suppressed immune response are largely believed to be the result of ketosis causing immune suppression. However in this study, AMIR categorization occurred prepartum. Given that ketosis is a postpartum disorder, it is not possible that ketosis caused a suppressed immune response during categorization. With change in BCS remaining in the model, prepartum BCS did not predispose cows to ketosis while influencing immune responsiveness. The odds for this effect followed a High < Medium < Low pattern.

The discovery of inverse associations between AMIR categorization and milk yield and fertility was an unexpected result. With regard to milk yield, these findings do not agree with previous literature in dairy cows (Wagter et al., 2003; Detilleux et al., 1995) or with other performance measures in swine (Mallard et al., 1998). However, possible explanations may arise from studies in other metabolically active species. Poultry selected for greater immune responsiveness have reported a reduction in growth performance (Klasing et al., 1987, 1998; Soler et al., 2003). This was explained by bodily function competition for available nutrients. The energy and nutrients required for maintenance and activation of a superior immune responder could otherwise be used for other phenotypic traits.

There is no known biological relevance for high immune responders to be predisposed to lower milk yields or decreased fertility. This finding may simply be the result of neglecting to

select or having inadequate tools to select for immune responsiveness while putting direct selection pressure on the metabolically demanding trait of milk yield.

There is another possible explanation for the unfavorable decline in fertility. This involves the maternal recognition of the conceptus by the immune system. During pregnancy the conceptus is a foreign body which otherwise would be subject to a maternal immune response followed by rejection. This rejection, however, is blocked due to various immune suppressive activities which are initiated during maternal recognition of pregnancy (Hansen, 1997). This relationship between the immune system and pregnancy brings the potential for an inappropriate immune response rejection of the conceptus. If it could be proven that cows who mount a superior antibody-mediated immune response are more likely to mount an inappropriate immune response against the conceptus, this could explain the present findings.

In hindsight of this study, there are certain techniques and strategies that could or should be implemented upon further research. Although it is difficult in a large scale dairy setting, and this aspect was accounted for in the present study, a tighter control on interval duration between antigen injection and blood collection could be practiced.

Another improvement would be in the use of antigen. A more representative categorization of AMIR could be obtained through two or more antigens. This philosophy has been practiced in other species (Mallard et al.,1998). To maintain the integrity of a broad based approach to improving immune response when selection pressure is applied for AMIR, it should be based on more than one antigen.

Using the antibody response to OVA around calving as a representative of the ability to mount an immune response during immune suppression may be partially confounded due to the fact that OVA was previously injected at wk-8 and wk-3. This is because by this point memory

lymphocytes have already formed for OVA and they are the cells being activated to mount the antibody response. These memory cells are much easier to activate and have greater affinity to antigen. For a true response during immune suppression primary exposure to antigen should occur closer to parturition. As a result, if using multiple antigens, exposure to these antigens could be staggered or initially introduced at different time points with one occurring at calving.

Due to the rising incidence of disease and the ever increasing necessity to produce milk as proficiently as possible, a more proactive and effective approach to disease resistance is required. Taking this initiative should also help alleviate concerns consumers have for animal welfare and usage of antibiotics.

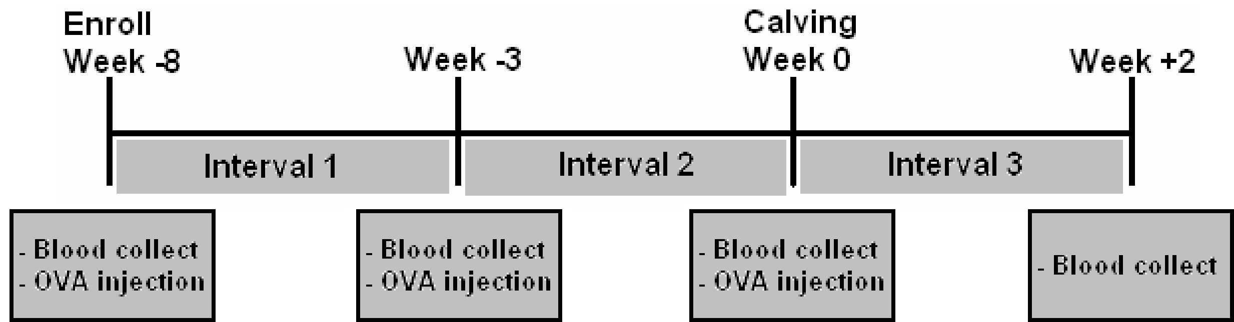

Figure 4-1. General outline of experimental design.

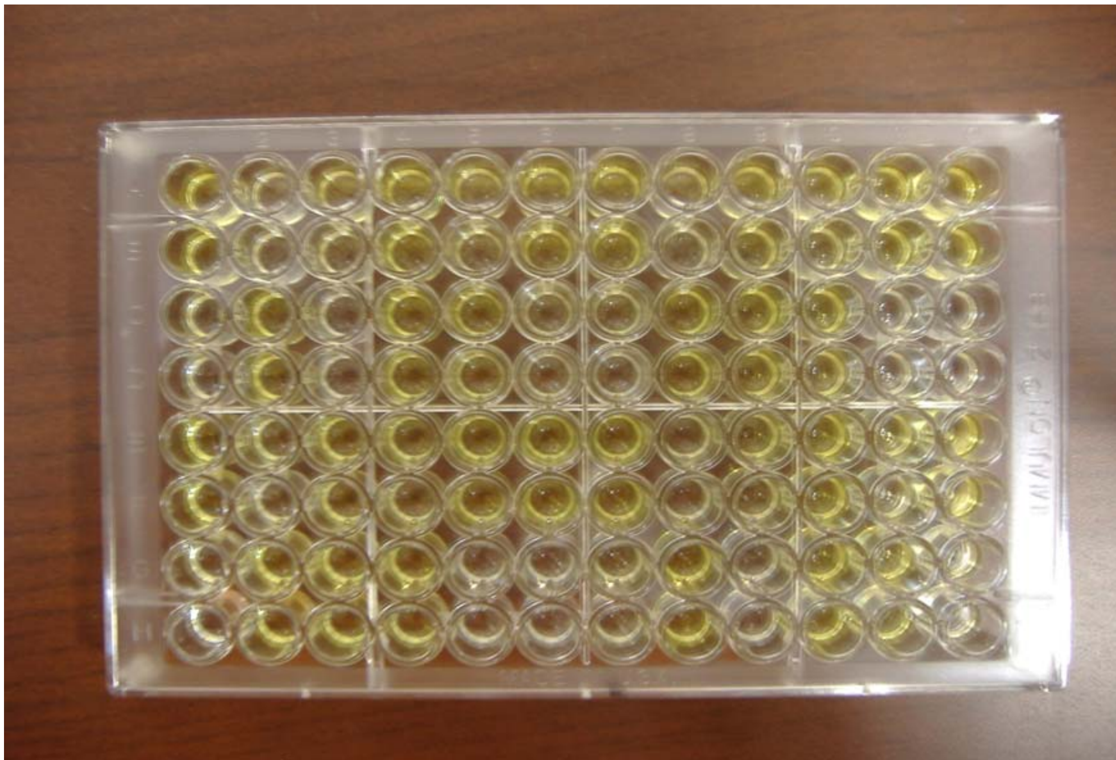

Figure 4-2. Polystyrene 96-well plate for enzyme linked immunosorbent assay.

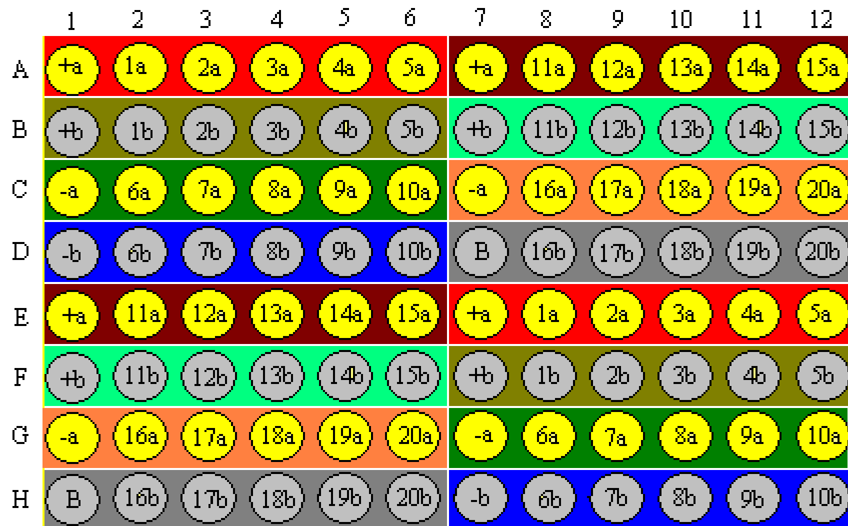

Figure 4-3. Diagram of the placement of test sera into 96 well polystyrene plate for enzyme-linked immunosorbent assay. Yellow wells identify 1/50 dilution, while grey wells identify 1/200 dilution. All positive and negative controls were run in columns 1 and 7. Well identified as “1a” is a 1/50 dilution of sample 1, which was run in column 2 of row A and duplicated in column 8 of row E. Sample identified as “1b” is a 1/200 dilution of sample 1 run in column 2 of row B and duplicated in column 8 of row F. Matching background row colors indicate half rows which were duplicated. Wells identified with “B” indicate blank wells for calibration of plate reader.

Table 4-1. Odds ratios of disease incidence for antibody response categorizations.

	Mastitis	Metritis	Ketosis	DA	RFM
Low vs Med & High	0.77 (0.55 - 1.07)	0.93 (0.56 - 1.54)	1.69 (1.06 - 2.69)	0.91 (0.45 - 1.85)	1.29 (0.86 - 1.94)
Low vs High	0.89 (0.49 - 1.64)	0.64 (0.27 - 1.56)	2.9 (1.10 - 7.62)	0.7 (0.19 - 2.6)	1.16 (0.56 - 2.42)
Med vs High	1.76 (1.08 - 2.89)	0.521 (0.23 - 1.19)	1.75 (0.68 - 4.45)	0.65 (0.20 - 2.15)	0.63 (0.31 - 1.27)

Values indicate the estimated odds ratios for incidence of disease for the following comparisons among antibody-mediated immune categorizations. Values in parenthesis represent the 95% confidence intervals for the odds ratio estimate.

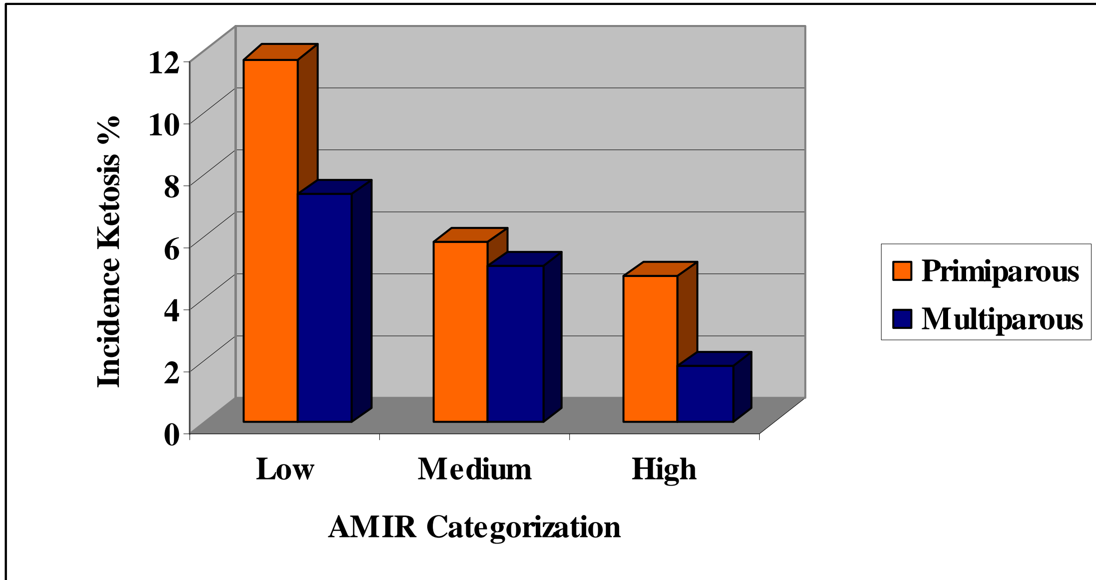

Figure 4-4. Incidence ketosis by AMIR categorization within parity.

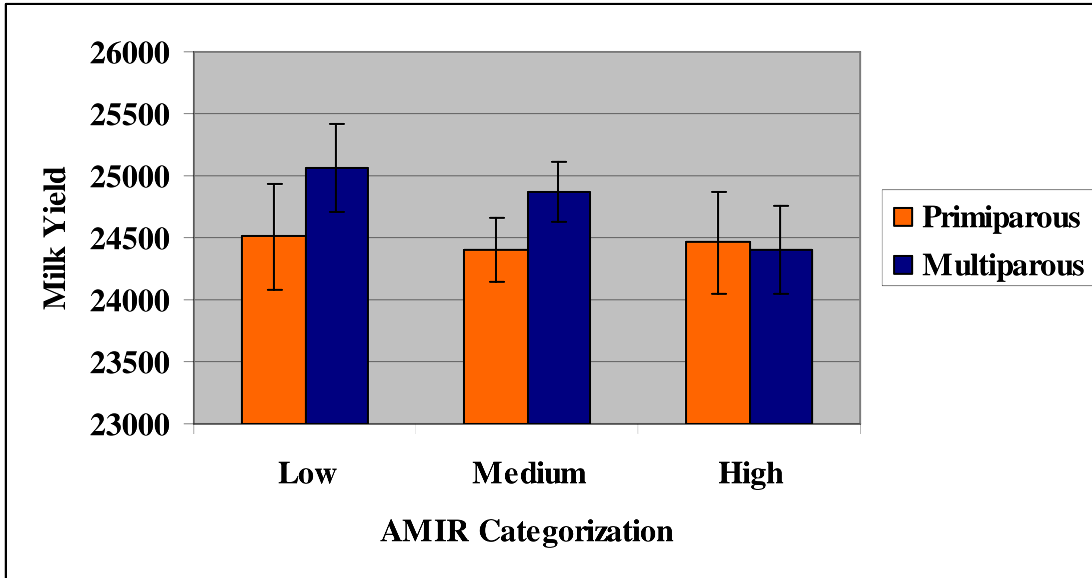

Figure 4-5. Graph for the effect of antibody-mediated immune response (AMIR) categorization on milk yield. Bars indicate SEM.

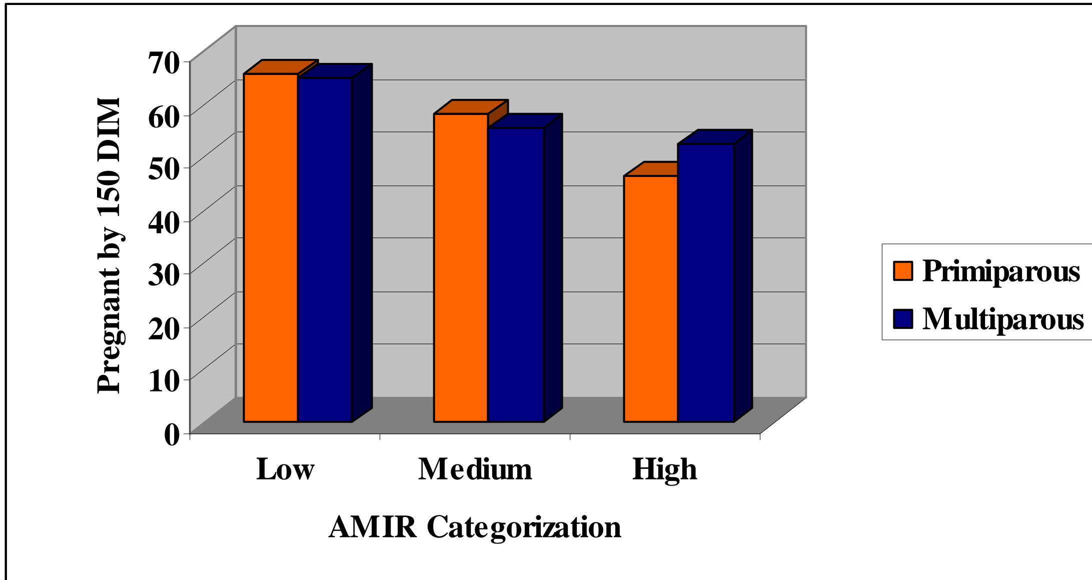

Figure 4-6. Graph for the effect of antibody-mediated immune response (AMIR) categorization on pregnancy by 150 DIM.

CHAPTER 5
CATEGORIZATION OF PERIPARTURIENT CELL-MEDIATED IMMUNE RESPONSE TO A TEST ANTIGEN AND ITS RELATIONSHIP WITH COMMON DISEASES AND PERFORMANCE MEASURES OF HOLSTEIN DAIRY CATTLE

Introduction

The increasing risk of disease for Holstein dairy cows (Harmon, 1994; Heringstad, 2000; Emanuelson, 1988) has sparked interest in genetic selection for disease resistance. Because the immune system is principally responsible for resisting the array of potential pathogens; many of the methods studied have analyzed the relationship between disease risk and immune function or an aspect of a particular part of the immune system. Park et al. (2004) studied the ratio of CD4+ to CD8+ T lymphocyte subsets and its relationship with mastitis incidence. Other studies have placed special attention on characteristics of the major histocompatability complex (MHC) (Rupp et al., 2007; Park et al., 2004; Aaerestrup et al., 1995). Some studies have used test antigen to quantify an individual's antibody-mediated immune responsiveness (AMIR) as a tool to predict the risk of disease (Wagter et al., 2000; Mallard et al., 1997).

An inverse relationship between cell-mediated immune response (CMIR) and AMIR has been documented (Rupp et al., 2007; Biozzi et al., 1979; de Vries, 1995). As a result, selection for increased AMIR without regard for CMIR may confer susceptibility to intracellular pathogens.

Being mediated by T_H1 cells, delayed-type hypersensitive reactions (DTH) are largely concerned with elimination of intracellular antigen. Therefore, DTH reactions have been previously used as a means to quantify CMIR (Mallard et al., 1998; Hernandez et al., 2003, 2005). However, few studies have analyzed the association between this measure of CMIR and disease susceptibility.

The objectives of this research were to categorize periparturient cow's using DTH reaction to a type 1 antigen and study the association it has with susceptibility to disease, namely; mastitis, metritis, retained fetal membrane (RFM), ketosis, and displaced abomasum (DA).The final objective was to examine for an effect of CMIR categorization on milk yield, somatic cell score (SCS), and fertility.

Materials and Methods

Research Sample Cows

This research study was given IACUC approval. In total, 875 Holstein cows/heifers were enrolled into the study population at 8 weeks (wk-8) prior to expected calving. In cows, this was the initiation of the dry period. Animals were enrolled if the expected dry period was not longer than 90 days, if reconfirmed pregnant at enrollment and also if found in good health with no obvious signs of disease. All test animals were from a single herd in north central Florida which maintains exceptional record keeping. All cows and heifers were: enrolled between September 9th and December 31st, 2004; calved between October 25th, 2004 and March 12th, 2005; and CMIR measurement occurred between November 2nd, 2004 and March 21st, 2005. All cows and heifers received a routine dry off, prefresh, and fresh cow protocol.

Animal Removal Criteria

Of the originally enrolled 875 cows and heifers, 13 were removed due to missing data, yielding 862. Interval 1 (Int_1) was defined as the time from enrollment (wk-8) to entry into the springer pen (wk-3). Interval 2 (Int_2) was defined as the time from entry into the springer pen (wk-3) to calving (wk0) (Figure 4-1). Due to the restrictions of a parallel study involving AMIR, animals were removed from the study if the period from wk-8 to wk0 (dry period length for cows) was more than 90 days. Additional animals were excluded if Int_1 or Int_2 was less than 12 days in length. As a result 50 cows were removed leaving the study population total at 812 with

362 heifers and 450 cows. For the rest of this paper heifers will be referred to as primiparous cows. Cows will be referred to as multiparous cows. Test animals were sired by over 237 different sires, which should provide adequate variation in the gene pool for a sire effect on disease resistance.

Body Condition Scoring

Body condition was scored in ¼ point increments using the 5-point scale by Ferguson et al. (1994). Body condition scoring (BCS) was grouped into high, medium, and low categories. For cows during wk-8, wk-3, and wk0, a BCS between 3.0 – 3.75 was coded medium, those below 3.0 were coded low and those above 3.75 were coded high. Heifers at wk-8, wk-3, and wk0 were coded medium if BCS was between 3.0 – 3.5. A BCS above 3.5 was considered high, and a BCS below 3.0 was considered low. At wk+2, all animals were coded normal if BCS fell in the range of 2.75 – 3.5.

It has been reported that for periparturient dairy cows, it was the loss of body condition, not just BCS alone that was responsible for alterations in lymphocyte function (Lacetera et al., 2005; Wentink et al., 1997; Kaneene et al., 1997). To account for this factor the interval change in BCS for various intervals was calculated. Interval 4 was defined as the period between wk-3 and wk+2, which serves as an indicator of BCS lost over the transition period. Interval 5 was defined as the period between wk-8 and wk0. Interval 6 was defined as the period between wk-8 and wk+2.

Delayed Type Hypersensitivity

To stimulate a DTH reaction, all cows received 0.5 mg *C. albicans* (raw whole cell material, Greer Laboratories, Lenoir, NC, USA) at three times; wk-8, wk-3 and wk0. This was also included with a standard *E. coli* J5 vaccination program on wk-8 and wk-3 along with the OVA for AMIR. Within 12hours of calving (wk0), animals received 0.5 mg *C. albicans* in 0.5

mg Quil A (Accurate Chemicals and Scientific Corp., Westbury, NY, USA) adjuvant suspended in 1 mL PBS along with OVA for AMIR.

At 1 week post-calving (wk+1), double skin-fold measurements were taken on the right and left skin folds under the base of the tail using a spring-loaded caliper (Harpenden skin-fold caliper, Creative Health Products, Inc., Ann Arbor, Michigan, USA). This was performed after raising the tail 90º to a horizontal position and repeated measurements were taken 3 times. The average measurement was then recorded as either the right (R0) or left (L0) time 0 h measurement. The locations of the two measurements were cleaned with 70% isopropyl alcohol. The right side received an intradermal injection of 0.1 mg candin (C. albicans allergen extract, Greer Laboratories, Inc.) suspended in 0.1 mL PBS. The left tail fold (control side) received 0.1 ml PBS intradermally. All injections were given with a 28 gauge needle. To identify the exact location of the measurement and injection, paper white-out solution marked the spot. Twenty-four hours later, injection sites were measured again to determine the increase in double skin-fold thickness for the right side (R24) and left side (L24) as an indicator for the magnitude of the DTH (CMIR) response.

Classification of Cell-Mediated Immune Response

To obtain a normal distribution, log transformations of the DTH measurements were performed. The magnitude of the DTH response was determined by the following (Eq. 5-1):

$$y = \ln(R24) - \ln(R0) \qquad (5\text{-}1)$$

A repeated measures preliminary analysis using the proc mixed procedure of SAS determined that multiparous cows tended to respond better than primiparous cows ($p < 0.064$) (Figure 5-1, Figure 5-2). Due to this effect of parity, determination of CMIR categorization occurred within parity.

To extrapolate CMIR categorizations the mean and standard deviation for the "y" values were configured for all cows respective of parity. Cows with "y" values above the mean plus one standard deviation were classified as high CMIR responders. Those cows below one standard deviation less than the mean were classified as low CMIR responders. All animals within one standard deviation of the mean were medium responders.

Diseases

Identification of disease was performed by farm personnel who were blinded to immune response categorizations. Disease information was collected for; mastitis, metritis, retained fetal membrane, ketosis, and displaced abomasum. All diseases were recorded as yes/no binary responses for the trial period of the current lactation.

Mastitis was recorded through 365 DIM as light, medium, or severe. Severity was recorded as light if there were no systemic signs and milk was slightly watery with minimal clots (gargot). Severity was medium if there were no systemic signs and a substantial amount of clots detected in the milk. Severity was severe if there were systemic signs, watery milk, and a substantial amount of clots in the milk.

Metritis was recorded through 30 DIM as light, medium or severe. Severity was light if there was an abnormal vaginal discharge and a palpable uterine lumen. Severity was medium if there was a purulent vaginal discharge, with an enlarged, not-flaccid uterus. Severity was severe if there was a purulent foul-smelling vaginal discharge, with an enlarged flaccid uterus.

Association between energy-related metabolic conditions and CMIR categorization were also considered. Ketosis was recorded through 30 DIM as light, medium, or severe. Ketosis was coded as light if the urine contained 15 mg/dL of ketone bodies. Severity was medium if the urine contained 40 mg/dL of ketone bodies. The severity was severe if the urine contained > 80 mg/dL of ketone bodies. Displaced abomasum was recorded through 16, and 50 DIM.

Due to the involvement of the immune system in determining the expulsion of fetal membranes (Kimura et al., 2002), this study also analyzed the association between RFM and CMIR categorization. Retained fetal membranes were identified if still retained 24 h postpartum.

Milk Yield, Somatic Cell Score, and Reproductive Efficiency

Milk yield data was gathered from the Dairy Herd Information Association (DHIA) records for the current lactation using ME305 which is an estimate of the milk yield for 305 DIM. For categorical data, low milk producers were identified if there 305 day milk was in the bottom 25% of the study group. High milk producers were those in the top 25% of the study group. The remaining middle 50% were classified as medium milk producers.

Somatic cell score (SCS) was obtained from DHIA records. The average SCS was determined for the first 3 test days, the first 6 test days, and for the first 10 test days, which are an indication of the average SCS for the first 90, 180, and 300 DIM respectively.

To test for associations between CMIR categorization and reproductive efficiency, a binary pregnancy term was used which simply indicated if a given cow was pregnant by 150 DIM. The number of number of days not pregnant (days open) and number of times bred were used as quantitative variables.

Statistics

To test for an association between CMIR categorization and the binary terms of specific disease risk and reproductive efficiency, a logistic regression model was used using the LOGISTIC procedure of SAS. Other than the main effect (CMIR categorization), possible explanatory variables include:

- BCS_x = categorical effect of body condition score for sampling week "x".
- ΔBCS_y = quantitative variable, change in body condition score over interval "y".
- Dex = binary effect, whether cow received dexamethasone prior to calving.
- Parity = binary effect, either primiparous or multiparous.

- RFM = binary effect, incidence of retained fetal membrane.
- CDiff = binary effect, if reported difficult calving.
- Ketosis = binary effect, incidence of ketosis.
- SCSavg = somatic cell score average over 10 monthly test days.

For analyzing the relationship between CMIR categorization and the quantitative variables for SCS, 305 day milk yield, and reproductive efficiency, linear regression was used with the REG procedure of SAS. All categorical variables were analyzed using logistic regression with the LOGISTIC procedure of SAS.

All relevant effects were put in the model. A backword elimination procedure was used to determine the final model. Explanatory variables remained in the model if they showed a tendency ($p < 0.10$) to predict the outcome variable. Statistical significance was determined by setting $\alpha = 0.05$.

Results

Mastitis

Incidence of all types of mastitis within 100 DIM was not significantly associated with CMIR status. However, when only medium and severe cases of mastitis within 365 DIM were considered (138 cases in multiparous cows and 42 cases in primiparous cows), CMIR status was significantly associated with mastitis occurrence (Type 3 analysis $p = 0.041$). Parity and SCSavg were also significant variables in the model. Those cows categorized as medium responders were 2.14 (CI = 1.13 – 4.08) times more likely to develop a medium or severe case of mastitis than high responders. When considering only multiparous cows, those categorized as low and medium responders were 2.80 (CI = 1.29 – 6.09) times more likely to develop a medium or severe case of mastitis than high responders.

Retained Fetal Membrane

There were 61 recorded cases of RFM (7.5%). Of these 61, primiparous cows accounted for 22 while multiparous cows accounted for 39. Contributing model effects were: BCS_0 and ΔBCS_5. The CMIR categorization was a significant predictor for the risk of RFM ($p = 0.0001$) (Table 5-1). Low cell-mediated immune responders were 6.68 (CI = 1.87 – 23.84) times more likely to have a case of RFM than high immune responders. Also, if only considering multiparous cows, low cell-mediated immune responders were 26.52 (2.30 – 306.11) times more likely to have an RFM than high cell-mediated immune responders (Figure 5-3).

Metritis

For this analysis there were 43 cases of light, medium or severe metritis within 30 DIM (5.3%). Primiparous cows accounted for 29 cases while multiparous cows accounted for 14. The remaining model contributing effects were: BCS_0, ΔBCS_5, Dex, Parity, and RFM. Although not significantly different, the medium immune responders were 7.40 (CI = 0.91 – 60.25) times more likely to develop metritis than high immune responders (Table 5-1).

Ketosis and Displaced Abomasum

There were 48 recorded incidences of light, medium, or severe ketosis within 30 DIM (6.9%). Twenty-seven cases were observed in primiparous cows and 21 cases in multiparous cows. Remaining model effects were Dex and ΔBCS_6. The categorization for CMIR was not a significant predictor for risk of ketosis ($p = 0.97$) (Table 5-1).

There were only 21 recorded incidences of DA, 13 in primiparous and 8 in multiparous cows. Contributing model effects were: Ketosis, ΔBCS_4, and Dex. The categorization for CMIR was not a significant predictor for incidence of DA (Table 5-1).

Milk Yield, Somatic Cell Score and Reproductive Efficiency

For the analysis of the effect of CMIR categorization on milk yield, the contributing model effects were parity, days open, and the binary variable mastitis. In this analysis, CMIR categorization was a significant predictor of milk yield ($p = 0.049$, $\beta = 508.08$) (Figure 5-4).

The analysis of the effect of CMIR on SCS included: ΔBCS_6, milk categorization, and a binary mastitis variable. The effect of CMIR on SCS was not a significant predictor of SCS ($p = 0.83$).

The model analyzing the effect of AMIR category on pregnancy by 150 DIM, included ΔBCS_4, and milk categorization. The effect of CMIR was not a significant predictor for pregnancy by 150 DIM ($p = 0.77$).

Discussion

The significant association between peripartum DTH response and mastitis identifies the role CMIR has on resisting infection in the mammary gland and the validity of using DTH to *Candida albicans* as a measure of CMIR. Although not significant, the estimated odds of metritis show promise for the ability of DTH to predict metritis infection (Table 5-1).

The results for RFM were in agreement with previous literature (Kimura et al., 2002). Although in the current study, because categorization of CMIR occurred early postpartum, one could speculate that RFM served to suppress immune response. However, in Kimura et al (2002), neutrophil activity was suppressed 15 days prepartum in cows with RFM.

The results for the association between milk yield and CMIR categorization prove that selection for improved CMIR does not predispose cows to lower milk production. In this study the significant effect of CMIR categorization on milk yield followed the pattern of High > Medium > Low. Because selection for improved immune response should include both CMIR and AMIR, the potential negative association between AMIR and milk yield should balance out

due to the positive effect of CMIR on milk yield. Previous studies in dairy cows have not studied the association between CMIR categorization and milk yield. Mallard et al. (1998) categorized CMIR in pigs and found greater growth rates in pigs with superior immune responsiveness.

Selection for decreased somatic cell score (SCS), increased productive life (PL), and improved structural traits of the udder, are all methods currently used to reduce incidence of disease. These methods, however, are largely based on fairly crude biological associations with disease risk and due not address the immune system which is principally responsible for host disease resistance. These traits are also not concerned with broad-based resistance to disease. Selection for improved immune responsiveness as a means to reduce the risk of disease should take a broad based approach. This is primarily due to the vast array of potential pathogens and the various virulence mechanisms employed to initiate disease. This broad based philosophy also becomes critically important due to the complexity of the immune system *in vivo*. Selection for specific improvements may prove inadequate, but may also confer unexpected disease susceptibility to additional varieties of pathogens. Inverse relationships between branches of adaptive immunity shed light on the potential for this to occur (de Vries, 1995; Biozzi et al., 1979; Rupp et al., 2007). If there is one thing that should be learned from the past 5 decades of applying selection pressure, it is that genetic selection should not focus or put too much pressure on specific traits. When this happens it is inevitable that unexpected unfavorable trends occur where selection pressure is neglected.

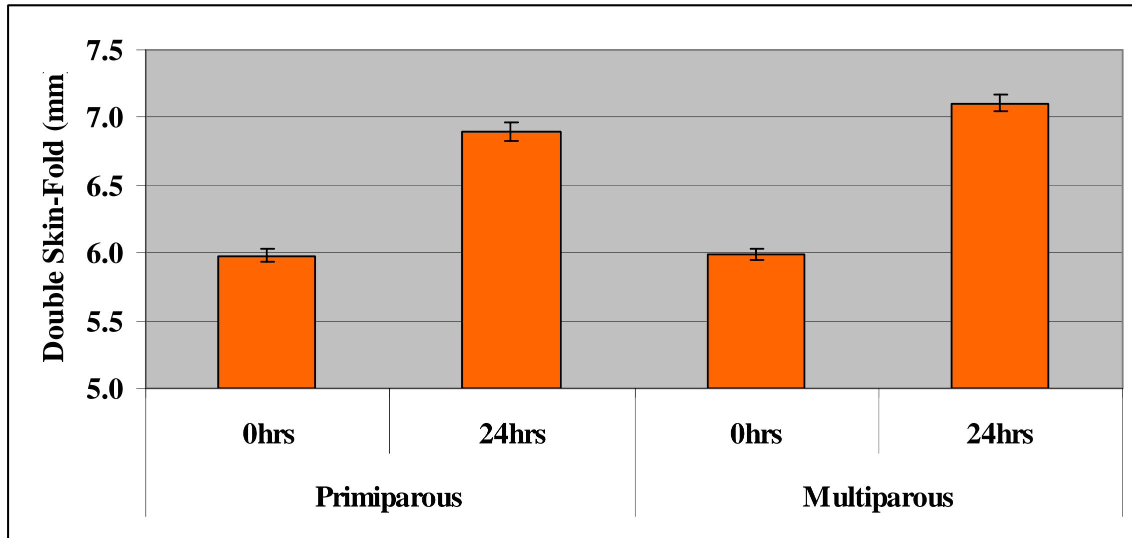

Figure 5-1. Graph showing increase in double skin-fold thickness respective of parity (multiparous or primiparous).Bars reflect SEM.

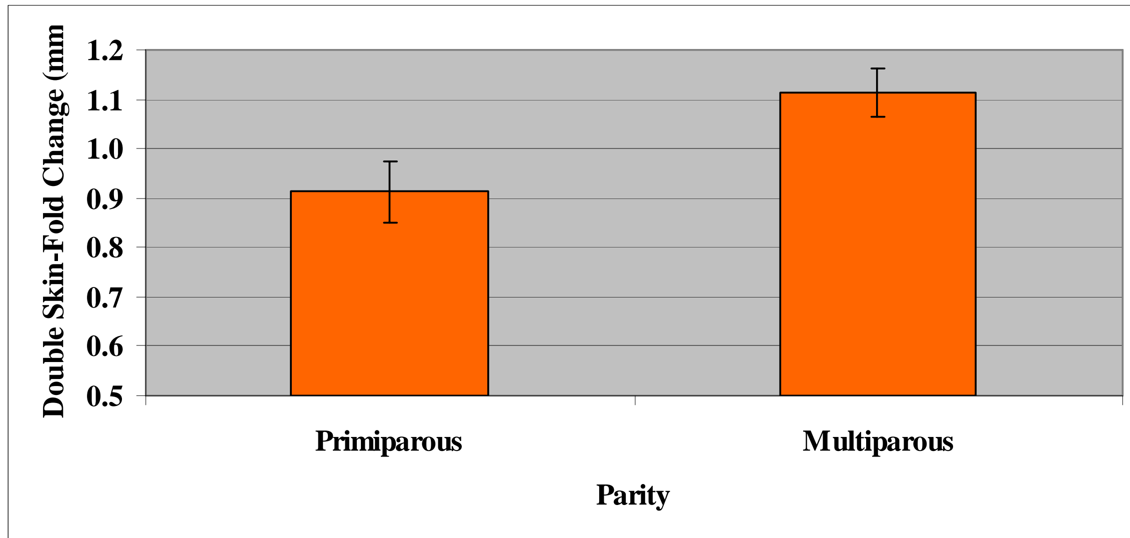

Figure 5-2. Graph revealing parity difference for cell-mediated immune response. Bars indicate SEM.

Table 5-1. Odds ratios of disease incidence for cell-mediated immune categorizations.

	Mastitis	Metritis	Ketosis	DA	RFM
Low vs Med & High	0.99 (0.64 - 1.53)	1.06 (0.39 - 2.92)	1.08 (0.56 - 2.06)	1.02 (0.30 - 3.52)	2.85 (1.67 - 4.86)
Low vs High	1.45 (0.63 - 3.31)	2.97 (0.29 - 30.51)	1.14 (0.33 - 3.95)	1.56 (0.12 - 20.21)	6.68 (1.87 - 23.84)
Med vs High	2.14 (1.13 - 4.08)	7.4 (0.91 - 60.25)	1.05 (0.40 - 2.78)	2.28 (0.27 - 19.28)	1.94 (0.58 - 6.45)

Values indicate the estimated odds ratios for incidence of disease for the following comparisons among cell-mediated immune categorizations. Values in parenthesis represent the 95% confidence intervals for the odds ratio estimate.

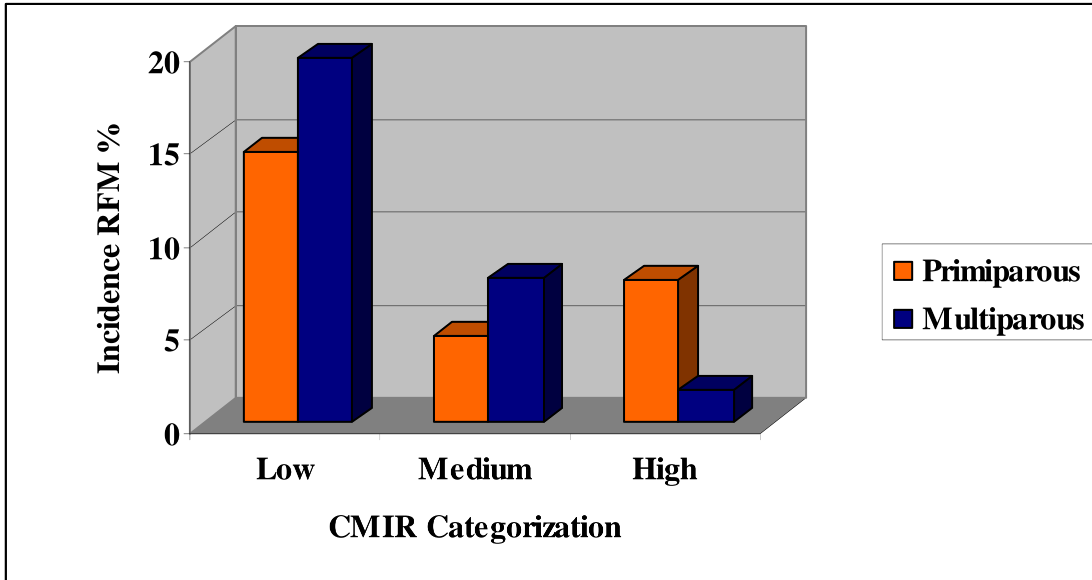

Figure 5-3. Graph indicating the difference in risk of RFM between high and low cell-mediated immune response categorization.

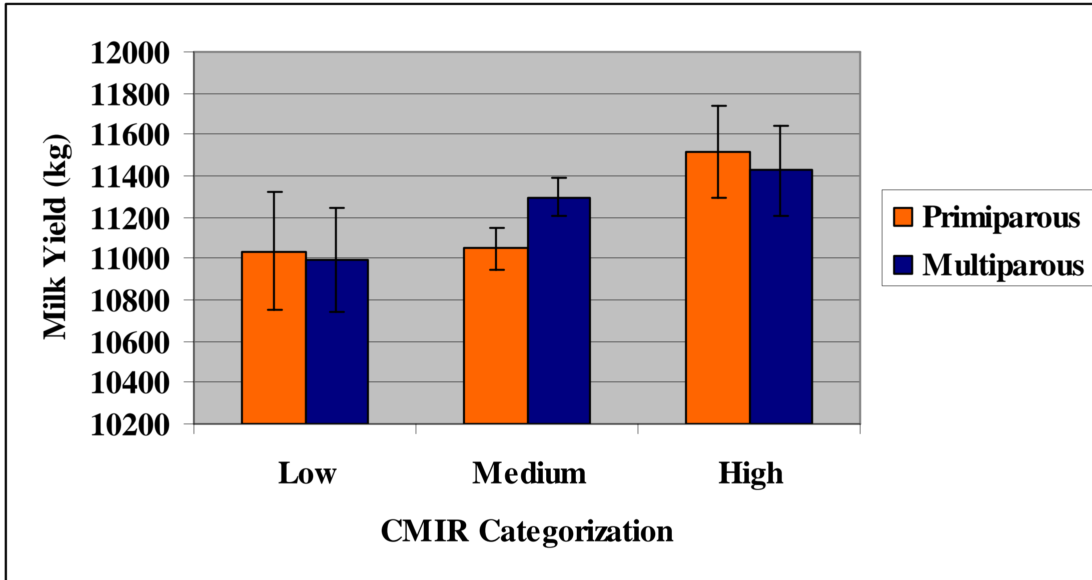

Figure 5-4. Graph for the effect of cell-mediated immune response (CMIR) categorization on milk yield. Bars indicate SEM.

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BIOGRAPHICAL SKETCH

Jason De La Paz was born in Tampa, Florida in 1977. He realized his interest in animals at an early age, and although never growing up on a farm he discovered a fascination for dairy cows while working toward an animal science degree at the University of Florida. During the four years of his undergraduate college education, he held a part-time position working as a veterinary technician. Jason received his bachelor's degree in 2001 and soon thereafter moved to Minnesota after accepting a position as a reproductive specialist for ABS Global, which is a bovine genetics company. In this position, he managed the reproductive concerns for several large farms in north central Minnesota. With family, warmer climate and saltwater fishing awaiting him back in Florida, he took a position with ABS Global which allowed him to move back. After working this position for a few years, Jason began pursuing a Master of Science degree at the University of Florida college of Veterinary Medicine. With Dr. Arthur Donovan as chair of Jason's supervisory committee, Jason received his Master of Science degree in August 2008 where he studied how to determine the immune response potential of individual Holstein dairy cows and how this is associated with disease risk.

For the years to come, Jason intends on continuing his focus on disease resistance through genetic selection for increased immune responsiveness. He has been married since 2002 to Amy, the woman he dated since high school. They now reside in Ocala, Florida with their two-year-old daughter Emily. His interests include gator football, fishing, and tennis.