IMMUNE FUNCTION, GENE EXPRESSION, BLOOD INDICES AND PERFORMANCE IN TRANSITION DAIRY COWS AFFECTED BY DIET AND INFLAMMATION

BY

DANIEL E. GRAUGNARD

DISSERTATION

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Doctoral Committee:

Associate Professor Juan J. Loor, Chair
Associate Professor James K. Drackley
Associate Professor Walter L. Hurley
Professor Giuseppe Bertoni, Universita Cattolica del Sacro Cuore, Piacenza, Italy

ABSTRACT

The transition period is associated with the peak incidence of production problems, metabolic disorders and infectious diseases in dairy cows. During this time the cow's immune system seems to be weakened; it is apparent that metabolic challenges associated with the onset of lactation are factors capable of affecting immune function. However, the reasons for this state are not entirely clear. The negative energy balance associated with parturition leads to extensive mobilization of fatty acids stored in adipose tissue, thus, causing marked elevations in blood non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHBA) concentrations. Prepartal level of dietary energy can potentially affect adipose tissue deposition and, thus, the amount of NEFA released into blood and available for metabolism in liver. The current feeding practices for pregnant non-lactating cows have been called into question because increasing amounts of moderate-to-high energy diets (i.e. those more similar to lactation diets in the content of energy) during the last 3 wk postpartum have largely failed to overcome peripartal health problems, excessive body condition loss after calving, or declining fertility. Current prepartal feeding practices can lead to elevated intakes of energy, which can increase fat deposition in the viscera and upon parturition lead to compromised liver metabolism. Our general hypothesis was that overfeeding dietary energy during the dry period, accompanied by the metabolic challenges associated with the onset of lactation would render the cow's immune function less responsive early postpartum. The chapters in this dissertation evaluated neutrophil function, metabolic and inflammation indices and gene expression affected by the plane of dietary energy prepartum and an early post-partum inflammatory challenge in dairy cows. The diet effect in this experiment was transcendental during the transition period and potentially during the entire lactation. Changes in energy balance were observed and provided a good model to study the challenges

associated with the onset of lactation. Overall the LPS model provided a consistent response representing an inflammation incident; however the changes in metabolic indices were sudden and hard to detect in most of the cases during the days following the challenge. In general overfeeding dietary energy during the dry period resulted in a less responsive immune function during the early postpartum. In other words, controlling the dietary energy prepartum has more benefits for the dairy cow during transition.

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INTRODUCTION

The transition period is associated with the peak incidence of production problems, metabolic disorders and infectious diseases in dairy cows (Drackley, 1999). During this time the cow's immune system seems to be weakened; it is apparent that metabolic challenges associated with the onset of lactation are factors capable of affecting immune function. However, the reasons for this state are not entirely clear (Goff, 2006). The negative energy balance associated with parturition leads to extensive mobilization of fatty acids stored in adipose tissue, thus, causing marked elevations in blood non-esterified fatty acids (**NEFA**) and β-hydroxybutyrate (**BHBA**) concentrations (Drackley et al., 2001). Prepartal level of dietary energy can potentially affect adipose tissue deposition and, thus, the amount of NEFA released into blood and available for metabolism in liver (Drackley et al., 2005). The current feeding practices for pregnant non-lactating cows has been called into question because increasing amounts of moderate-to-high energy diets (i.e. those more similar to lactation diets in the content of energy) during the last 3 wk postpartum have largely failed to overcome peripartal health problems, excessive body condition loss after calving, or declining fertility (Beever, 2006). Current prepartal feeding practices can lead to elevated intakes of energy, which can increase fat deposition in the viscera and upon parturition lead to compromised liver metabolism (Beever, 2006, Drackley et al., 2005). Our general hypothesis was that overfeeding dietary energy during the dry period, accompanied by the metabolic challenges associated with the onset of lactation would render the cow's immune function less responsive early postpartum. The chapters in this dissertation evaluated neutrophil function, metabolic and inflammation indices and gene expression affected by the plane of dietary energy prepartum and an early post-partum inflammatory challenge in dairy cows.

LITERATURE REVIEW

The Transition Period

The transition period is defined as the stage in the lactation cycle where the cow undergoes a transition from being pregnant and non-lactating into the beginning of lactation after parturition (Drackley, 1999). The duration of the dry period varies according to management strategies and has been divided into the "far-off" (generally the first 4 to 6 wk after dry off) and the "close-up" (generally the last 3 wk before expected parturition) (Dann et al., 2006). The length of the transition period encompasses the last 3 wk of the dry period (from the close-up) until 3 wk after parturition (Drackley, 1999, Grummer, 1995).

Nutrient demands for support of fetal growth and initiation of milk synthesis are increased during the transition period (Grummer, 1995). In contrast, this period is characterized by a 30% reduction in dry matter intake (Kedmi and Peer) at 5 to 7 d prepartum followed by a steady increase from 0 to 21 (but also after it) d postpartum (Bertics et al., 1992). Marked changes in endocrine status occur to prepare parturition and lactogenesis (Grummer, 1995). Progesterone concentrations during gestation remain elevated to maintain pregnancy but concentrations decline rapidly approximately 2 d before calving; in the same fashion, estrogen increases in plasma during late gestation but decreases immediately at calving (Chew et al., 1979). Growth hormone increases gradually as the cow progresses from late gestation into early lactation; however, the peak occurs at parturition (Kunz et al., 1985). Plasma NEFA increase slightly around 2 wk before parturition and the concentration increases dramatically around and after parturition (Grummer, 1995). Plasma thyroxine and triiodothyronine concentrations gradually increase during late gestation, decreasing substantially at calving and increasing once again few weeks after parturition (Kunz et al., 1985). Prolactin increases shortly before

parturition (Farmer and Petitclerc, 2003) and Glucocorticoid and prolactin concentrations increase on the day of calving and return to near prepartum concentrations soon after parturition (Edgerton and Hafs, 1973). The increase in nutrient demand, the drastic changes in endocrine status and the decrease in DMI during late gestation influence metabolism; in particular the metabolism of lipid (Grummer, 1995). Adipose tissue, liver, gut, and mammary gland are key components of the adaptations that dairy cows experience to achieve the necessary balance to adapt to the onset of lactation (Drackley, 1999).

The transition period is considered the most important phase during the lactation cycle since a successful transition can effectively determine a profitable lactation (Drackley, 1999). However, immunosuppression during this period leads to increased susceptibility to invading pathogens (Mallard et al., 1998) and the incidence of health problems during this time relative to the rest of the lactation cycle is significantly greater (Drackley, 1999). In addition, the transition period is where the risk for mammary infections, displaced abomasums, milk fever, ketosis, retained fetal membranes and metritis is at a peak (Shaver, 1997, Smith et al., 1985). Besides infectious diseases it is also important to mention that a high susceptibility for metabolic disorders occurs during this time (Drackley, 1999) and these can be also responsible of pro-inflammatory cytokine raise with consequent negative effects (Bertoni et al., 2008).

Controlling Energy during the Dry Period

Energy consumption (or dry matter intake) may be the determining factor for the success of the transition period (Drackley, 1999). Conventionally at dry-off, cows are fed high forage rations with higher fiber content compared to the lactation diet; this change affects the bacterial population, the absorptive capacity and size of the rumen papillae and consequently the

absorption capacity of VFA in the rumen (Goff and Horst, 1997). Cows remain on high fiber diets until the close-up period when rations of higher energy and nutrient density are fed ("steam-up" diets) in an effort to adapt the rumen microbial population and papillae to the high-grain diets fed after calving (Grummer, 1995). Regardless of the diet adjustments during the dry-off and close-up periods, there is evidence that dairy cows can easily consume more energy than required during these times (Dann et al., 2006, Janovick and Drackley, 2010) except the fiber, energy and protein contents are specifically and well balanced (Bertoni and Trevisi, 2008). Overall, research data is unsuccessful demonstrating that steam-up diets reliably and repeatedly improve production, body condition, or the immune status of the transition cow.

Our group at the University of Illinois has been doing extensive research whether controlling energy intake during the dry period might lead to a better transition (Dann et al., 2006, Douglas et al., 2006, Janovick and Drackley, 2010) The strategy used is to formulate and feed rations with relatively low and diluted energy density (1.30 – 1.39 Mcal NE_L/kg DM) during the entire dry period. The incorporation of low energy ingredients (straw or low quality grass hays) is key in these types of rations since they allow cows to consume *ad libitum* without exceeding their daily energy requirements (Janovick and Drackley, 2010). Controlling energy intake, with high fiber rations, seems to improve DMI after parturition avoiding excessive adipose tissue mobilization (Douglas et al., 2006). Milk production appears to be similar to the yield obtained with higher energy close-up programs (Douglas et al., 2006, Janovick and Drackley, 2010). Some of the benefits of feeding low energy- high fiber rations include a reduction in the incidence of displaced abomasum resulting from greater rumen fill that is maintained for longer periods, thus, helping regulate feed intake. Body condition, reproductive success and foot health also may be improved (Janovick and Drackley, 2010). From a practical

standpoint, feeding a single low energy- high fiber diet simplifies dry cow management avoiding social stress due to group changes (Cook and Nordlund, 2009) and a single group feeding instead of the two stage group approach (Dann et al., 2006). Finally, in the majority of the cases straw is readily available and likely the cost of the ration will be similar or less expensive than feeding far-off and close-up diets.

Energy Balance in the Periparturient Cow

In general, animals attempt to achieve energy equilibrium regardless of physiological and environmental circumstances using the available energy in the diet and the tissue reserves (Baile and Forbes, 1974). In the case of dairy cows during transition, as mentioned before, there is a marked decrease in DMI which in turn limits the consumption of dietary energy, and has a negative impact on the energy balance equilibrium (Bertics et al., 1992). At the same time, nutrient demands for fetus needs and for mammary gland development as well as for initiation of milk synthesis are increased aggravating the energy balance status (Grummer, 1995). After parturition, as milk production increases, the energy needed for milk production increases resulting in a stage of negative energy balance (**NEB**). To meet the energy requirements of this period, dairy cattle rely on mobilization of adipose and muscle tissue (Drackley, 1999). This period of NEB lasts until the yield of milk starts to decline (6 – 10 wk after parturition) and the energy from the DMI becomes sufficient to meet the cow's requirements (Roche et al., 2009). The degree of NEB that cows experience most likely would be a function of the milk yield since high producing dairy cows would require a greater amount of energy for lactation (Detilleux et al., 1994). A period of severe NEB, where extended mobilization of adipose tissue has occurred, could result in the incidence of metabolic disorders such as ketosis and fatty liver (Drackley, 1999).

A central area in the biology of the transition cow is related to the metabolism of lipids. In the process of mobilization of energy, the adipose, mammary and liver tissues are key components for the adaptations dairy cows undergo. Briefly, high NEFA concentrations in the bloodstream are a consequence of hydrolyzed adipose tissue triacylglycerol by the action of hormone sensitive lipase (Zammit, 1984). At this point NEFA can be utilized as an energy source by other tissues (Drackley, 1999). However, the liver is the most important site for removal of NEFA from circulation (Bell, 1979); NEFA that reach the liver are extracted in a concentration-dependent manner and converted to acyl-CoA by the enzyme acyl-CoA synthethase. Carnitine plays an important role in the transport of acyl-CoA into the mitochondria matrix, where β-oxidation will occur (Zammit, 1984). In the mitochondria, acyl-CoA is oxidized through β-oxidation into acetyl-CoA and can be further oxidized for energy in the citric acid (**TCA**) cycle. However, if the amounts of acetyl-CoA generated in fatty acid β-oxidation challenge the processing capacity of the TCA cycle or if activity in the TCA cycle is low due to low amounts of intermediates such as oxaloacetate, acetyl-CoA is then used for biosynthesis of ketone bodies (Drackley, 1999). Partially oxidized acetyl-CoA is converted into the ketone bodies acetoacetate (**ACAC**), BHBA, and acetone, which are released from the liver into the blood (Bell, 1979). Ketones can be used as an alternate water-soluble fuel source by many tissues (e.g., heart and skeletal muscle) when glucose is limited (Leslie et al., 2000). However, if the rate of lipid mobilization exceeds the rate of ketone body utilization, then ketones accumulate and may adversely affect the health and productivity of the cow (Ingvartsen and Andersen, 2000).

Ketosis and Liver Lipidosis

Ketosis is characterized by marked increases in circulating ketones and primarily occurs within a few weeks after parturition; this problem is closely related with the typical decrease of DMI that occurs before parturition and in many cases is aggravated when feed intake is significantly reduced after calving (Ingvartsen and Andersen, 2000). Ketosis can occur as either a sub-clinical or clinical condition. Sub-clinical ketosis is characterized by greater than normal concentrations of ketones in circulation (1000-12001400 µmol/L) with no adverse effects observed (Duffield et al., 2009). Clinical ketosis is characterized by even higher concentrations of circulating BHBA along with physical signs such as loss of appetite (decreased DMI), a decrease in blood pH and a drop in body weight and body condition score. With the drop in DMI, there is commonly observed a drop in milk yield, and an increase in susceptibility to infectious diseases (Gerloff, 2000). Constant supervision and treatment is necessary in the clinical cases. In the worst cases culling or even death occurs (Geishauser et al., 1998, Kremer et al., 1993). Nearly 50% of high-producing cows experience a case of sub-clinical ketosis and approximately 6% of the sub-clinical cases proceed to clinical ketosis in early lactation (Geishauser et al., 1998, Grohn et al., 1989).

Another fate NEFA can undergo in the liver is to be esterified and exported as triglycerides within very low density lipoproteins (**VLDL**) to extra hepatic tissues such as the mammary gland, where the fatty acids are incorporated into milk fat TG (Smith et al., 1997). However, this last outcome occurs to a limited extent in dairy cows and when cows consume excess energy during the dry period it frequently results in the metabolic disorder of fatty liver (Drackley, 1999). During the transition period the ability of the liver to utilize NEFA and secrete triglycerides decreases as the duration and seriousness of NEB increases (Morrow, 1976). A

further cause of lipidosis can be a lower synthesis of apo-lipoproteins in case of serious inflammations at calving time (Bertoni et al., 2004). Therefore, triglycerides tend to build up in the liver, decreasing liver function and eventually causing liver lipidosis (Drackley, 1999). When the amount of lipid infiltration in the liver becomes severe dairy cows are more susceptible to other pathologies and at the same time, when a treatment is functional the recovery period is prolonged (Herdt, 1988).

Immune cells and Mechanisms of Defense

The immune system is integrated by a diversity of cells and molecules that are capable of recognizing and eliminating invading foreign microorganisms in a specific manner (Baumann and Gauldie, 1994). There are two mechanisms of defense: innate and specific. Both mechanisms interact in order to recognize and discriminate between foreign substances and the host's own molecules (Kehrli and Harp, 2001). Among the most common immune cells are the macrophages; they are the first line of defense against invading microorganisms and play a key role in the innate immune response. Macrophages evolve from monocytes. They are originated in the bone marrow during hematopoiesis, and then enter the blood where they differentiate into mature monocytes. Within 8 h, circulating monocytes will enlarge and migrate into tissues where they further differentiate into tissue-specific macrophages (Goldsby et al., 2000). They detect and recognize non-specific foreign pathogens locally and produce cytokines that initiate the immediate sets of reactions that are known as the acute phase response (Baumann and Gauldie, 1994). Also they recruit other immune cells to the site of infection and are the bridge between the innate and specific immune responses through antigen presentation to prime T cells (Rainard and Riollet, 2006)

The neutrophils are known as polymorphonuclear (**PMN**) leukocytes for their multilobed nucleus and granulated cytoplasm; these cells constitute up to 70% of the circulating white blood cells (Goldsby et al., 2000). After initiation of the inflammatory response, PMN become the predominant cell type observed during an infection. Like macrophages, PMN are originated during hematopoiesis in bone marrow, which takes approximately 10 to 14 d for PMN to mature (Bainton et al., 1971). In circulation, the half-life of PMN is short (8.9 h), and once in tissues, PMN function for only 1 to 2 d (Paape et al., 2002). The life span of PMN is tightly regulated because these cells can cause tissue and blood vessel damage through the production of reactive oxygen metabolites during a "respiratory burst", as well as granular secretion of antimicrobial proteins. Therefore, PMN undergo apoptosis once they are released from the bone marrow to minimize host tissue damage (Capuco et al., 1986). During an infection, PMN migrate from blood to the tissue in response to chemoattractants secreted from either macrophages or the epithelial cells within the tissue (Goldsby et al., 2000). During diapedesis (migration from blood into the tissue), chemoattractants stimulate endothelial cells to express specific molecules (i.e., E-selectin and P-selectin) that allow the PMN to adhere to the endothelial cell surface.

Lymphocytes are produced in bone marrow by white blood cells through hematopoiesis and become activated due to response to a local antigenic stimulation (Asai et al., 1998). They proliferate and recognize foreign antigens through membrane receptors. Lymphocytes consist of T and B lymphocytes. The T cells can be sub divided into T-helper and T-cytotoxic (**CTL**) lymphocytes. The T-helper cells produce cytokines, such as interleukin- 2 (**IL-2**) and interferons (**IFN**), which are crucial for an effective cell mediated immune response. The B lymphocytes differentiate to produce proteins called antibodies or immunoglobulins (**Ig**) and effector B cells, or plasma cells (Sordillo et al., 1997). Plasma cells are important for the specific immunity; one

of the main functions is the production of antibodies. Immunoglobulins, such as IgG_1, IgG_2, and IgM, are the primary defense mechanisms for specific immunity (Paganelli et al., 1984).

The Acute Phase Response

In the presence of injury, trauma or infection the host organism in an effort of preventing further damage, executes a series of vascular reactions that cause inflammation. The beginning of this cascade of events is part of the innate immunity and is commonly associated with tissue macrophages. Activated macrophages release a broad spectrum of mediators in which cytokines interleukin-1 (**IL-1**) and tumor necrosis factor (**TNF**) play an important role recruiting immune cells (Baumann and Gauldie, 1994). These cytokines perform different functions locally and systemically. Locally they act on stromal cells causing a release of a secondary wave of cytokines; some of these cytokines might have been released earlier by the macrophages, however, it is this secondary wave the one that increases the homeostatic signal that initiates the acute phase response (**APR**). The secondary wave of cytokines released by the local tissues includes interleukin-8 (**IL-8**) and monocyte chemoattractant protein (**MCP**). These chemokines (cytokines with chemotactic capacity), appear to control the migration of immune cells. Migration from the blood to the affected tissue takes place by diapedesis (Suriyasathaporn et al., 1999). The main cells that respond to the innate response are PMN and monocytes that will control in a specific manner any invading microorganisms (Paape et al., 1991). During the progressing APR, leukocytes also synthesize and release their own particular set of cytokines within the tissue. At this point there is an alteration of the temperature set point in the hypothalamus generating a febrile response that will inhibit the growth of pathogens (Baumann

and Gauldie, 1994). If the infection persists, at this point the specific immunity, also known as humoral or acquired immunity, takes control (Mallard et al., 1998).

Phagocytosis

Some of the immune cells in the innate and specific system perform phagocytosis. This process consists in the ingestion of microorganisms by phagocytic cells (Paape et al., 1979). Phagocytosis first requires the adherence and tagging for ingestion and destruction (opsonization) of macrophages or PMN to the cell wall of bacteria. Cell surface receptors found on phagocytic cells that are specific for certain opsonins, such as certain classes of antibodies and components of complement, also can enhance adherence and phagocytosis (Goldsby et al., 2000). Once the microorganism is completely surrounded and enclosed by the pseudopodia, it then enters the cytosol as a membrane-bound structure called a phagosome. The phagosome then fuses with a lysosome, which contains enzymes that digest engulfed material, to form a phagolysosome. During this process, a burst of oxidative metabolism called the respiratory burst occurs in activated phagocytes via the activation of a membrane-associated nicotinamide adenine dinucleotide phosphate (**NADPH**) linked oxidase that catalyzes the reduction of oxygen to superoxide anion ($\mathbf{O_2^-}$). After the respiratory burst, the digested contents of the microorganism are eliminated by exocytosis (Burvenich et al., 2004).

The Liver Systemic Response to Inflammation

The liver is a central organ during an inflammatory response in the organism. It is responsible for determining the level of essential metabolites during the critical stages of stress. In addition, the liver synthesizes the necessary components for immediate defense at the site of

tissue damage (Baumann and Gauldie, 1994). During an ongoing APR the liver synthesizes certain plasma proteins known as acute phase proteins (**APP**) (Koj et al., 1988). The acute phase proteins posses a pathogen pattern recognition capacity and their main role is the activation of the complement system cascade that concludes in pathogen destruction (Goldsby et al., 2000). The concentration of most of the APP increase during inflammation (positive APP); this group includes fribrinogen (**FBG**), α1-acid glycoprotein (**AGP**), haptoglobin (**HP**), α1-proteinase inhibitor (**API**), α1-antichymotrypsin (**ACT**), C-reactive protein (**CRP**), C3 complement (**C3C**), serum amyloid A protein (**SAA**), α2-macroglobulin (**A2M**) and α1-cysteine proteinase inhibitor (**CPI**). Conversely, the concentration of some APP decrease (negative APP) during inflammation; these proteins include albumin (**BSA**) and transferrin (Koj et al., 1988). However the physiological function of many APP is still not completely understood but some of these proteins are part of the innate immune system (Goldsby et al., 2000). In cattle, the most sensitive acute phase proteins are HP and SAA; the concentration of which in serum can increase over 100-fold showing a substantial rise in the response to acute inflammation. Also, a moderate APP in cattle is AGP, which has a lower relative rise indicating chronic conditions (Pyorala, 2003).

Regulation of APP synthesis in the liver is accomplished in parallel with the recruitment of immune cells by inflammatory mediators. The mediators fall into four major categories: Interleukin-6 (**IL-6**) type cytokines, IL-1 type cytokines, glucocorticoids and growth factors (Baumann and Gauldie, 1994). Interleukin-6 type cytokines include IL-6, interleukin-11, leukemia inhibitor factor (**LIF**), oncostatin M (**OSM**) and ciliary neutrophic factor (**CNTF**); this group of cytokines, especially IL-6, has been recognized to be the principal regulator of most APP including HP, FBG, API and A2M (Mackiewicz, 1992). IL-1 type cytokines include IL-1α, IL-1β, TNFα and TNFβ. These cytokines regulate AGP, SAA and CRP (Baumann and Gauldie,

1994). Glucocorticoids stimulate the expression of APP directly (AGP) and indirectly by enhancing the effect of the IL-1 and IL-6 types (Sayers et al., 1990). Finally the growth factors include insulin-like growth factor (**IGF**), insulin, hepatocyte growth factor (**HGF**), fibroblast growth factor (**FGF**) and transforming growth factor-β1 (**TGF-β1**). These mediators are able to suppress IL-1 and IL-6 type cytokines, having an indirect impact on the APP (Mackiewicz et al., 1990).

Mastitis

Mastitis is defined as mammary gland (**MG**) inflammation; it is frequently associated with the presence of a pathogen (Bradley, 2002).The decrease in profit that results from mastitis is associated with the loss in milk production, treatment costs, removal of milk from the bulk tank after treatment was administered, veterinary costs, increased labor, premature culling, and sometimes even death of the animal (Miller et al., 1993). Mastitis can be classified into sub-clinical and clinical depending on severity and duration of the inflammation. During sub-clinical mastitis, there are no visual signs of an infection, somatic cell counts (**SCC**) in milk are usually elevated, and milk production is decreased (Sordillo et al., 1997). Clinical mastitis is characterized by an elevated SCC in milk and visual signs of an infection can be observed such as clumpy, watery, bloody and/or yellowish colored milk. In addition, clinical mastitis causes a decrease in milk production and feed intake, swelling of the udder, and, in extreme cases, septicemia caused by mastitis can cause death (Constable and Morin, 2003). Milk composition can also be affected, including altered salinity, electrical conductivity, acidity, appearance, and flavor, as well as increased SCC and decreased casein and fat content (Gill et al., 1990).

A high proportion of intra-mammary infections occur during the first wk of the dry period when milk flow ceases and bacteria invade from the streak canal before the gland is fully involuted (Sordillo et al., 1997). However, mammary infections at this time often do not result in clinical mastitis. Although many of the pathogens are eliminated by the immune cells during the dry period, some are simply held in check until the beginning of the next lactation. Clinical mastitis is most likely to occur during the first mo of lactation (Oviedo-Boyso et al., 2007) and in many cases results from an infection established during the dry period or during early lactation (Goff and Horst, 1997). Once a pathogen is detected by the receptors in the epithelial cells of the MG the APR response begins, the immune system is activated to eliminate the pathogen. This defense mechanism includes anatomical, cellular, and soluble factors that act in coordination and are crucial to the modulation of the MG resistance and susceptibility to infection (Oviedo-Boyso et al., 2007).

The pathogens associated with mastitis can cause significant and irreversible damage to the MG. In most of the cases the pathogens consist primarily of bacteria but, in rare cases, yeasts can cause infection. Mastitis pathogens can be environmental or contagious (Anderson et al., 1982). Environmental pathogens are commonly found in the environment such as manure, soil, and bedding. These organisms enter the MG, proliferate, and trigger a host immune response that kills the invading bacteria in most cases. The major environmental bacteria are coliforms such as *Escherichia coli*, *Klebsiella* species, and environmental *Streptococcus* and *Enterococcus*; however, *Streptococcus uberis* can persist and become a chronic infection (Sordillo et al., 1997). One of the most important environmental bacteria that cause bovine mastitis is *Escherichia coli.* Mastitis caused by *E. coli* can be resolved in a few days and it is characterized by pain, inflammation of one or all MG quarters, fever, and milk with clots and abnormal appearance

(Burvenich et al., 2003). Apparently, the severity of mastitis depends on the bacterial strain that infects the MG, and the speed with which neutrophils move from the bloodstream to the MG depends on the severity of mastitis. Neutrophil recruitment to an MG infected with *E. coli* occurs with a delay of 16 h post-infection (Oviedo-Boyso et al., 2007).

Contagious mastitis is transferred by organisms that live and proliferate on or within the host and generally do not survive outside of the animal's body. They are primarily spread at or around the time of milking from one cow to another. The major contagious pathogens that colonize the MG are *Staphylococcus aureus*, *Streptococcus dysgalactiae*, *Mycoplasma* and *Streptococcus agalactiae* (Dinsmore, 2002).

Recognition of Lipopolysaccharide

When mastitis occurs, the first detection event of the immune response occurs when the invading pathogen interacts with membrane receptors of the host capable of precisely discriminating between self and non-self. These molecular sensors (e.g. Toll like receptors, CD14) are known as pathogen recognition receptors and are capable of distinguishing structures that are part of microbial species and in particular recognize molecular patterns. When such patterns are found on pathogens, they are called pathogen associated molecular pattern (**PAMP**) (Goldsby et al., 2000). Pathogens that cause mastitis have cell wall structures that are recognized by the host molecular sensors. These structures are lipopolysaccharide (Small et al., 2000), peptidoglycan (**PGN**), and lipoteichoic acid (**LTA**), which constitute the PAMP (Bannerman et al., 2004). These PAMP are recognized by Toll like receptors (**TLR**). It is now known that TLR4 recognizes the LPS of Gram-negative bacteria (e.g. E. coli) and molecules such as fibrinogen,

heat shock proteins, and polypeptides, whereas TLR2 is implicated in recognition of LTA and PGN from Gram-positive bacteria (e.g. S. aureus) (Takeuchi et al., 2000).

In acute mastitis caused by coliforms, recognition of LPS is fundamental to the MG immune response. LPS and other bacterial cell wall structural components are recognized by the TLR4 and the plasma membrane CD14 receptors. CD14 is a glycoprotein expressed on the surface of monocytes, macrophages, and neutrophils (Rietschel et al., 1998). CD14 has been assigned a functional role, serving as a receptor for LPS in association with the lipopolysaccharide-binding protein (**LBP**) (Wright et al., 1990). LPS-binding protein is an acute-phase reactant produced by the liver. In the presence of LBP, CD14 has been implicated as a high-affinity LPS receptor, facilitating LPS-induced macrophage activation. In addition, several alternate LPS receptors have been described which may participate in the host response to endotoxin (e.g. CD18 and p73) (Rietschel et al., 1998).

Several studies have used LPS to evaluate the effect of the APR in production variables as well on immune response (e.g. leukocyte function). There is evidence that the LPS model generates a local and systemic action involving immune cells and the liver (Mehrzad et al., 2001). Findings indicate that the liver produces inflammatory cytokines and SAA and HP after mastitis induced with LPS (Vels et al., 2009).

Gene Expression Technology

New molecular biology approaches can be used to understand the basics of the models used in dairy cattle research with promising prospects for the future (Drackley et al., 2006). The technique broadly known as microarray allows direct and simultaneous comparison of multiple samples (Liang et al., 2002) so that relative changes in gene expression between and within

individuals can readily be identified. Microarray based technologies for gene expression are widely popular in the scientific community and provide several advantages over other techniques (Schena et al., 1995). In particular, they allow the global assessment of gene expression. It is therefore possible to asses not only the expression status of a particular gene, but also the expression pattern of that gene relative to others, which clearly represents an useful approach for understanding polygenic regulation of complex processes. There are a variety of microarray platforms that have been developed (Cummings and Relman, 2000). Basically a glass slide or membrane is spotted or arrayed with DNA fragments or oligonucleotides that represent specific coding regions of genes. Construction of microarrays is generally dependent on information gained from genome sequencing or EST (expressed sequence tag) projects that provide large sets of annotated clones and sequences (Rimm et al., 2001). The construction of cDNA microarray slides is based on fragments of candidate genes provided by the investigator. PCR products or oligonucleotides are directly spotted or printed onto glass slides to develop a microarray (Kauraniemi et al., 2001). Oligonucleotides can be designed and purchased from a number of commercial providers. Purified RNA is then fluorescently or radioactively labeled and hybridized to the slide/membrane. In some cases, hybridization is done simultaneously with "reference" RNA to facilitate comparison of data across multiple experiments. Signal intensity data for each spot on a microarray is obtained by laser scanning or autoradiographic imaging. At this point, the data may then be entered into a database and analyzed.

The University of Illinois has developed a 13,257 oligonucleotide bovine microarray, which essentially represented an expansion of the 7,000 cDNA microarray platform developed originally (Everts et al., 2005). Details of the development of the microarray plataform used can be found in the Supplementary Materials and Methods from (Loor et al., 2007). Briefly, an

embryonic bovine cDNA library and 38,732 high-quality expressed sequence tag (EST) sequences based on the cattle 7,872 cDNA array (Everts et al., 2005) were filtered for repeats as well as sequences of viral, bacterial, or mitochondrial origin using RepeatMasker (Smit and Green, 1999). Subsequently, seventy-base long (i.e., 70-mers) oligos from the unique cluster and singlet sequences were designed. Sequence alignments of designed oligos (Table 1) were done by BLASTN similarity searches against human RefSeq, mouse RefSeq, bovine RefSeq, human UniGene, mouse UniGene, bovine UniGene, bovine TIGR and the bovine genome using an E-value cut-off of $E \leq e^{-5}$ and scoring threshold of 40 bp (Everts et al., 2005). Best hits were used to annotate the cattle sequences. NCBI UniGene and Gene databases were used for functional annotation (e.g., gene symbol, gene name, function, OMIM number, PubMed identification numbers) and Gene Ontology (**GO**) annotation (Everts et al., 2005). This microarray contains ca. 97% unique elements.

Different studies have explored and linked large-scale liver (Loor et al., 2005, Loor et al., 2006, Loor et al., 2007) and mammary (Moyes et al., 2010, Piantoni et al., 2010) tissue gene expression data with typical blood metabolite, performance and liver composition data to study tissue function under different physiological conditions. The integration of functional genomics technology with measurements of metabolism obtained by conventional methods is particularly promising to find new answers (Drackley et al., 2006). Findings of the experiments previously mentioned have revealed genes that could play key roles in hepatic metabolic adaptations to negative energy balance. Coupling metabolic and performance data with gene expression allowed the development of an integrative model of liver function during ketosis (Loor et al., 2007). In the mammary gland, gene expression results have provided novel information about the early signaling and activation of pro-inflammatory pathways associated with the innate

immune response to infection. These pathways have been associated with the inhibition of lipid synthesis and PPAR signaling that could partially explain the inverse relationship between immune response and milk fat synthesis (Moyes et al., 2010). In this thesis proposal, the use of the current microarray technology is applied to actual problems of dairy cattle biology in order to uncover answers that could serve to improve the dairy industry.

Summary

The transition period is considered the most important phase during the lactation cycle (Drackley, 1999). The increase in nutrients demand, the drastic changes in endocrine status and the decrease in DMI during late gestation influence metabolism rendering dairy cows to a state of immunosuppression that leads to increased susceptibility to mammary infections (Mallard et al., 1998) and metabolic disorders (Drackley, 1999). Energy consumption may be a determinant factor for the success of the transition period; regardless of the diet adjustments during the dry-off and close-up, there is evidence that dairy cows can easily consume more energy than required during these periods (Dann et al., 2006). During NEB the cow relies on adipose tissue mobilization leading to greater incidence of metabolic disorders like ketosis and fatty liver (Drackley, 1999). The incorporation of low energy ingredients (straw or low quality grass hay) is key in the formulation of dry period rations since it allows the cows to consume *ad libitum* without exceeding their daily energy requirements. Controlling energy with high fiber rations seems to improve DMI after parturition avoiding excessive adipose tissue mobilization. Milk production appears to be similar to the yield obtained with higher energy close-up programs (Janovick and Drackley, 2010). It also simplifies the dry cow management avoiding social stress due to group changes (Cook and Nordlund, 2009).

The transition period also represents an enormous opportunity to study the acute phase response and the mechanisms of defense of the immune system. The LPS model has been used extensively to study the immune system response. Recognition of LPS is fundamental to the MG immune response. LPS is recognized by the toll-like receptor 4 and the plasma membrane CD14 receptors that work in concert with acute proteins synthesized in the liver (LPS-binding protein) in order to activate monocytes, macrophages, and neutrophils (Rietschel et al., 1998). However our group is particularly interested in finding relationships between the intensity of lipid mobilization, plasma NEFA, and bovine immune cell function through the use of microarrays developed at the University of Illinois. This resource has been used in different studies of liver (Loor et al., 2005, Loor et al., 2006, Loor et al., 2007) and mammary (Moyes et al., 2010, Piantoni et al., 2010). Data have been linked with typical blood metabolite, performance and liver composition to better understand the fundamental changes occurring in the animal.

Table 1. Annotation of the bovine microarray using Tera-BLAST. Blast searches were non-redundant. Annotation was based on $E < e^{-5}$ and/or an extension threshold of 40 (Loor et al., 2007)

Database search	No. of hits	No. of unique hits
Human RefSeq, March 2007	9,655	9,332
Mouse RefSeq, March 2007	286	285
Bovine RefSeq, March 2007	725	703
Human UniGene 201	656	641
Mouse UniGene 162	32	31
bovine UniGene 83	1,460	1,411
bovine TIGR 12	166	165
Bovine genome 3.1	223	223
No hit (putative novels)[1]	54	54
Total no. of sequences	13,257	12,845

[1]Includes 5 control sequences (soybean *MSG*, *CAB*, *RBS*, and scrambled control oligos 1 and 2).

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CHAPTER 1:

Blood Polymorphonuclear Leukocyte Function and Metabolic and Inflammation Indices in Peripartal Dairy Cows Fed Two Levels of Dietary Energy Prepartum

D. E. Graugnard,*† M. Bionaz,*† E. Trevisi, # K. M. Moyes,*† J. L. Salak-Johnson,† R. L. Wallace,‡ J. K. Drackley,†§ G. Bertoni, # and J. J. Loor*†§

*Mammalian NutriPhysioGenomics, †Department of Animal Sciences, ‡College of Veterinary Medicine, and §Division of Nutritional Sciences, University of Illinois, Urbana, IL 61801; and #Istituto di Zootecnica, Facolta di Agraria, Universita Cattolica del Sacro Cuore, 29122 Piacenza, Italy

INTRODUCTION

Peripartal cows experience some degree of negative energy balance (**NEB**) and immunosuppression (Goff, 2006). The latter is characterized by an impairment of neutrophil trafficking, phagocytosis, and killing capacity (Kehrli et al., 1989). Lymphocyte numbers decrease around parturition as a function of reduced proliferation (Kehrli et al., 1989). In addition, the cytokine and hormonal changes around parturition are closely related to neutrophil development and immunity-related activities, although these changes are poorly understood (Burton et al., 2005). It is not entirely clear why most cows experience immunosuppression around the time of calving, but it is apparent that metabolic challenges associated with the onset of lactation are factors capable of affecting immune function (Goff, 2006).

The NEB associated with parturition leads to extensive mobilization of fatty acids stored in adipose tissue, thus, causing marked elevations in blood NEFA and BHBA concentrations (Drackley et al., 2001). Prepartal dietary energy intake can potentially affect adipose tissue deposition and, thus, the amount of NEFA released into blood and available for metabolism in liver (Drackley et al., 2005). Elevated blood NEFA and BHBA as well as reduced concentrations of antigen-binding antibodies (van Knegsel et al., 2007) during peripartal NEB all can contribute to immunosuppression (Goff, 2006). Ketone body concentrations similar to those observed around parturition impair the phagocytic and bactericidal capacity of polymorphonuclear leukocytes or neutrophils (**PMN**) *in vitro* (Suriyasathaporn et al., 1999). Similarly, high concentrations of NEFA negatively affect bovine PMN *in vitro* (Scalia et al., 2006). High ketone bodies or NEFA might reduce udder defense mechanisms against mastitis pathogens.

The efficacy of current NRC (NRC, 2001) feeding practices for pregnant non-lactating cows has been called into question because increasing amounts of moderate- to high-energy diets (i.e., those more similar to lactation diets in the content of energy) during the last 3 wk postpartum have largely failed to overcome peripartal health problems, excessive body condition loss after calving, or declining fertility (Beever, 2006). Current prepartal feeding practices can lead to elevated intakes of energy, which can increase fat deposition in the viscera and upon parturition lead to compromised liver metabolism (Beever, 2006, Drackley et al., 2005). In addition, prepartal feeding practices can affect indices of metabolism and inflammation which can potentially influence immature immune cells in the peripheral circulation (Lacetera et al., 2005).

During the first wk of lactation there is a significant risk for development of new mastitis infections in the udder and new cases of clinical mastitis (Hogan et al., 1989). Our general hypothesis is that overfeeding dietary energy during the dry period, accompanied by the metabolic challenges associated with the onset of lactation, would render immune function less responsive early postpartum. The main objective of this study was to evaluate the effect of level of dietary energy prepartum on peripartal PMN phagocytosis, chemotaxis, and blood indices of metabolism and inflammation during the first wk after parturition.

MATERIALS AND METHODS

Animals and Diets

All procedures were conducted under protocols approved by the University of Illinois Institutional Animal Care and Use Committee (protocol # 06145). Twenty Holstein cows entering their second or greater lactation were enrolled in the study. The average composite SCC

was ~128,000 ± 108,000 during the previous lactation. Cows were assigned randomly (n = 10/diet) to a control diet (controlled energy, high fiber), which was fed for ad libitum intake to provide approximately 100% of calculated NE_L (1.34 Mcal/kg diet DM, Control group), or were fed a diet to provide at least 150% of calculated NE_L requirements (Overfed group, 1.62 Mcal/kg DM) during the entire 45-d dry period (NRC, 2001). Ingredient composition of the diets is reported in Table 2. Samples of feed ingredients and TMR were obtained weekly and analyzed for DM content to maintain desired ingredient ratios. Weekly samples of individual ingredients were frozen at −20°C and were composited monthly. Composite samples were sent to a commercial laboratory (Dairy One, Ithaca, NY, USA) for analysis of DM, CP, NDF, ADF, Ca, P, Mg, and K (Table 2). Diets were fed as TMR once daily (0600 h) using an individual gate feeding system (American Calan, Northwood, NH, USA).

Cows were housed in a ventilated enclosed barn during the dry period (Photoperiod 8 h light:16 h dark) and had access to sand-bedded free stalls until 5 d before expected calving date, when they were moved to an individual maternity pen bedded with straw. After parturition, cows were moved to a tie-stall barn and were fed a common lactation diet (NE_L = 1.69 Mcal/kg DM) as TMR once daily (0600 h). Cows were milked twice daily (0400 and 1600 h). Diets were mixed in a Keenan Klassik 140 mixer wagon (Richard Keenan & Co., Ltd., Borris, County Carlow, Ireland) equipped with knives and serrated paddles; straw in large square bales was chopped directly by the mixer without preprocessing.

Body weight was measured for each cow wkly. Milk weights were recorded daily and samples were obtained from consecutive a.m. and p.m. milkings. Milk samples were composited in proportion to milk yield at each sampling and preserved (800 Broad Spectrum Mirotabs II; D&F Control Systems, Inc., San Ramon, CA, USA). Composite samples were analyzed for fat,

protein, lactose, urea N, and SCC using midinfrared procedures (AOAC., 1995) at a commercial laboratory (Dairy One, Ithaca, NY, USA).

Energy Balance Calculations and Estimates

Energy balance was calculated individually for each cow using equations described previously (NRC, 2001). Briefly, net energy intake (NE_I; Mcal/d) was determined by multiplying DMI by the calculated mean NE_L density of the diet. The NE_L value of each individual feed, provided by Dairy One was used to calculate the mean NE_L content of the diet. The net energy required for maintenance (NE_M) was calculated as $BW^{0.75} \times 0.08$. Net energy requirement for pregnancy (NE_P; Mcal/d) was calculated as $[(0.00318 \times \text{day of gestation} - 0.0352) \times (\text{calf birth weight}/45)]/0.218$. Milk net energy requirement (NE_{MILK}; Mcal/kg) was calculated as $(0.0929 \times \text{fat\%} + 0.0563 \times \text{protein\%} + 0.0395 \times \text{lactose \%}) \times \text{milk yield}$. The equation used to calculate prepartum energy balance (EB_{PRE}; Mcal/kg) was $EB_{PRE} = NE_I - (NE_M + NE_P)$. The equation used to calculate postpartal energy balance (EB_{POST}; Mcal/d) was $EB_{POST} = NE_I - (NE_M + NE_{MILK})$.

Blood Metabolites

Blood was sampled from the coccygeal vein or artery at -14 (± 3 d) and 7 d relative to parturition. Samples were collected at 1200 h into evacuated tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) containing either ethylenediaminetetra acetic acid (**EDTA**) or lithium heparin for plasma and a clot activator for serum. After blood collection, tubes with EDTA and lithium heparin were placed on ice while tubes with clot activator were kept at room temperature until centrifugation (~30 min). Serum and plasma were obtained by centrifugation at

1,900 × *g* for 15 min. Aliquots of serum and plasma were frozen (-20°C) until further analysis. Measurements of NEFA and BHBA were performed using commercial kits in an auto-analyzer at the University of Illinois Veterinary Diagnostic Laboratory (Urbana, IL, USA). Other parameters were measured in lithium heparin samples at the Istituto di Zootecnica at the Università Cattolica del Sacro Cuore in Piacenza (Italy). Glucose, albumin, cholesterol, bilirubin, creatinine, urea, and glutamic-oxalacetic transaminase (**GOT**) were determined using kits purchased from Instrumentation Laboratory (IL Test) following the procedures previously described by Bionaz et al. (Bionaz et al., 2007) in a clinical auto-analyzer (ILAB 600, Instrumentation Laboratory, Lexington, MA, USA) . Triacylglycerol (**TAG**) was measured using a commercial kit (LabAssay^{TM} Triglyceride, Wako Chemicals Inc., USA). Haptoglobin and ceruloplasmin were analyzed using methods described by Bertoni et al. (Bertoni et al., 2008) adapted to the ILAB 600 conditions. Plasma vitamin A, vitamin E, and β-carotene were extracted with hexane and analyzed by reverse-phase HPLC using Allsphere ODS-2 3μm in a 150 × 4.6 mm column (Grace Davison Discovery Science, Deerfield, IL, USA), a UV detector set at 325 nm (for vitamin A), 290 nm (for vitamin E), or 460 nm (for β-carotene), and 80:20 methanol:tetrahydrofurane as the mobile phase. Total plasma reactive oxygen metabolites (**ROM**) were measured using the analytical method patented by Diacron International s.r.l. (Grosseto, Italy). Plasma insulin concentrations were measured by a double antibody radioimmunoassay, using a kit for human insulin following the procedures from the vendor (Diagnostic Systems Laboratories, Inc., Webster, TX, USA). The detection limit of the assay was 1.3 mU/ml; the coefficients of variation averaged 7.5% within assay and 9.5 % between assays.

Liver Tissue Composition

Liver was sampled via puncture biopsy (Dann et al., 2006) from cows under local anesthesia at approximately 0730 h on d −14 (± 3) and 7 relative to parturition. Liver was frozen immediately in liquid nitrogen and stored until further analysis for contents of total lipids and TAG (Dann et al., 2006).

Polymorphonuclear Leukocyte Isolation

Samples of blood (20 mL/tube) were collected at ~0700 h from the coccygeal vein or artery in evacuated tubes containing EDTA for chemotaxis and sodium heparin for phagocytosis at -14 (± 3 d) and 7 d relative to parturition. After blood collection, tubes were placed on ice (~30 min) until isolation (Auchtung et al., 2004, Moyes et al., 2009, Salak et al., 1993). Samples were centrifuged at 600 × *g* for 15 min at 4 °C. The buffy coat and approximately one-fourth of red blood cells were removed and discarded. The remaining sample was poured into a 50-mL tube. Twenty milliliters of deionized water at 4 °C were added to lyse red blood cells followed by addition of 5 mL 5X PBS at 4 °C to restore an iso-osmotic environment. Samples were centrifuged at 200 × *g* for 10 min at 4°C. Three subsequent washings using 1X PBS at 4 °C were performed with samples centrifuged at 500 × *g* for 3 min at 4 °C. Isolated PMN were resuspended in 1 mL 1X PBS at 4 °C and kept on ice. Cells were counted using a Beckman Coulter Counter after addition of Zap-OGlobin II Lytic Reagent (cat. #13020, Beckman Coulter) to lyse any remaining red blood cells. A total of 3 x 10^6 cells/mL of Roswell Park Memorial Institute (**RPMI**) 1640 media with 5% FBS were used for chemotaxis and 2 x 10^6 cells/mL for phagocytosis.

Chemotaxis

Chemotaxis was assessed using a method previously described (Auchtung et al., 2004, Salak et al., 1993) with modifications (Moyes et al., 2009). The assay was conducted in a 48-well Micro AP48 Chemotaxis Chamber (P48AP30, Neuro Probe, USA). Thirty microliters of 100 ng/mL RPMI 1640 (without FBS) containing human interleukin-8 (I1645, Sigma, USA), 10^{-8} M of human complement C5a (C5788, Sigma, USA) in RPMI 1640 (without FBS), or RPMI 1640 (without FBS, control) were added to each of 4 wells per sample (quadruplicate). A PVP-free filter (5 µm pore size, 25 × 88 mm; cat# 416306, Neuro Probe, USA) was mounted in each chamber. The chamber was incubated in 5% CO_2: 95% humidity at 37 °C for 10 min for equilibration. Fifty microliters of 3×10^6 cells/mL were added in each chamber and incubated in quadruplicate in 5% CO_2: 95% humidity at 37°C for 1 h. The membrane was then removed using forceps. To remove not migrated cells the side of the membrane in contact with the original cell suspension was carefully dipped in PBS solution (i.e., the other surface was not allow to get in contact with the PBS) and the cells removed by scraping against a sharp plastic surface. The removal of non-migrating cells was repeated 3 times. After cleaning, the membrane was allowed to dry and then was fixed with Hema 3 Hematology Staining Solution II (122-952, Fisher Scientific, USA). The number of cells in each well was counted using an inverted microscope. Cell counts were corrected based on viability (see below) and background (i.e., control or cell migrated with only RPMI).

Phagocytosis

Phagocytosis was conducted in quadruplicate in 1 mL RPMI 1640 media following addition of a 1:10 ratio of Fluoresbrite latex Carboxy Yellow-Green 1.75 µm Microspheres

(2.5%, #17687, Polysciences, Inc., USA). Samples were then incubated for 2 h in 5% CO_2: 95% humidity at 37 °C. A control sample was incubated for 2 h at 4 °C. After incubation, cells were rinsed twice with 1X PBS (via centrifugation at 1000 × *g* for 5 min at 4 °C), fixed with 150 μL 4% paraformaldehyde (P6148, Sigma, USA), and preserved at 4 °C until reading using flow cytometry.

Cell Viability and Differential Counts

Aliquots (20 μL) of the cell suspension from each sample for chemotaxis and phagocytosis assays was used to determine viability using a Burke chamber after 2 min incubation with a solution of Trypan blue. The average percentage of viable PMN was 71.7 ± 7.8; viability data were used to correct data on chemotaxis and phagocytosis. Aliquots (50 μL) of cell suspension from the samples used for the chemotaxis assay were fixed in a microscope slide to determine cell differentials; overall, the average percentage of PMN in the differential was 56.0 ± 5.5.

Statistical Analysis

Each variable of interest was evaluated for normal distribution using the Shapiro-Wilk test (SAS Inst. Inc.) and normalized by logarithmic transformation when necessary prior to statistical analysis. After analysis log-transformed data were back-transformed to be included in the tables. The MIXED procedure of SAS (SAS Institute, Inc., Cary, NC, USA) was used for statistical analysis. The fixed effects included diet (control or overfed energy), time (-14 and 7 d relative to parturition), and the interaction of diet × time. The random effect was cow within

diet. A repeated measures analysis using an AR(1) structure was used. All means were compared using the PDIFF statement of SAS (SAS Institute, Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

The immune status of dairy cows during the first wk of lactation is of importance during the transition period because there is a significant risk for development of new mastitis infections in the udder and new cases of clinical mastitis (Hogan et al., 1989). Dairy cows during the transition period normally experience a marked decrease in DMI some days before parturition, which in turn limits the consumption of dietary energy and has a negative impact on the energy balance equilibrium (Bertics et al., 1992). At the same time, nutrient demands for initiation of milk synthesis are increased, which aggravates the energy balance status (Grummer, 1995). After parturition, as milk production increases the energy needed for milk production also increases resulting in a state of NEB.

Table 3 reports the DMI, milk yield and milk composition. We observed higher (diet $P = 0.07$) DMI from d -14 to the day of parturition in overfed compared to control cows. As designed, DMI in the overfed group exceeded energy requirements during the prepartal period (-4 to -1 wk relative to parturition) resulting in significantly higher (diet $P < 0.01$) energy balance when compared to the control group (Table 3). However, both groups were in NEB during wk 1 after calving with a larger drop (expressed a percentage of calculated requirements) observed in the overfed group (from ~159% prepartum to ~83% postpartum, Table 3). These data confirmed a previous study from our group (Janovick et al., 2010) where cows fed to meet or exceed (100% or 150% of NE_L) prepartal energy requirements experienced a drastic decrease in energy balance from 160% of requirements at wk −3 relative to parturition to less than 72% during the first wk

of lactation. That study also observed similar DMI and milk yield during wk 1 to 3 postpartum between overfed and control cows.

Previous data from our laboratory suggested that over-consumption of energy during the dry period can result in poorer transitions, including lower post-partum DMI and slower starts in milk production (Dann et al., 2005, 2006). Cows fed diets to meet ca. 80% of NE_L requirements throughout the dry period were able to adapt better after parturition and were in more positive energy balance than cows fed to exceed ca. 150% of NE_L requirements (Dann et al., 2006). Milk yield and DMI during the first wk postpartum also were greater in prepartal energy-restricted cows. Another study comparing diets to meet 80%, 100%, or 150% NE_L requirements during the dry period revealed that cows fed 80% or 100% of NE_L requirements during the first 30 d of the dry period had greater DMI and improved energy balance coupled with lower serum NEFA and BHBA during the first 10 d postpartum compared with overfed cows (Dann et al., 2005).

Our data, although limited to the first wk after parturition, partly support previous observations suggesting that cows overfed during the dry period are more likely to be in greater NEB postpartum. To meet the energy requirements during the period of NEB, dairy cattle rely on mobilization of adipose TAG and muscle tissue (Drackley, 1999). Concentrations of NEFA in the blood are a consequence of hydrolyzed adipose tissue TAG and can be taken up by liver and oxidized to produce ketone bodies (e.g. BHBA) (Zammit, 1984). In our study, despite differences in energy balance but consistent with the comparable milk production and similar DMI (Table 3), there were no statistical differences in NEFA and BHBA concentrations between diets (Table 4). Furthermore, the observed concentrations of NEFA were within a non-pathological range (Bertoni et al., 2008). The blood NEFA pattern around parturition is dynamic and it is likely, based on previous data with similar dietary treatments (Dann et al., 2006;

Janovick et al., 2011), that treatment differences would have been more apparent if additional times would have been sampled. The numerically greater NEFA postpartum in overfed cows agrees with the more severe NEB in those cows; nevertheless the absolute values of NEFA and BHBA do not suggest a ketotic state.

Although NEFA concentrations prepartum were not different due to diet, blood insulin concentration was markedly greater (diet × time $P < 0.01$) prepartum in cows overfed energy but decreased sharply by wk 1 postpartum in this group (Table 4). In addition, blood glucose pre- and postpartum was greater ($P < 0.04$) in the overfed cows (Table 4). Together, the prepartal data are consistent with a greater potential for insulin-driven adipose lipid deposition. Our group has recently shown that overfeeding dry non-pregnant cows with a similar energy density as in the present study (i.e., NE_L 1.61 Mcal/kg DM) for 8 wk increased the deposition of visceral fat by ca. 70% compared with cows fed a controlled-energy/high-fiber diet similar to the present study (Nikkhah et al., 2008).

Urea concentration was significantly lower (diet × time $P < 0.01$) in overfed cows before parturition (Table 4). Considering that overfed cows ate more and received a diet with a higher protein content (Table 3), that means a more crude protein intake than control cows, it is possible to explain the different plasma urea concentration between groups with a difference in the diet fermentability. Thus, the difference in plasma urea concentration is likely not indicative of decreased hepatic functionality, as also confirmed by the low level of TAG in the liver (Table 4). The level of creatinine prepartum was lower (diet × time $P < 0.01$) in overfed compared to control cows, but the postpartum pattern was opposite between the two groups, i.e. increased in overfed and decreased in control cows (Table 4). (Reynolds and Kristensen, 2008). The very high insulin concentrations observed in overfed cows before calving might have affected

skeletal muscle protein turnover (Bolster et al., 2004) with a consequent reduction of protein catabolism. Therefore, the increase in serum creatinine postpartum, an index of muscle mass, indicates enhanced muscle synthesis in overfed cows occurring before calving (Baxmann et al., 2008).

As for urea and creatinine, overfed cows had lower plasma TAG prepartum, but in control cows there was a decrease in TAG postpartum while in overfed cows there was a numerical increase (Table 4). Although speculative given that we have data at a single time point, the lower blood TAG prepartum in overfed cows might have been a consequence of the high insulin levels causing chylomicron-TAG hydrolysis and NEFA uptake by the adipose tissue or may have been a consequence of lower rates of very low density lipoproteins (**VLDL**) export from liver. Interestingly, serum glucose concentration, which is the main source of energy for lymphocytes (Pithon-Curi et al., 2004) was higher in overfed cows during the whole study.

Parameters of liver function (i.e. serum bilirubin, albumin and cholesterol), acute-phase proteins (i.e., ceruloplasmin, haptoglobin), oxidative stress (i.e. ROM), and GOT were not different between groups. Furthermore, all those parameters indicated "normal" and satisfactory liver activity during the peripartal period (Bertoni et al., 2008). Among measured vitamins, vitamin A was significantly higher (diet × time $P = 0.02$) prepartum in overfed vs. control cows (Table 4). The greater concentration of vitamin A together with other indices of liver activity (see above) indicated that overfeeding cows did not result in significant impairment of liver function prepartum and at least through the first wk postpartum. Vitamin E is an important antioxidant that binds free radicals and prevents lipid peroxidation, which may have importance to the immune response (Bendich, 1993). In our study we observed that vitamin E concentration was greater (diet $P = 0.09$) in the overfed cows both pre- and post-calving. Higher vitamin E

concentration in blood has been associated with lower incidence of clinical mastitis during the first wk of lactation (Weiss et al., 1997), and lower concentrations seem to hamper immune function (Grasso et al., 1990). However, neutrophil phagocytosis capacity appears independent of blood vitamin E concentration (Hogan et al., 1990), which seems to agree with results from our study.

The transition period is accompanied by reduced immunological capacity from 2 to 3 wk before parturition (Goff, 2006, Mallard et al., 1998). After parturition, dramatic changes in patterns of leukocyte trafficking occur including increased cell counts and an increased number of immature cells; at the same time chemotaxis and the oxidative burst are impaired (Burvenich et al., 2003). These changes lead to impairment of a number of immunological parameters around parturition (Waller, 2000). In our study no significant differences in chemotaxis were observed between prepartal treatments. However, a significant decrease (time $P = 0.02$) in chemotaxis was observed after parturition regardless of diet (Table 5).

Phagocytosis capacity of PMN was greater (diet × time, $P < 0.01$) prepartum in the control group than in the overfed group; after parturition phagocytosis capacity in the control group remained constant while in the overfed group phagocytosis increased to levels similar to the control. In "healthy" cows PMN function (e.g., phagocytosis, superoxide anion generation, chemotaxis) declines gradually as parturition approaches. Lowest function is often reached soon after parturition and continues through 15 d postpartum (Gilbert et al., 1993, Kimura et al., 2002), after which PMN function increases through at least 6 wk postpartum (Gilbert et al., 1993, Moya et al., 2008). The increase in phagocytosis capacity in the overfed group from -14 to 7 d might be explained by the higher overall glucose concentration. Glucose rather than amino acids, ketone bodies, or fatty acids has been shown to be the preferred metabolic fuel for immune

cells (Pithon-Curi et al., 2004). However, at -14 d the concentrations of glucose and insulin were greater in the overfed group but the phagocytosis capacity was lower compared to control cows. Based on human experiments we speculate that the high insulin concentration prepartum coupled with higher glucose in overfed cows were indicative of insulin insensitivity, thus reducing the ability of immune cells to fully utilize glucose in order to execute the immune response (Saiepour et al., 2006).

CONCLUSION

Overall, our data indicated that the more positive energy status prepartum and the ensuing surge of insulin had a transient but significant effect on metabolism. We obtained evidence that the greater insulin concentration decreased muscle protein turnover and perhaps increased the circulating TAG uptake by adipose tissue prior to parturition. However, elevated insulin concentration also could have impaired phagocytic capacity of the PMN prior to calving. The underlying mechanisms for insulin's impairment of PMN function is not evident and warrants further studies. At least through the first wk postpartum, our blood data showed some indications of negative carry over effects of overfeeding energy to the point of calving, i.e. concentrations of haptoglobin, bilirubin, and ROM were increased. In that context, the more severe NEB observed during the first wk postpartum could potentially render cows more susceptible to immunosuppression and infectious diseases as well as metabolic disorders (Goff, 2006). The effect of overfeeding dietary energy prepartum might be more noticeable in the long-term as the demands for milk production and reliance on adipose mobilization continue to increase.

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Table 2. Ingredients and chemical composition of experimental diets.

	Prepartum		
	Overfed	Control	Lactation
Ingredients, % of DM			
Wheat straw	-	41.9	-
Corn silage	50.3	29.3	29.9
Alfalfa silage	18.0	10.0	14.8
Soybean meal	3.54	9.64	2.39
Ground shelled corn	13.9	3.59	-
Alfalfa hay	6.06	3.35	5.55
Magnesium sulfate	0.63	0.64	-
Magnesium oxide	0.43	0.42	0.13
Vitamin E	0.24	0.27	-
Mineral and vitamin mix[1]	0.18	0.18	0.22
Magnesium chloride	0.35	0.17	0.00
Urea	-	0.17	0.13
Salt	0.24	0.15	0.13
Sodium phosphate	-	0.13	-
Vitamin A	0.01	0.01	-
Vitamin D	0.01	0.01	-
Whole cottonseed	5.03	-	5.55
Calcium carbonate	0.9	-	0.56
Corn ground	-	-	20.3
Wet brewer's grain	-	-	12.9
Soybean hulls	-	-	5.55
Sodium bicarbonate	-	-	0.83
Dicalcium phosphate	-	-	0.54
Vitamin H	-	-	0.28
Chemical composition			
DM, %	50.0	51.9	60.5
$NE_{L,}$ Mcal/kg DM	1.62	1.34	1.69
CP, % DM	15.0	12.0	17.4
AP, % DM	14.3	11.2	11.9
ADICP, % DM	0.73	0.70	5.53
NDF, % DM	36.6	53.4	34.1
ADF, % DM	25.7	36.6	21.8
Ca, % DM	0.73	0.67	0.80
P, % DM	0.31	0.24	0.43
Mg, % DM	0.57	0.50	0.33
K, % DM	1.28	1.45	1.16
S %DM	0.25	0.21	0.21
Na % DM	0.09	0.07	0.29
Fe, ppm	339	305	203
Zn , ppm	80.0	66.6	65.8
Cu, ppm	14.6	13.0	10.9
Mn, ppm	70.3	72.0	67.0

[1]Mineral and vitamin mix: zinc = 60 ppm, copper = 15 ppm, manganese = 60 ppm, selenium 0.3 ppm, iodine = 0.6 ppm, iron = 50 ppm, and cobalt = 0.2 ppm. Rumensin:360mg/day in the lactation diet.

Table 3. Intake of DM and energy balance prepartum (-28 d to calving) and early postpartum (7 d) and milk production and composition postpartum in cows fed a control diet (n = 10; 1.34 Mcal/kg DM) or overfed diet (n = 9; 1.62 Mcal/kg DM) during the entire dry period.

	Prepartal energy			*P*-value		
	Overfed	Control	SEM[1]	Diet	Time	Diet × time
DMI						
% BW						
-28 to -14 DIM[2]	1.82	1.70	0.18	0.59	0.93	0.68
-14 to 0 DIM	2.03	1.66	0.22	0.21	0.40	0.98
-7 to 0 DIM	2.10	1.72	0.22	0.21	0.40	0.98
1 to 7 DIM	2.01	2.07	0.16	0.78	0.01	0.84
kg/d						
-28 to -14 DIM[2]	14.2	12.1	1.3	1.00	0.91	0.73
-14 to 0 DIM	15.3	11.7	1.5	0.07	0.98	0.90
-7 to 0 DIM	15.4	11.9	1.4	0.07	0.79	0.76
1 to 7 DIM[2]	14.0	13.1	0.8	0.41	0.01	0.31
Milk yield, kg 1 to 7 d[2]	27.3	25.3	3.2	0.61	0.01	0.97
Fat, % wk 1	3.60	3.95	0.25	0.32	-	-
Protein, % wk 1	3.58	2.98	0.34	0.23	-	-
Lactose, % wk 1	4.82	4.91	0.08	0.45	-	-
Energy balance						
Prepartum, -4 to -1 wk						
Mcal/d	8.85	0.06	2.21	0.01	0.29	0.40
% requirements	159	102	14	0.01	0.31	0.46
Postpartum, wk 1						
Mcal/d	-7.04	-3.89	1.44	0.10	-	-
% requirements	83.3	89.8	3.4	0.15	-	-

[1]Largest SEM is shown.
[2]Data were log-transformed prior to statistics and back-transformed for inclusion in tables.

Table 4. Prepartum (-14 d) and early postpartum (7 d) blood and liver parameters in cows fed a control diet (1.34 Mcal/kg DM) or overfed energy diet (1.62 Mcal/kg DM) during the entire dry period

	Prepartal energy							
	Overfed		Control			*P*-value		
Item	-14	7	-14	7	SEM[1]	Diet	Time	Diet × time
n =	10	10	9	9				
Metabolism								
NEFA, mEq/L[2]	0.140	0.521	0.136	0.320	0.111	0.25	0.01	0.30
BHBA, mmol/L[2]	0.514	0.631	0.554	0.528	0.066	0.61	0.45	0.23
Glucose, mmol/L[2]	4.24	3.49	4.00	3.30	0.12	0.04	0.01	0.95
Insulin, µIU/mL[2]	16.7[a]	3.70[c]	3.94[b]	2.31[d]	2.61	0.01	0.01	0.01
Glucose:insulin[2]	0.25[c]	0.94[b]	1.02[b]	1.43[a]	0.22	0.01	0.01	0.01
Urea, mmol/L	4.63[b]	5.53[a]	6.38[a]	5.49[a]	0.49	0.15	0.98	0.01
Creatinine, µmol/L[2]	100.5[b]	105.7[b]	117.9[a]	101.8[b]	3.6	0.09	0.04	0.01
Triacylglycerol, mg/dL[2]	4.00[b]	4.64[b]	8.57[a]	3.86[b]	1.02	0.03	0.01	0.01
Liver function								
Bilirubin, µmol/L	0.76[b]	4.67[a]	1.71[b]	3.61[a]	0.50	0.94	0.01	0.02
Albumin, g/L	36.6	36.2	37.0	37.2	0.60	0.31	0.74	0.51
Cholesterol, mmol/L[2]	3.02	2.54	2.83	2.52	0.13	0.46	0.01	0.51
Acute-phase proteins								
Ceruloplasmin, µmol/L	2.44	3.16	2.24	2.94	0.13	0.20	0.01	0.81
Haptoglobin, g/L[2]	0.33	0.64	0.24	0.49	0.12	0.14	0.01	0.91
Oxidative stress								
ROM[3], mg H_2O_2/100 mL	11.3	14.1	10.5	13.3	0.6	0.25	0.01	0.90
Liver injury								
GOT[4], U/L[2]	73.8	119.0	82.8	125.4	10.9	0.29	0.01	0.66
Antioxidants-antiinflammation								
Vitamin A, µg/100 mL	47.3[a]	27.5[b]	36.4[b]	32.4[b]	4.1	0.53	0.01	0.02
Vitamin E, µg/mL[2]	4.78	2.75	3.88	2.41	0.42	0.09	0.01	0.57
β-carotene, mg/100 mL	0.20	0.12	0.17	0.12	0.01	0.31	0.01	0.14
Liver tissue, % wet weight								
Lipid	4.52	5.98	4.21	5.21	0.42	0.23	0.01	0.55
Triacylglycerol[2]	0.49	1.80	0.48	0.87	0.93	0.35	0.03	0.40

[a-d]Means within a row with different superscripts differ (Diet × Time $P < 0.05$).
[1]Largest SEM is shown.
[2]Data were log-transformed prior to statistics and back-transformed for inclusion in tables.
[3]Reactive oxygen metabolites.
[4]Glutamic-oxalacetic transaminase.

Table 5. Prepartum (-14 d) and early postpartum (7 d) polymorphonuclear leukocytes (PMN) chemotaxis and phagocytosis in cows fed a control diet (1.34 Mcal/kg DM) or overfed energy diet (1.62 Mcal/kg DM) during the entire dry period

	Prepartal energy							
	Overfed		Control			*P*-value		
Item	-14	7	-14	7	SEM[1]	Diet	Time	Diet × time
n =	9	9	10	8				
Chemotaxis, cells/cm²								
Complement C5a	23.9	65.4	27.6	71.4	36.4	0.89	0.22	0.97
Interleukin-8	144.0	80.8	149.0	40.3	37.0	0.60	0.02	0.50
Phagocytosis, %	32.7[b]	48.4[a]	46.5[a]	50.0[a]	4.66	0.20	0.01	0.01

[a-b]Means within a row with different superscripts differ (Diet × Time $P < 0.05$).
[1]Largest SEM is shown.
[2]Data were log-transformed prior to statistics and back-transformed for inclusion in tables.

CHAPTER 2:

The Effect of an *E. coli* Lipopolysaccharide Intra-Mammary Challenge on Cow Performance, Metabolic and Inflammation indices and Immune Response of Dairy Cattle during Early-Lactation

D. E. Graugnard*†, M. Bionaz*†, E. Trevisi#, M. Mukesh*†, M. Ordonez*†, K. M. Moyes*†, J. L. Salak-Johnson†, R. L. Wallace‡, J. K. Drackley†§, G. Bertoni#, and J. J. Loor*†§.

*Mammalian NutriPhysioGenomics, †Department of Animal Sciences, ‡College of Veterinary Medicine, and §Division of Nutritional Sciences, University of Illinois, Urbana, IL 61801; and #Istituto di Zootecnica, Facolta di Agraria, Universita Cattolica del Sacro Cuore, 29122 Piacenza, Italy

INTRODUCTION

The transition period is considered the most important phase during the lactation cycle since a success transition, can effectively determine a profitable lactation (Drackley, 1999). However, immunosuppression during the this period leads to increased susceptibility (Mallard et al., 1998) and the incidence of health problems during this time is significantly elevated relative to the rest of the lactation cycle (Drackley, 1999). Is the major risk period for mammary infections (Shaver, 1997, Smith et al., 1985). A high proportion of the intra-mammary infections occur during first mo of lactation (Oviedo-Boyso et al., 2007) and in many cases results from an infection established during the dry period or during early lactation (Goff and Horst, 1997). Once a pathogen is detected by the receptors in the epithelial cells of the mammary gland the acute phase response begins, the immune system is activated to eliminate the pathogen.

Lipopolysaccharide (**LPS**) is a major component in the outer membrane of gram-negative bacteria; it acts as an endotoxin eliciting a strong acute phase immune response in mammals (Small et al., 2000). The LPS is recognized by Toll like receptor-4 (**TLR4**), which is located on the intracellular membranes (Rosenberger and Finlay, 2003). Several studies have used LPS to evaluate the effect of the acute phase response in production variables as well on immune response (e.g. leukocyte function). There is evidence that the LPS model generates a local and systemic action involving immune cells and the liver (Mehrzad et al., 2001). In addition the effect of LPS challenge has been investigated during early and late lactation. Data indicates that the LPS challenge generate a more severe response during early lactation; immune cells function seems to be decreased and impaired during early lactation compared the late lactation LPS challenge (Lehtolainen et al., 2003). Using and LPS challenge during early lactation represents a common situation dairy cows undergo when mastitis occur. This model allows us to study in

controlled manner the effect of the acute phase response on the performance of dairy cows that are already under the immunosuppression state due to transition.

OBJECTIVE

The main objective of this study was to evaluate the effect of an early-lactation *E. coli* lipopolysaccharide intra-mammary challenge on performance, metabolic and inflammation indices and immune function of dairy cattle.

MATERIALS AND METHODS

Animals and Diets

All procedures were conducted under protocols approved by the University of Illinois Institutional Animal Care and Use Committee (protocol # 06145). Twenty Holstein cows entering their second or greater lactation were enrolled in the study. Cows averaged composite somatic cell count (SCC) of ~128,000 ± 108,000 during the previous lactation. Cows were fed a diet providing ~159% calculated NE_L requirements (Overfed diet, 1.62 Mcal/kg DM) during the entire 45-d dry period. The diet was fed as TMR once daily (0600 h) using an individual gate feeding system (American Calan, Northwood, NH, USA). Cows were housed in a ventilated enclosed barn during the dry period and had access to sand-bedded free stalls until 5 d before expected calving date, when they were moved to an individual maternity pen bedded with straw. After parturition, cows were moved to a tie-stall barn and were fed a lactation diet (NE_L = 1.69 Mcal/kg DM) as TMR once daily (0600 h) and milked twice daily (0400 and 1600 h). The diet was mixed in a Keenan Klassik 140 mixer wagon (Richard Keenan & Co., Ltd., Borris, County Carlow, Ireland) equipped with knives and serrated paddles.

Samples of feed ingredients and TMR were obtained weekly and analyzed for DM content to maintain desired ingredient ratios. Weekly samples of individual ingredients were frozen at -20°C and were composited monthly. Composite samples were analyzed for contents of DM, CP, NDF, ADF, Ca, P, Mg, and K using wet chemistry methods (Dairy One, Ithaca, NY, USA). Body weight was measured for each cow weekly. Milk weights were recorded daily and samples were obtained from consecutive a.m. and p.m. milkings. Milk samples were composited in proportion to milk yield at each sampling and preserved (800 Broad Spectrum Mirotabs II; D&F Control Systems, Inc., San Ramon, CA, USA). Composite samples were analyzed for fat, protein, lactose, urea-N, and SCC using midinfrared procedures (AOAC International., 1995) at a commercial laboratory (Dairy One, Ithaca, NY, USA).

Energy Balance Calculations and Estimates

Energy balance was calculated individually for each cow using equations described previously (NRC, 2001). Net energy intake (NE_I; Mcal/d) was determined by multiplying DMI by the calculated mean NE_L density of the diet. The NE_L value of each individual feed (Dairy One, Ithaca, NY, USA) was used to calculate the mean NE_L content of the diet. The NE_M was calculated as $BW^{0.75} \times 0.08$. Milk net energy requirement (NE_{MILK}; Mcal/kg) was calculated as $(0.0929 \times \text{fat\%} + 0.0563 \times \text{protein\%} + 0.0395 \times \text{lactose \%}) \times \text{milk yield}$. The equation used to calculate postpartal energy balance (EB_{POST}; Mcal/d) was $EB_{POST} = NE_I - (NE_M + NE_{MILK})$.

Lipopolysaccharide Challenge

At ~7 DIM, cows (10/treatment) were assigned to receive an intra-mammary *E. coli* lipopolysaccharide (LPS) challenge (200 μg, strain 0111:B4, cat. # L2630, Sigma Aldrich, St.

Louis, MO) or to serve as controls (Non-LPS, these cows did not receive any control infusion). Prior to LPS challenge (~2 days), foremilk samples from all quarters of each cow were cultured and confirmed to be bacteriologically negative. LPS was dissolved in 20 mL of 0.09% sterile physiological saline (Hospira, Lake Forest, IL). Immediately after milking (0530 h), one rear mammary quarter was disinfected with cotton wool pre-soaked in 70% ethanol and the LPS was infused via a sterile disposable syringe fitted with a sterile teat cannula using the full insertion infusion method. The quarter was thoroughly massaged.

Blood Metabolites

Blood was sampled from the coccygeal vein or artery at 2, 7, 10, 14, and 21 d relative to parturition. Samples were collected at 1200 h, except during d 7 when samples were collected before receiving the LPS challenge. Blood was collected into evacuated tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) containing either EDTA or lithium heparin for plasma and a clot activator for serum. After blood collection, tubes with EDTA and lithium heparin were placed on ice while tubes with clot activator were kept at room temperature until centrifugation (~30 min). Serum and plasma were obtained by centrifugation at 1,900 × *g* for 15 min. Aliquots of serum and plasma were frozen (-20°C) until further analysis. Measurements of NEFA and BHBA were performed using commercial kits in an auto-analyzer at the University of Illinois Veterinary Diagnostic Laboratory (Urbana, IL, USA). Other parameters were measured at the Istituto di Zootecnica at the Università Cattolica del Sacro Cuore in Piacenza (Italy). Glucose, albumin, β-carotene, cholesterol, bilirubin, creatinine, urea, and glutamic-oxalacetic transaminase (**GOT**) were determined using kits purchased from Instrumentation Laboratory (IL Test) following the procedures previously described by Bionaz et al. (Bionaz et al., 2007) in a

clinical auto-analyzer (ILAB 600, Instrumentation Laboratory, Lexington, MA, USA) . Triacylglycerol (**TAG**) was measured using a commercial kit (LabAssay^{TM} Triglyceride, Wako Chemicals Inc.). Haptoglobin and ceruloplasmin were analyzed using methods described by Bertoni et al. (Bertoni et al., 2008) adapted to the ILAB 600 conditions. Plasma vitamin A, vitamin E and β-carotene were extracted with hexane and analyzed by reverse-phase HPLC using Allsphere ODS-2 3μm in a 150 × 4.6 mm column (Grace Davison Discovery Science, Deerfield, IL, USA); a UV detector set at 325 nm (for vitamin A) or 290 nm (for vitamin E) or 460 nm (for β-carotene); and 80:20 methanol:tetrahydrofurane as the mobile phase. Total plasma reactive oxygen metabolites (**ROM**) were measured using the analytical method patented by Diacron International s.r.l. (Grosseto, Italy). Plasma insulin concentrations were measured by a double antibody radioimmunoassay, using a kit for human insulin (Diagnostic Systems Laboratories, Inc., Webster, TX, USA). The detection limit of the assay was 1.3 mU/ml; the coefficients of variation averaged 7.5% within assay and 9.5 % between assay; parallelism between standard curve and scalar dilution of bovine plasma did not show significant differences.

Liver Tissue Composition

Liver was sampled via puncture biopsy (Dann et al., 2006) from cows under local anesthesia at approximately 0730 h on d −14 (± 3), 7, 14 and 30 relative to parturition. During d 7 the liver sample was collected ~2.5 h after LPS challenge. Liver was frozen immediately in liquid nitrogen and stored until further analysis for contents of total lipids and TAG (Dann et al., 2006).

Neutrophil Isolation

Samples of of blood (20 mL/tube) were collected from the coccygeal vein or artery in vacutainer tubes containing EDTA for chemotaxis and sodium heparin for phagocytosis at -14 (± 3), 7, 14, 30, 60 and 120 d relative to parturition. Samples were collected at ~0700 h, except during d 7 when samples were collected before receiving the LPS challenge. After blood collection, tubes were placed on ice (~30 min) until isolation (Auchtung et al., 2004, Moyes et al., 2009, Salak et al., 1993). Samples were centrifuged at 600 × *g* for 15 min at 4 °C. The buffy coat and approximately one-fourth of red blood cells were removed and discarded. The remaining sample was poured into a 50 mL tube. Twenty milliliters of deionized water at 4 °C were added to lyse red blood cells followed by addition of 5 mL 5X PBS at 4 °C to restore an iso-osmotic environment. Samples were centrifuged at 200 × *g* for 10 min at 4°C. Three subsequent washings using 1X PBS at 4 °C were performed with samples centrifuged at 500 × *g* for 3 min at 4 °C. Isolated neutrophils were resuspended in 1 mL 1X PBS at 4 °C and kept on ice. Cells were counted using a Beckman Coulter Counter after addition of Zap-OGlobin II Lytic Reagent (cat. #13020, Beckman Coulter) to lyse any remaining red blood cells. A total of 3 x 10^6 cells/mL of RPMI 1640 media with 5% FBS were used for chemotaxis and 2 x 10^6 cells/mL for phagocytosis.

Chemotaxis

Chemotaxis was assessed using a method previously described (Auchtung et al., 2004, Salak et al., 1993) with modifications (Moyes et al., 2009). The assay was conducted in a 48-well Micro AP48 Chemotaxis Chamber (P48AP30, Neuro Probe, USA). Thirty microliters of 100 ng/mL RPMI 1640 (without FBS) containing human interleukin-8 (I1645, Sigma, USA), 10^-

^{8} M of human complement C5a (C5788, Sigma, USA) in RPMI 1640 (without FBS), or RPMI 1640 (without FBS, control) were added to each of 4 wells per sample (quadruplicate). A PVP-free filter (5 µm pore size, 25 × 88 mm; cat# 416306, Neuro Probe, USA) was mounted in each chamber. The chamber was incubated in 5% CO_2: 95% humidity at 37 °C for 10 min for equilibration. Fifty microliters of 3×10^6 cells/mL from each incubated in quadruplicate in 5% CO_2: 95% humidity at 37°C for 1 h. The membrane was then removed using forceps. To remove not migrated cells the side of the membrane in contact with the original cell suspension was carefully dipped in PBS solution (i.e., the other surface was not allow to get in contact with the PBS) and the cell removed by scrapping against a sharp plastic surface. The removal of non-migrating cells was repeated 3 times. After cleaning, the membrane was let dry and fixed with Hema 3 Hematology Staining Solution II (122-952, Fisher Scientific, USA). The number of cells in each well was counted using an inverted microscope. Cell counts were corrected based on viability and background (i.e., control or cell migrated with only RPMI).

Phagocytosis

Phagocytosis was conducted in quadruplicate in 1 mL RPMI 1640 media following addition of a 1:10 ratio of Fluoresbrite latex Carboxy Yellow-Green 1.75 µm Microspheres (2.5%, #17687, Polysciences, Inc., USA). Samples were then incubated for 2 h in 55% CO_2: 95% humidity at 37 °C. A control sample was incubated for 2 h at 4 °C. After incubation, cells were rinsed twice with 1X PBS (via centrifugation at 1000 × *g* for 5 min at 4 °C), fixed with 150 µL 4% paraformaldehyde (P6148, Sigma, USA), and preserved at 4 °C until reading using flow cytometry.

Cell Viability and Differential Counts

Aliquots (20 μL) of the cell suspension from each sample for chemotaxis and phagocytosis assays was used to determine viability using a Burke chamber after 2 min incubation with a solution of Trypan blue. The average percentage of viable neutrophils was 71.7 ± 7.8; viability data were used to correct data on chemotaxis and phagocytosis. Aliquots (50 μL) of cell suspension from the samples used for the chemotaxis assay were fixed in a microscope slide to determine cell differentials; overall, the average percentage of neutrophils in the differential was 56.0 ± 5.5.

Statistical Analysis

Each variable of interest was evaluated for normal distribution using the Shapiro-Wilk test (SAS Inst. Inc.) and normalized by logarithmic transformation when necessary prior to statistical analysis. The MIXED procedure of SAS (SAS Institute, Inc., Cary, NC, USA) was used for statistical analysis. The fixed effects included treatment (LPS or non-LPS), time, and interaction treatment × time. The random effect was cow within treatment. A repeated measures analysis using an AR(1) structure was used. All means were compared using the PDIFF statement of SAS (SAS Institute, Inc.).

RESULTS AND DISCUSSION

It has been well-known that peripartal cows are immunosuppressed and it appears that the metabolic changes associated with the beginning of lactation are capable of affecting immune function (Goff, 2006). During an intra-mammary infection, the host defense mechanism in themammary gland immune system is activated to eliminate the pathogen or toxin (Oviedo-

Boyso et al., 2007). This defense mechanisms include anatomical, cellular, and soluble factors that act in coordination and are crucial to the modulation of mammary gland resistance and susceptibility to infection (Chaneton et al., 2008). The peak in systemic signs, including elevated rectal temperature and heart rate, loss of appetite, and discomfort during an induced LPS challenge occurs between 4 and 8 h postchallenge regardless of whether the animal is challenged soon after parturition (Waldron et al., 2006) or at early- or mid-lactation (Waldron et al., 2006); systemic signs start to decrease and are almost at baseline levels by 24 h after the LPS challenge (Lehtolainen et al., 2003). We performed an intra-mammary bacterial LPS challenge with a higher dose (200 vs. 100 μg) than used previously (Waldron et al., 2006) in order to better understand the systemic response that animals might experience due to environmental or pathogenic microorganisms in the mammary gland.

Table 7 shows the effect of LPS challenge (i.e. LPS or Non-LPS) at 7 DIM on DMI, milk production, and energy balance in cows fed a moderate energy diet (1.62 Mcal/kg DM) during the entire dry period. Regardless of LPS challenge, DMI increased (time $P < 0.05$; % BW and kg/d) between the time of challenge through 41 DIM. However, average DMI as % BW from 7 to 41 DIM was greater (diet $P < 0.05$) for the Non-LPS cows than LPS cows. Milk yield increased (time $P < 0.05$) from 7 to 41 DIM with no differences observed between treatments. Milk composition was not markedly affected by LPS challenge. Estimated EBAL before (week 1) as well as during the first 6 weeks postpartum was not different due to treatment or treatment × time. It is noteworthy, however, that cows challenged with LPS appeared to be in more severe NEB during the 5 weeks after challenge. Over time, both groups of cows were able to improve EBAL. We present evidence that LPS challenge early postpartum had long-term carry over effects on DMI and potentially energy balance, i.e., LPS-challenged cows consumed less DM as

a proportion of their body weight and appeared to be in more negative EBAL for the first 6 weeks postpartum (Table 7).

Figure 1shows liver lipid and TAG concentration in the liver. In both parameters greater concentrations were observed in the LPS challenged group at d 7 (treatment × time $P < 0.08$ and $P < 0.05$ for lipid and TAG respectively). At 14 d greater concentrations of TAG were observed in the LPS challenged group (treatment × time $P < 0.05$).

Figure 2 shows concentrations of blood NEFA, BHBA, glucose and insulin. After LPS challenge day NEFA concentrations were lower (treatment × time $P < 0.05$) in the Non-LPS group (10 and 14 d) when compared to the LPS group that maintained the concentration similar to the challenge day. In contrast to NEFA, blood BHBA and glucose did not differ due to LPS challenge. However Insulin concentration was higher ($P = 0.06$) in the control group at 10 and 21 DIM when compared to challenged cows. Other studies showed that during the very early stages (2 to 4 h) of an intra-mammary LPS-challenge in peripartal cows, estimated glucose production, plasma glucose concentration, and plasma insulin concentration were increased but plasma NEFA and BHBA were unchanged despite minimal DMI during this period (Waldron et al., 2006). Except for plasma insulin, values returned to pre-challenge levels by 6-8 h post-LPS challenge, which may explain why we observed no changes in glucose concentration during the 14 d after LPS challenge (Figure 2). Contrary to results from Waldron et al. (2006), we observed differences in blood NEFA during the days following the LPS challenge.

Figure 3 shows concentrations of haptoglobin, bilirubin, urea, ceruloplasmin, albumin and GOT. The concentration of bilirubin decreased (time $P < 0.05$) consistently through from 2 to 21 DIM. An overall treatment difference was observed in plasma urea concentration (treatment $P = 0.07$), however the difference was observed before LPS treatment. Regardless of

LPS challenge, the concentration of urea decreased by 10 DIM and then was stable through 21 DIM. An interaction of treatment × time ($P < 0.05$) was observed for albumin concentration due to greater concentration at 14 DIM in cows challenged with LPS, a response brought about by a decrease in albumin between 7 and 14 DIM in Non-LPS cows. Ceruloplasmin increased over time regardless of the LPS challenge from 2 to 21 d ($P < 0.05$). However, the concentrations of haptoglobin and GOT increased from 2 through 7 (GOT) and 10 d (haptoglobin) DIM then decreased consistently through 21 DIM regardless of treatment. In a recent study, oral dosing with interferon (IFN)-α to induce a pro-inflammatory response during the peripartal period resulted in similar concentrations of NEFA but greater BHBA soon after parturition (Trevisi et al., 2009). We observed that NEFA remained higher by 10 and 14 DIM in cows challenged with LPS at 7 DIM and experienced a numerical decrease (diet × time P = 0.17) in blood BHBA over time (Figure 1). In another study where cows were challenged with LPS during early and late lactation, blood urea concentration was lower in the challenged cows in early lactation (Lehtolainen et al., 2003). In our study, the group challenged with LPS had lower urea concentration during the first 21 DIM, thus, confirming previous relationships. In two studies (Trevisi et al., 2009) where cows were treated with low doses (i.e., 0.5 or 10 IU /kg body weight) of IFN-α from the last 2 weeks prepartum through parturition or 5 DIM, it was reported that blood haptoglobin and ceruloplasmin increased more markedly but albumin, cholesterol, and vitamin A concentrations (i.e., negative acute-phase proteins) increased less rapidly in LPS challenged vs. Non-LPS cows. However, treated cows maintained numerically higher ROM in blood through 21 DIM when values were greater (ca. 15 vs. 12 mg H_2O_2/100 mL) compared with Non-LPS.

Figure 4 shows temporal concentrations of ROM, cholesterol, vitamin A, vitamin E, β-carotene and creatinine. An interaction treatment × time ($P < 0.05$) was observed for vitamin A concentration due to greater concentration in cows receiving the LPS challenge at 21 DIM. Concentration of ROM increased (time $P < 0.05$) through 10 DIM, and then it remained relatively stable through 21 DIM. This indicated that prepartal energy overfeeding per se rendered these cows more susceptible to oxidative stress. Cholesterol concentration was constant from 2 to 7 DIM; thereafter, a gradual increase (time $P < 0.05$) was observed through 21 DIM. Concentration of vitamin E was constant from 2 to 7 DIM, subsequently an increase over time ($P < 0.05$) was observed until 10 DIM and was followed by another increase from 14 through 21 DIM. β-carotene concentration was constant from 2 to 14 DIM, after which concentration increased ($P < 0.05$) by 21 DIM. Creatinine concentration decreased gradually over time from 2 to 21 DIM (time $P < 0.05$). These temporal responses and the actual concentrations resembled more those observed in cows receiving the inflammatory challenge with IFN-α, i.e., in our study other factors besides LPS probably were responsible for the sustained oxidative stress. As suggested by others (Trevisi et al., 2009), the concomitant increase in ROM and haptoglobin, regardless of LPS, might be the consequence of inflammatory auto-amplification through nuclear factor kappa B activation induced potentially by ROM released during inflammation itself. Although the above inflammation indices did not entirely reflect the profiles of positive and negative acute-phase proteins, a global comparison with data from Trevisi et al. (2009) suggest that cows fed moderate energy were under a sustained inflammatory state during the first 10 DIM regardless of LPS. We speculate that excessive adipose tissue deposition during early lactation probably induced a mild but chronic inflammatory state as commonly observed in overweighed/obese human subjects. Together with DMI and energy

balance data, the blood NEFA data from cows challenged with LPS are suggestive of longer-term metabolic adaptations caused by the inflammatory challenge soon after parturition.

Chemotaxis was not affected by LPS challenge in cows fed moderate energy prepartum (Figures 5A and 5B). Overall, a significant (treatment × time $P < 0.05$) difference was observed for total neutrophil phagocytosis due to greater responses at 14 DIM in control vs. LPS-challenged cows (Figure 5C). The neutrophils play an important role in the intra-mammary defense against an invading pathogen (Burvenich et al., 1994). High neutrophils counts and function in blood (Dosogne et al., 1997) as well as high somatic cell count (SCC) in milk (Shuster et al., 1996) and lower levels of lymphocytes in blood (Mehrzad et al., 2008) correlate positively with the severity of infection. Phagocytic capability of blood neutrophils and monocytes typically increase between early postpartum and peak lactation to values that are greater than prepartum (Moya et al., 2008). In a previous study, basal amounts of blood neutrophils and lymphocytes in blood were not different in cows during early (3.53×10^6/mL; 6 to 15 DIM) or mid-lactation (3.05×10^6/mL; 137 to 77 d before next parturition) (Lehtolainen et al., 2003). However, following an LPS challenge it was reported that blood neutrophil counts increased to a greater extent in early lactation compared with late lactation (Lehtolainen et al., 2003). They also reported numerically-greater PMN phagocytosis and respiratory burst activity (by 8-h post LPS challenge) and lower numbers of lymphocytes (12 through 24 h post LPS challenge) in late-lactation vs. early-lactation cows. Those parameters returned to pre-LPS challenge levels by 32 (phagocytosis) to 72 h (blood neutophils) post-LPS regardless of stage of lactation (Lehtolainen et al., 2003). Similar results were observed in cows that were challenged with LPS at 20-35 DIM (Mehrzad et al., 2001). Unlike cows at 20 to 35 DIM (Mehrzad et al.,

2001), the decrease in total neutrophil phagocytosis between 7 and 14 DIM in our study might have been associated with the hormonal and metabolic environment characteristic of this period, e.g., blood NEFA, BHBA, cortisol, pro-inflammatory cytokines, and glucose all of which could affect immune function in some fashion (Burvenich et al., 2007). In fact, blood NEFA increased nearly two-fold after LPS challenge in early lactation (Lehtolainen et al., 2003) and in our study NEFA remained higher through 14 DIM in LPS-challenged cows (Figure 2). Judging from work with non-ruminant lymphocytes, the energy needs of immune cells seem to rely almost exclusively on glucose availability (Fox et al., 2005) suggesting that any shortfalls in glucose availability between 7 to 14 DIM might have compromised neutrophil function. Blood glucose was not affected by LPS challenge but it remains to be determined if insulin sensitivity is affected by inflammatory conditions after parturition. The contrasting temporal patterns for the total percentage of neutrophils phagocytosing (Figure 5A) particularly in LPS-challenged cows was indicative of a substantial recovery of phagocytic capacity as cows moved from the peripartal period through peak and mid-lactation. This type of response also is suggestive of a more precarious and stressful situation for cows undergoing a pro-inflammatory challenge early postpartum (Bertoni et al., 2008) in terms of their ability to fight invading pathogens. It also was evident that neutrophils from cows undergoing the LPS challenge might not have been able to recover their full biological activity for several weeks, underscoring the need for adequate management during the recovery phase.

The presence of an intra-mammary *E. coli* LPS challenge during early lactation represent rapid changes in metabolic indices that can affect the dairy cow and might represent adaptations in a longer term that can negatively affect performance. In our study the changes caused by the

LPS challenge seemed to happen fast however causing important changes in immune function during the days following.

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Table 6. Ingredients and chemical composition of experimental diets.

	Overfed	Lactation
Ingredients		
Corn silage	50.3	29.9
Alfalfa silage	18.0	14.8
Soybean meal	3.54	2.39
Ground shelled corn	13.9	-
Alfalfa hay	6.06	5.55
Magnesium sulfate	0.63	-
Magnesium oxide	0.43	0.13
Vitamin E	0.24	-
Mineral and vitamin mix[1]	0.18	0.22
Magnesium chloride	0.35	0.00
Urea	-	0.13
Salt	0.24	0.13
Vitamin A	0.01	-
Vitamin D	0.01	-
Whole cottonseed	5.03	5.55
Calcium carbonate	0.9	0.56
Corn ground	-	20.3
Wet brewer's grain	-	12.9
Soybean hulls	-	5.55
Sodium bicarbonate	-	0.83
Dicalcium phosphate	-	0.54
Vitamin H	-	0.28
Chemical composition		
DM, %	50.0	60.5
$NE_{L,}$ Mcal/kg DM	1.62	1.69
CP, % DM	15.0	17.4
AP, % DM	14.3	11.9
ADICP, % DM	0.73	5.53
NDF, % DM	36.6	34.1
ADF, % DM	25.7	21.8
Ca, % DM	0.73	0.80
P, % DM	0.31	0.43
Mg, % DM	0.57	0.33
K, % DM	1.28	1.16
S %DM	0.25	0.21
Na % DM	0.09	0.29
Fe, ppm	339	203
Zn , ppm	80.0	65.8
Cu, ppm	14.6	10.9
Mn, ppm	70.3	67.0

[1]Mineral and vitamin mix: zinc = 60 ppm, copper = 15 ppm, manganese = 60 ppm, selenium 0.3 ppm, iodine = 0.6 ppm, iron = 50 ppm, and cobalt = 0.2 ppm.
Rumensin: 360mg/day in lactation diet.

Table 7. The effect of intra-mammary LPS challenge at 7 d postpartum on DMI, milk production, and energy balance in cows fed a moderate-energy diet (1.62 Mcal/kg DM) during the entire dry period.

	Treatment			P value		
Item	Non-LPS	LPS	SEM[1]	Trt	Time	Trt × time
DMI[2]						
% BW						
7 to 14	2.67	2.20	0.26	0.16	0.001	0.61
7 to 41	3.38	2.89	0.18	0.04	0.001	0.81
kg/d						
7 to 14	18.5	15.9	1.5	0.18	0.001	0.47
7 to 41	21.4	20.0	1.2	0.34	0.001	0.92
Milk[2]						
kg/d						
7 to 14	36.4	35.1	3.5	0.77	0.55	0.77
7 to 41	43.9	41.9	3.5	0.63	0.001	0.69
Fat, %						
wk 1	3.55	3.35	0.33	0.65	--	--
wk 1 to 6	3.81	3.77	0.34	0.94	0.27	0.86
Protein, %						
wk 1	4.02	3.06	0.53	0.18	--	--
wk 1 to 6	3.31	2.99	0.13	0.06	0.16	0.29
Lactose, %						
wk 1	4.80	4.75	0.11	0.70	--	--
wk 1 to 6	4.86	4.79	0.05	0.25	0.66	0.22
Energy balance[2]						
Postpartum						
wk 1						
Mcal/d	-7.8	-11.3	2.7	0.35	--	--
% requirements	78.6	66.3	7.2	0.22	--	--
wk 1 and 2						
Mcal/d	-6.5	-9.1	2.6	0.46	0.02	0.48
% requirements	82.3	74.4	6.7	0.37	0.001	0.19
wk 1 to 6						
Mcal/d	-5.9	-8.3	1.3	0.16	0.21	0.80
% requirements	85.3	80.3	3.0	0.22	0.01	0.89

[1]Largest SEM is shown.
[2]Day or wk relative to parturition.

Figure 1. Liver lipid and liver triglyceride (TAG) in cows fed a moderate-energy diet (1.62 Mcal/kg DM) during the entire dry period with (LPS) or without (Non-LPS) an intra-mammary LPS challenge at 7 d postpartum. Superscript letters (a, b) denote significant (Treatment × time P < 0.05) differences between treatment at a specific time point.

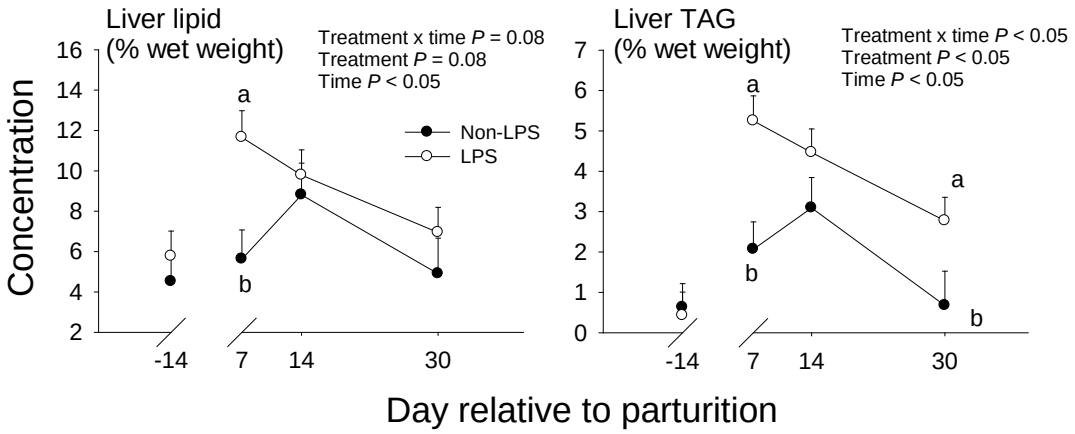


Figure 2. Blood concentrations of NEFA, BHBA, glucose and insulin in cows fed a moderate-energy diet (1.62 Mcal/kg DM) during the entire dry period with (LPS) or without (Non-LPS) an intra-mammary LPS challenge at 7 d postpartum. Superscript letters (a, b) denote significant (Treatment × time P < 0.05) differences between treatment at a specific time point.

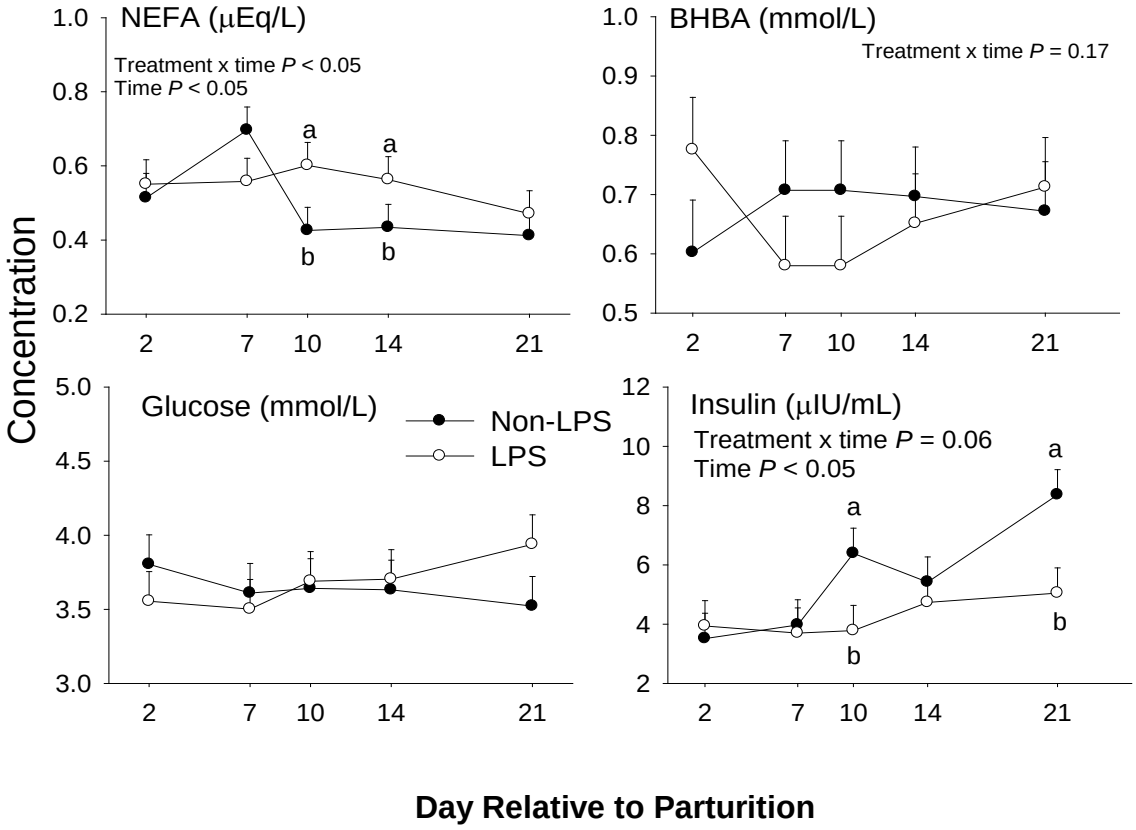


Figure 3. Blood concentrations of haptoglobin, bilirubin, urea, ceruloplasmin, albumin and GOT in cows fed a moderate-energy diet (1.62 Mcal/kg DM) during the entire dry period with (LPS) or without (Non-LPS) an intra-mammary LPS challenge at 7 d postpartum. Superscript letters (a, b) denote significant (Treatment × time P < 0.05) differences between treatment at a specific time point.

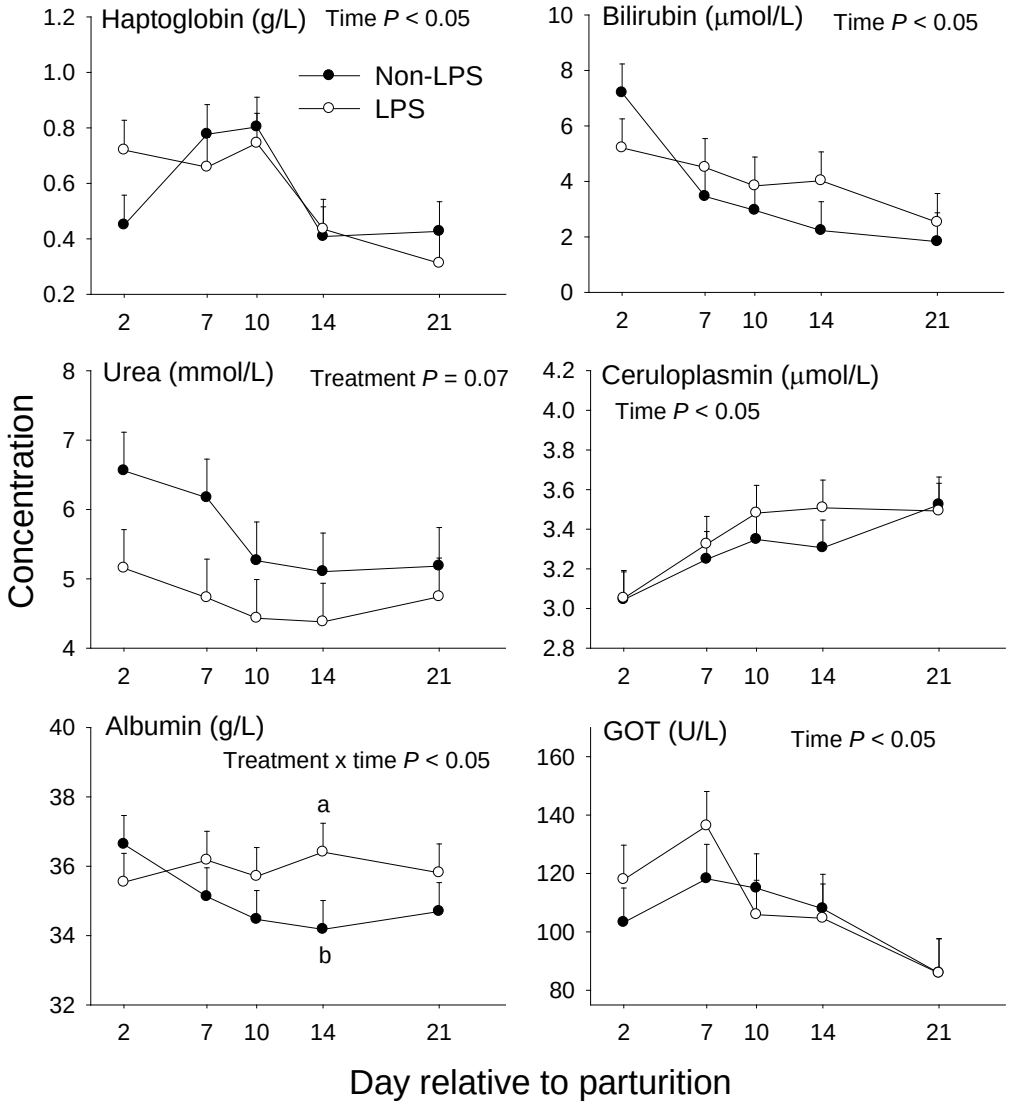


Figure 4. Blood concentrations of ROM, cholesterol, vitamin A, vitamin E, β-carotene and creatinine in cows fed a moderate-energy diet (1.62 Mcal/kg DM) during the entire dry period with (LPS) or without (Non-LPS) an intra-mammary LPS challenge at 7 d postpartum. Superscript letters (a, b) denote significant (Treatment × time $P < 0.05$) differences between treatment at a specific time point.

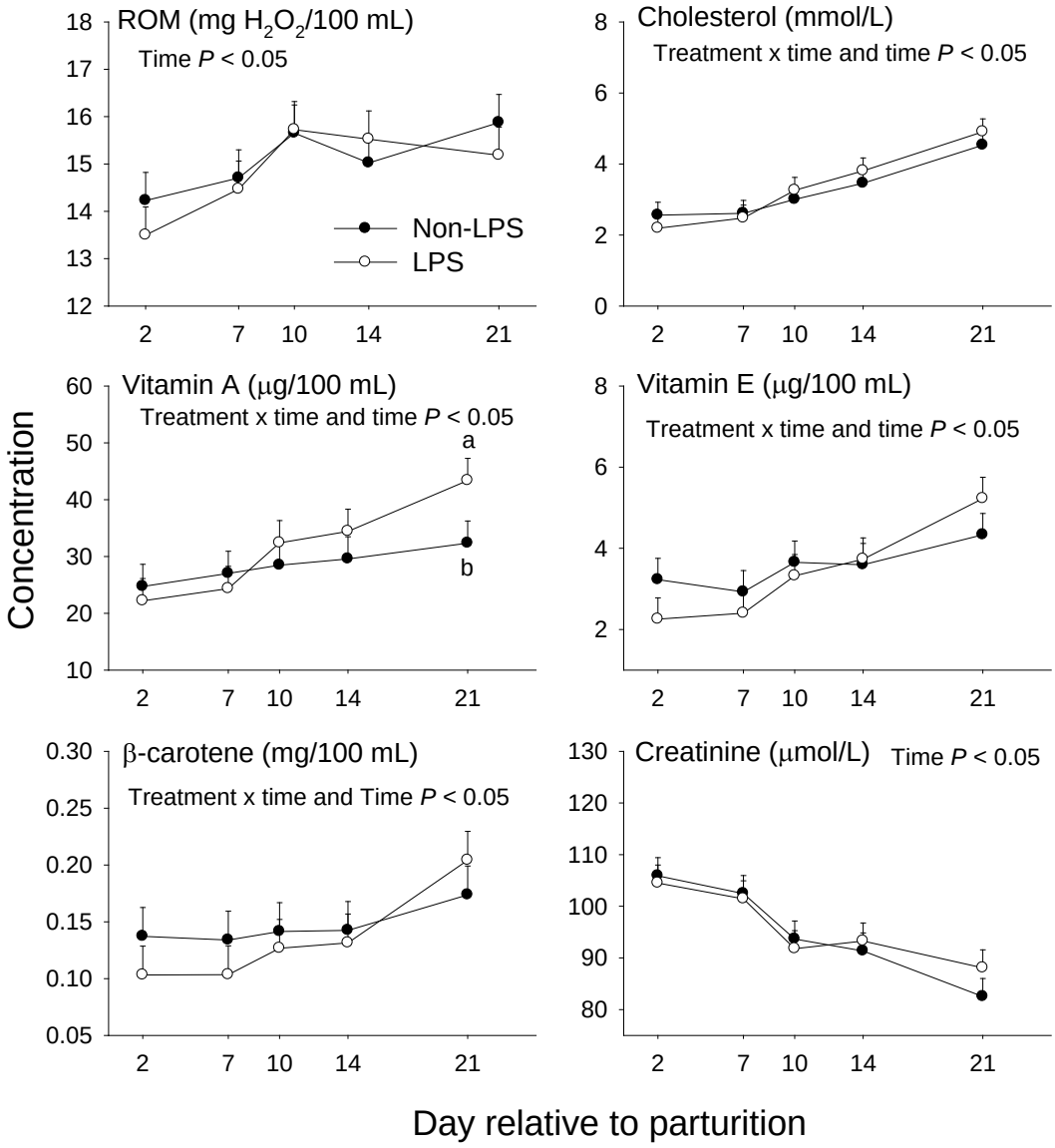
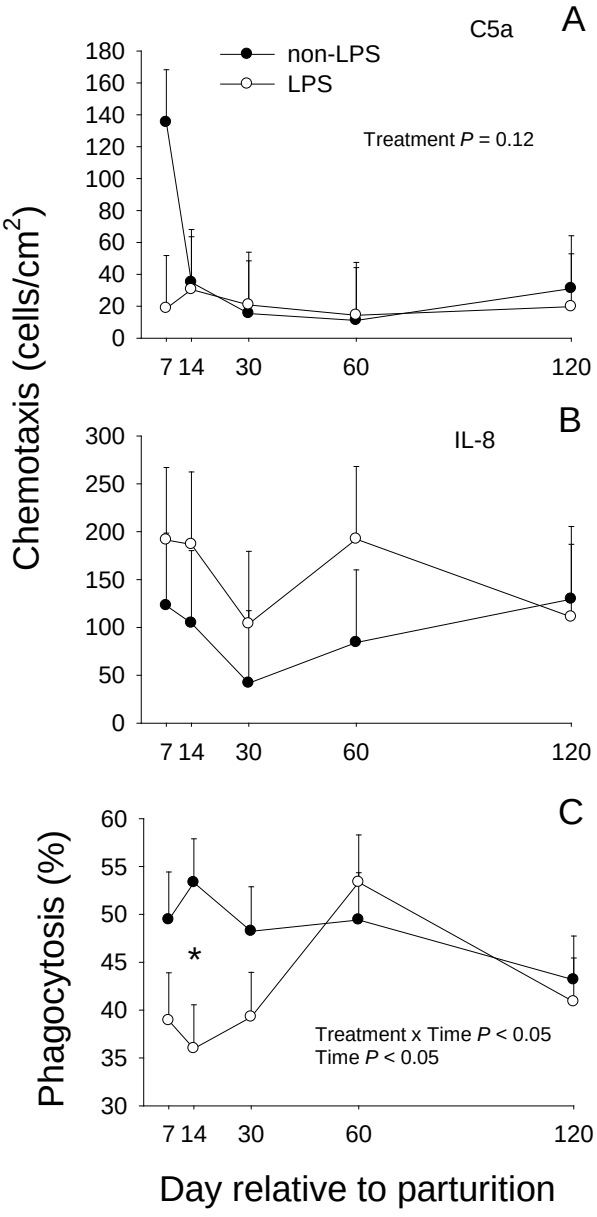


Figure 5. Blood neutrophils chemotaxis assessed with C5a (A) and human IL-8 (B) and total phagocytosis (C) in cows with (LPS) or without (non-LPS) an intra-mammary LPS-challenge at 7 d postpartum. Asterisks denote significant (Treatment × time $P < 0.05$) differences between treatments at a specific time point.



CHAPTER 3:

Immunometabolic Indices and Hepatic Gene Expression are Altered by Level of Dietary Energy Prepartum and Postpartum Inflammatory Challenge in Dairy Cows

D. E. Graugnard,*† M. Bionaz,*† E. Trevisi, ‡ J. M. Khan,*† D. Keisler,§ K. M. Moyes,*† J. Salak-Johnson,† J. K. Drackley,†‖ G. Bertoni, ‡ and J. J. Loor*†

*Mammalian NutriPhysioGenomics, †Department of Animal Sciences and ‖Division of Nutritional Sciences, University of Illinois, Urbana, IL 61801

‡Istituto di Zootecnica, Facolta di Agraria, Universita Cattolica del Sacro Cuore, 29122 Piacenza, Italy

§Department of Animal Science, University of Missouri, Columbia, MO 65211

INTRODUCTION

Most high-producing dairy cows experience a significant number of production (e.g., fatty liver and ketosis) and infectious diseases that may impair reproductive performance, consequently resulting in financial losses to farmers and reduced welfare (Mulligan and Doherty, 2008). The majority of these diseases are caused by factors including a level of production inconsistent with nutrient intake, provision of an inadequate diet pre- and post-partum, an unsuitable environment or various combinations of these factors (Mulligan and Doherty, 2008). Health problems experienced by cows during the peripartal period can hamper production in the long-term and lead to early culling from the herd (Mulligan and Doherty, 2008).

The negative energy balance (**NEB**) experienced by cows soon after calving has been associated with impaired neutrophil trafficking, phagocytosis, and killing capacity (Goff, 2006; Sordillo et al., 2009). The NEB associated with parturition leads to extensive mobilization of fatty acids stored in adipose tissue, thus, causing marked elevations in blood non-esterified fatty acids and hydroxybutyrate (**BHBA**) concentrations. Prepartal level of dietary energy can potentially affect adipose tissue deposition and, thus, the amount of NEFA released into blood and available for metabolism in liver (Drackley et al., 2005). Elevated blood NEFA and BHBA as well as reduced concentrations of antigen-binding antibodies (van Knegsel et al., 2007) during peripartal NEB all can contribute to immunosuppression. Ketone body concentrations similar to those observed around parturition impair the phagocytic and bactericidal capacity of neutrophils or polymorphonuclear leukocytes (**PMN**) in vitro, an effect that may lead to reduced udder defense mechanisms against mastitis pathogens (Sordillo and Aitken, 2009).

Current prepartal feeding practices can lead to elevated intakes of energy and have largely failed to overcome peripartal health problems or declining fertility (Beever, 2006).

Uncontrolled intake of diets with energy content resembling lactation diets can increase fat deposition in the viscera (Nikkhah et al., 2008) and upon parturition lead to compromised liver metabolism (Beever, 2006; Drackley et al., 2005). Although different studies have evaluated effects of prepartal manipulation of body condition score (**BCS**), overfeeding or underfeeding energy, and lipid supplementation on measures of metabolism and performance in peripartal dairy cows (Dann et al., 2006; Dann et al., 2005; Douglas et al., 2006), the present study sought to expand on the available body of knowledge accumulated regarding the concept of "feed-to-fill" (Dann et al., 2006; Janovick and Drackley, 2010), which has clearly shown that overfeeding moderate-energy diets prepartum results in greater metabolic stress and incidence of disorders postpartum (Janovick et al., 2011).

Our general hypothesis was that overfeeding a moderate-energy diet during the dry period would render the cow's immune function less responsive to an intramammary inflammatory challenge early postpartum. The main objectives of this study were to evaluate the effect of prepartal energy overfeeding on peripartal PMN function, metabolic and inflammation indices in blood, liver tissue lipid composition, and liver gene expression after an intramammary challenge with *Escherichia coli* (*E. coli*) lipopolysacharide (**LPS**) during the first wk postpartum.

MATERIALS AND METHODS

Animals and Diets

All procedures were conducted under protocols approved by the University of Illinois Institutional Animal Care and Use Committee # 06145. Twenty Holstein cows entering their second or greater lactation were used. Cows averaged composite SCC of ~128,000 ± 108,000 during the previous lactation and were assigned (n = 10/diet) to a control diet (high-wheat straw diet); which was fed ad libitum intake to provide at least 100% of calculated net energy for

lactation (NE_L) = 1.34 Mcal/kg or an overfed diet to provide 150% of calculated NE_L = 1.62 Mcal/kg during the entire dry period (~45 d) (Table 8). During the dry period, cows were fed diets as a total mixed ration (**TMR**) once daily (0600 h) using an individual Calan (American Calan, Northwood, NH) gate feeding system and were housed in a ventilated enclosed barn. Cows had access to sand-bedded free stalls until 5 d before expected calving date, when they were moved to an individual maternity pen bedded with straw. After parturition, cows were moved to a tie-stall barn and were fed a common lactation diet post-partum (NE_L = 1.69 Mcal/kg DM) and milked twice daily (0400 and 1600 h). Diets were mixed in a Keenan Klassik 140 mixer wagon (Richard Keenan & Co., Ltd., Borris, County Carlow, Ireland) equipped with knives and serrated paddles; straw in large square bales was chopped directly by the mixer without preprocessing.

Samples of feed ingredients and TMR were obtained weekly and analyzed for DM content to adjust the ration. Weekly samples of individual ingredients were frozen at -20°C and were composited monthly. Composite samples were analyzed for contents of DM, crude protein (**CP**), neutral detergent fiber, acid detergent fiber, Ca, P, Mg, and K using wet chemistry methods (Dairy One, Ithaca, NY, USA). Body weight was measured for each cow weekly. Milk weights were recorded daily and samples were obtained from consecutive a.m. and p.m. milkings. Consecutive a.m. and p.m. samples were composited in proportion to milk yield at each sampling and preserved (800 Broad Spectrum Mirotabs II; D&F Control Systems, Inc., San Ramon, CA). Composite samples were analyzed for fat, protein, lactose, urea-N, and SCC using midinfrared procedures at a commercial laboratory (Dairy One, Ithaca, NY, USA).

Energy Balance Calculations and Estimates

Energy balance was calculated individually for each cow using equations described previously (NRC, 2001). Net energy intake (NE_I; Mcal/d) was determined by multiplying DM intake (Kedmi and Peer) by the calculated mean NE_L density of the diet. The NE_L value of each individual feed (Dairy One, Ithaca, NY, USA) was used to calculate the mean NE_L content of the diet. Net energy required for maintenance (NE_M) was calculated as $BW(kg)^{0.75} \times 0.08$ (Mcal/kg). Net energy requirement for pregnancy (NE_P; Mcal/d) was calculated as [(0.00318 × day of gestation − 0.0352) × (calf birth weight/45)]/0.218. Milk net energy requirement (NE_{LAC}; Mcal/kg) was calculated as (0.0929 × fat% + 0.0563 × protein% + 0.0395 × lactose%) × milk yield. The equation used to calculate prepartal energy balance (EB_{PRE}; Mcal/kg was $EB_{PRE} = NE_I - (NE_M + NE_P)$. The equation used to calculate postpartal energy balance (EB_{POST}; Mcal/d) was $EB_{POST} = NE_I - (NE_M + NE_{LAC})$.

Lipopolysaccharide Challenge

At ~7 d in milk (**DIM**), all the cows received an intramammary *E. coli* LPS challenge (200 µg, strain 0111:B4, cat. # L2630, Sigma Aldrich, St. Louis, MO). Prior to LPS challenge (~2 days), foremilk samples from all quarters of each cow were cultured and confirmed to be bacteriologically negative. LPS was dissolved in 20 mL of 0.09% sterile physiological saline (Hospira, Lake Forest, IL). Immediately after milking (0530 h), one rear mammary quarter was disinfected with cotton wool pre-soaked in 70% ethanol and the LPS was infused via a sterile disposable syringe fitted with a sterile teat cannula using the full insertion infusion method. The quarter was thoroughly massaged.

Liver Biopsy and Chemical Composition

Liver was sampled via puncture biopsy (Dann et al., 2006) from cows under local anesthesia at approximately 0800 h on d -14, 7, 14 and 30 relative to parturition. Liver was frozen immediately in liquid N, and later analyzed for contents of total lipids, TAG and RNA extraction. Lipids and TAG were analyzed following protocols described before (Dann et al., 2006).

Chemotaxis

Twenty mL of blood for neutrophil isolation were collected at ~0700 h from the coccygeal vein or artery in vacutainer tubes containing EDTA at -14 (± 3 d) , 7 (prior to LPS), 14, 30, 60 and 120 DIM. After blood collection, tubes were placed on ice (~30 min) until isolation (Auchtung et al., 2004, Moyes et al., 2009, Salak et al., 1993). Samples were centrifuged at 600 × *g* for 15 min at 4 °C. The buffy coat and approximately one quarter of red blood cells were removed and discarded. The remaining sample was poured into a 50 mL tube prior to chemotaxis assay. Twenty mL deionized water at 4 °C were added to lyse red blood cells followed by addition of 5 mL 5X PBS at 4 °C to repristinate an iso-osmotic environment. Samples were centrifuged at 200 × *g* for 10 min at 4°C. Three subsequent washings using 1X PBS at 4 °C were performed (centrifuge was set at 500 × *g* for 3 min at 4 °C). Isolated neutrophils were resuspended in 1 mL 1X PBS at 4 °C and kept on ice. Cells were counted using a Beckman Coulter Counter after addition of Zap-OGlobin II Lytic Reagent (cat. #13020, Beckman Coulter) to further lyse any remaining red blood cells.

Chemotaxis was assessed using a method previously described (Auchtung et al., 2004, Salak et al., 1993) with modifications (Moyes et al., 2009). A total of 3×10^6 cells/mL were resuspended in 1 mL of RPMI 1640 media (R0883, Sigma) with 5% FBS. The assay was

conducted in a 48-well Micro AP48 Chemotaxis Chamber (P48AP30, Neuro Probe). Thirty µL of 10 ng/mL RPMI 1640 (without FBS) of human interleukin-8 (I1645, Sigma), 1 ng/mL RPMI 1640 (without FBS) of human complement C5a (C5788, Sigma), and RPMI 1640 (without FBS, control) were added to each of 4 wells per sample (quadruplicate). A PVP-free filter (5 µm pore size, 25 × 88 mm; cat# 416306, Neuro Probe) was mounted in each chamber. The chamber was incubated in 5% CO_2 at 37 °C for 10 min for equilibration. Fifty µL of 3×10^6 cells/mL from each incubated in quadruplicate in 5% CO_2 at 37°C for 1 h. The membrane was then removed and fixed with Hema 3 Hematology Staining Methanol Fixative (122-929, Fisher Scientific). Cell membranes were stained with Hema 3 Hematology Staining Solution I (122-937, Fisher Scientific) and then cell nuclei were stained with Hema 3 Hematology Staining Solution II (122-952, Fisher Scientific). The number of cells in each well was counted using an inverted microscope. Lastly, cell counts were corrected based on viability and background (RPMI values were subtracted).

Phagocytosis

Twenty mL of blood for neutrophils isolation were collected at ~0700 h by coccygeal vein or artery in vacutainer tubes containing sodium heparin at -14 (± 3 d), 7 (prior to LPS), 14, 30, 60 and 120 DIM. After blood collection, tubes were placed on ice (~30 min) until isolation (Moyes et al., 2009). Samples were centrifuged at 600 × *g* for 15 min at 4 °C. The buffy coat and approximately one quarter of red blood cells were removed and discarded. The remaining sample was poured into a 50 mL tube prior to phagocytosis assay. Twenty mL deionized water at 4 °C were added to lyse red blood cells followed by addition of 5 mL 5X PBS at 4 °C to repristinate an iso-osmotic environment. Samples were centrifuged at 200 × *g* for 10 min at 4°C. Three subsequent washings using 1X PBS at 4 °C were performed (centrifuge was set at 500 × *g* for 3

min at 4 °C). Isolated neutrophils were resuspended in 1 mL 1X PBS at 4 °C and kept on ice. Cells were counted using a Beckman Coulter Counter after addition of Zap-OGlobin II Lytic Reagent (cat. #13020, Beckman Coulter) to further lyse any remaining red blood cells.

Phagocytosis (2×10^6 cells/mL) was conducted in quadruplicate in 1 mL RPMI 1640 media following addition of a 1:10 ratio of Fluoresbrite latex Carboxy Yellow-Green 1.75 μm Microspheres (2.5%, #17687, Polysciences, Inc.). Samples were then incubated for 2 h in 5% CO_2 at 37 °C. A control sample was incubated for 2 h at 4 °C. After incubation, cells were rinsed twice with 1X PBS (via centrifugation at 1000 × *g* for 5 min at 4 °C), fixed with 150 μL 4% paraformaldehyde (P6148, Sigma), and preserved at 4 °C until reading using flow cytometry.

Cell Viability and Differential

Twenty μL of the cell suspension from each sample for chemotaxis and phagocytosis assays was used to determine viability using a Burke chamber after 2 min incubation with a solution of Trypan blue. The average of viable neutrophils was 71.69 ± 7.87; data for chemotaxis and phagocytosis was corrected with the observed viability. Fifty μL of cell suspension from the samples used for the chemotaxis assay were fixed in a microscope slide to determine cell differentials; overall the average of neutrophils in the differential was 55.98 ± 5.49.

Blood Metabolites

For the immediate time post-LPS challenge, blood was sampled from the coccygeal vein or artery at 1200 h during d 2, 10, 14, and 21 relative to parturition. During d 7 (LPS challenge day) the blood samples were collected before the LPS challenge (at 530 = 0 h), 2, 6 and 12 h after LPS. For data at 2, 7, 10, 14, and 21 d relative to parturition blood was collected at 1200 h. In both cases samples were collected into evacuated tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) containing either ethylenediaminetetra acetic acid (**EDTA**)

or lithium heparin for plasma and a clot activator for serum. After blood collection, tubes with EDTA and lithium heparin were placed on ice while tubes with clot activator were kept at room temperature until centrifugation (~30 min). Serum and plasma were obtained by centrifugation at 1,900 × *g* for 15 min. Aliquots of serum and plasma were frozen (-20°C) until further analysis. Measurements of serum NEFA and BHBA were performed using commercial kits in an auto-analyzer at the University of Illinois Veterinary Diagnostic Laboratory (Urbana, IL, USA). Other parameters were measured in lithium heparin samples at the Istituto di Zootecnica at the Università Cattolica del Sacro Cuore in Piacenza (Italy). Glucose, albumin, cholesterol, bilirubin, creatinine, urea, and glutamic-oxalacetic transaminase (**GOT**) were determined using kits purchased from Instrumentation Laboratory (IL Test) following the procedures previously described (Bionaz et al., 2007) in a clinical auto-analyzer (ILAB 600, Instrumentation Laboratory, Lexington, MA, USA) . Triacylglycerol (**TAG**) was measured using a commercial kit (LabAssay^{TM} Triglyceride, Wako Chemicals Inc., USA). Haptoglobin and ceruloplasmin were analyzed using methods previously described (Bertoni et al., 2008) adapted to the ILAB 600 conditions. Plasma vitamin A, vitamin E, and β-carotene were extracted with hexane and analyzed by reverse-phase HPLC using Allsphere ODS-2 3μm in a 150 × 4.6 mm column (Grace Davison Discovery Science, Deerfield, IL, USA), a UV detector set at 325 nm (for vitamin A), 290 nm (for vitamin E), or 460 nm (for β-carotene), and 80:20 methanol:tetrahydrofurane as the mobile phase. Total plasma reactive oxygen metabolites (**ROM**) were measured using the analytical method patented by Diacron International s.r.l. (Grosseto, Italy) (Bertoni et al., 2008). Plasma insulin concentrations were measured by a double antibody radioimmunoassay, using a kit for human insulin following the procedures from the vendor (Diagnostic Systems

Laboratories, Inc., Webster, TX, USA). The detection limit of the assay was 1.3 mU/ml; the coefficients of variation averaged 7.5% within assay and 9.5 % between assays.

There is no commercially available bovine-specific Angptl4 ELISA kit. Therefore, we validated a human Angptl4 Elisa kit (RayBiotech Inc., Norcross, GA) for use with bovine samples. The specificity of the anti-human Angptl4 detection antibody from RayBio kit was evaluated by Western blot analysis of bovine serum protein and recombinant bovine Angptl4. Recombinant bovine Angptl4 was produced by GenScript Corporation (Piscataway, NJ). The ArcticExpress (DE3) RP E. coli strain was transformed a pET-15b vector construct encoding an N-His tag and the mature bovine Angptl4 protein (NCBI accession NP_001039508.1, region 24 - 410). Purity and immunoreactivity of the isolated recombinant bAngptl4 were verified by a previously-validated Western blot assay (Mamedova et al., 2010). Serum or standard samples (1 µL) were diluted with 19 µL Laemmli sample buffer (Bio-Rad, Richmond, CA). Recombinant bovine and human Angptl4 standards were used at 200 pg and 20 pg concentrations. Samples were heated at 90°C for 5 min, cooled, vortexed, and loaded onto a 4-20% Tris-HCl gel for electrophoresis. Samples were separated by SDS-PAGE and dry-transferred onto nitrocellulose membranes (iBlot, Invitrogen, Carlsbad, CA). Membranes were blocked for 2 h in blocking buffer (5% dry milk in Tris-HCl buffer, pH 7.5, with 0.05 % Tween 20). After incubation with blocking buffer, the membranes were washed 3 times for 5 min each with washing buffer (phosphate-buffered saline, pH 7.5, containing 0.05% Tween 20), then incubated for overnight with biotinylated anti-human Angptl4 (RayBiotech Inc) diluted 20,000-fold in blocking buffer. After incubation, membranes were rinsed 3 times with washing buffer and then incubated for 1 h with horseradish peroxidase-labeled streptavidin diluted 50,000-fold in blocking buffer. Immunodetection was performed by chemiluminescence (West-Dura; Thermo Scientific,

Waltham, MA). Western blot showed that the detection antibody used in the Angptl4 ELISA detected a single band in serum with the expected molecular weight of hAngptl4 (~55 kDa).

RNA extraction, quantitative PCR (qPCR), and design and evaluation of primers

RNA samples were extracted from frozen tissue using established protocols in our laboratories (e.g. Loor et al., 2007). Briefly, liver tissue sample was weighed (~0.3-0.5 g) and straightway was put inside a 15 ml centrifuge tube (Corning Inc. ®, Cat. No. 430052) with 1 µl of Linear Acrylamine (Ambion® Cat. No. 9520) as a co-precipitant, and 5 ml of ice-cold Trizol reagent (Invitrogen Corp.) to proceed with RNA extraction according to the manufacturer. This extraction procedure also utilizes acid-phenol chloroform (Ambion® Cat. No. 9720, Austin, TX, USA), which removes residual DNA. Any remaining genomic DNA was removed from RNA with DNase using RNeasy Mini Kit columns (Qiagen, Germany). RNA concentration was measured using a Nano-Drop ND-1000 spectrophotometer (Nano-Drop Technologies). The purity of RNA (A_{260}/A_{280}) for all samples was above 1.81. The quality of RNA was evaluated using the Agilent Bioanalyzer system (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA). The average RNA integrity number (RIN) value for samples was 8.0 ± 0.4.

For qPCR, cDNA was synthesized using 100 ng RNA, 1 µg dT18 (Operon Biotechnologies, Huntsville, AL, USA), 1 µL 10 mmol/L dNTP mix (Invitrogen Corp., CA, USA), 1 µL random primer $p(dN)_6$ (Roche® Cat. No. 11 034 731 001, Roche Diagnostics GmbH, Mannheim, Germany), and 10 µL DNase/RNase free water. The mixture was incubated at 65 °C for 5 min and kept on ice for 3 min. A total of 6 µL of master mix composed of 4.5 µL 5X First-Strand Buffer, 1 µL 0.1 M dithiothreitol, 0.25 µL (50 U) of SuperScript™ III RT (Invitrogen Corp.CA, USA), and 0.25 µL of RNase Inhibitor (10 U; Promega, Madison, WI, USA) was added. The reaction was performed in an Eppendorf Mastercycler® Gradient using the

following temperature program: 25 °C for 5 min, 50 °C for 60 min and 70 °C for 15 min. cDNA was then diluted 1:4 (v:v) with DNase/RNase free water.

Quantitative PCR (qPCR) was performed using 4 μL diluted cDNA (dilution 1:4) combined with 6 μL of a mixture composed of 5 μL 1 × SYBR Green master mix (Applied Biosystems, CA, USA), 0.4 μL each of 10 μM forward and reverse primers, and 0.2 μL DNase/RNase free water in a MicroAmp™ Optical 384-Well Reaction Plate (Applied Biosystems, CA, USA). Each sample was run in triplicate and a 6 point relative standard curve plus the non-template control (NTC) were used (User Bulletin #2, Applied Biosystems, CA, USA). The reactions were performed in an ABI Prism 7900 HT SDS instrument (Applied Biosystems, CA, USA) using the following conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C (denaturation) and 1 min at 60 °C (annealing and extension). The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures to 95 °C for 15 s plus 65 °C for 15 s. Data were calculated with the 7900 HT Sequence Detection Systems Software (version 2.2.1, Applied Biosystems, CA, USA). The final data were normalized using the geometric mean of ubiquitously-expressed transcript (*UXT*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and ribosomal protein S9 (*RPS9*). Sequence and primer information of *GAPDH* and *RPS9* were published by Janovick et al. (2007).

Genes selected for transcript profiling in this study are listed in Table 10. Primers were designed using Primer Express 2.0 or 3.0 with minimum amplicon size of 80 bp (when possible amplicons of 100-150 bp were chosen) and limited 3' G+C (Applied Biosystems, CA). When possible, primers were designed to fall across exon–exon junctions. Primers were aligned against publicly available databases using BLASTN at NCBI and UCSC's Cow (Bos taurus) Genome

Browser Gateway. Prior to qPCR, primers were tested in a 20 μL PCR reaction using the same protocol described for qPCR except for the final dissociation protocol. For primer testing we used a universal reference cDNA (RNA mixture from 5 different bovine tissues) to ensure identification of desired genes. Five μL of the PCR product were run in a 2% agarose gel stained with ethidium bromide (2 μL). The remaining 15 μL were cleaned using QIAquick® PCR Purification Kit (QIAGEN) and sequenced at the Core DNA Sequencing Facility of the Roy J. Carver Biotechnology Center at the University of Illinois, Urbana-Champaign. Only those primers that did not present primer-dimer, a single band at the expected size in the gel, and had the right amplification product (verified by sequencing) were used for qPCR (Tables 11-13). The accuracy of a primer pair also was evaluated by the presence of a unique peak during the dissociation step at the end of qPCR.

Efficiency of PCR amplification for each gene was calculated using the standard curve method [$E = 10^{(-1/slope)}$] (Table 14). Relative mRNA abundance among measured genes was calculated as previously reported (Bionaz and Loor 2008), using the inverse of PCR efficiency raised to ΔCt (gene abundance = $1/E^{\Delta Ct}$, where ΔCt = Ct sample - geometric mean Ct of 3 internal control genes; Table 14). Overall mRNA abundance for each gene among all samples measured was calculated using the median ΔCt. Use of this technique for estimating relative mRNA abundance among genes was necessary because relative mRNA quantification was performed using a standard curve (made from a mixture of RNA) which precluded a direct comparison among genes. Together, use of Ct values corrected for the efficiency of amplification plus internal control genes as baseline overcome this limitation.

Statistical Analysis

A total of 10 cows from each prepartal dietary group (from 20/group that started the study) with the most complete data set including production, blood indices, and PMN function were used for the present report. Eleven of the original 40 cows were removed from statistical analysis due to lameness and/or bacteriologically-positive quarters after parturition. The MIXED procedure of SAS (SAS Institute, Inc., Cary, NC, USA) was used for statistical analysis. The fixed effects included prepartal diet (control or overfed), time (7, 14, 30, 60, and/or 120 d relative to parturition; or 0, 2, 6, and 12 h post-LPS), and interaction diet × time. The random effect was cow within diet. A repeated measures analysis using an AR(1) structure was used. Each variable of interest was evaluated for normal distribution using the Shapiro-Wilk test (SAS Inst. Inc.) and normalized by logarithmic transformation when necessary prior to statistical analysis. Normalized gene expression data were log-2 transformed prior to statistical analysis. All means were compared using the PDIFF statement of SAS (SAS Institute, Inc., Cary, NC, USA).

RESULTS

Performance

Average DMI (% or kg/d) over the last 4 wk prepartum did not differ ($P > 0.10$) due to diet (Table 9). We observed greater DMI as % of BW during the first 41 d postpartum in the control group (Table 9). The overall consumption of DM was similar for both groups when expressed in kg/day, thus, differences in DMI as % of BW were due to lower BW in control cows when compared to overfed (Table 9). Both groups increased DMI (kg/d or as % of BW) gradually over time (Time $P < 0.05$; Figure 6). Milk yield also increased (Time $P < 0.05$; Figure 6) for both groups from 7 to 41 DIM with no differences among diets; there were no differences

among diets in milk fat percentage resulting in similar fat-corrected milk (**FCM**) yield and apparent energetic efficiency.

Average BW and BCS during the last 4 wk prepartum were greater ($P < 0.05$) in overfed than control cows (Table 9). In terms of postpartal BCS there was no overall difference ($P > 0.10$) due to diet. However, we observed an expected decrease ($P < 0.05$) in BCS between wk 1 to 6 after parturition. As expected, energy balance (Mcal/d or % of requirements) during the last 4 wk prepartum was greater ($P < 0.05$) in cows overfed the moderate-energy diet. We also observed clear differences in energy balance status during the first wk after parturition due to diet, i.e. cows in the control group were in more positive energy balance status (i.e. before LPS; $P < 0.05$). There was an overall increase (Time $P < 0.05$) in energy balance between wk 2 to 6 regardless of diet. Calf birth weight was not affected ($P > 0.10$) by prepartal dietary energy level.

Acute Response to Lipopolysaccharide

Figure 15 shows the temporal changes in rectal temperature and blood concentration of NEFA, BHBA, TAG and bilirubin before (0 h) and during the first 12 h post-LPS challenge. There were no temperature differences among dietary prepartum treatments; however, there was an obvious and expected temperature increase (Time $P < 0.05$) that peaked at 6 h post LPS challenge. Intramammary LPS challenge regardless of diet increased ($P < 0.05$) circulating NEFA and TAG by 2 h post-challenge after which concentrations returned to pre-challenge values. Opposite to NEFA and TAG, there was a gradual decrease (Time $P < 0.05$) in blood BHBA concentration regardless of prepartal diet. Bilirubin increased (Time $P < 0.05$) 2 h after the infusion of LPS regardless of prepartal diet and was maintained until the last sampling time at 12 h post challenge.

Lipid and TAG Accumulation in Liver

Figure 7 shows the concentrations of liver lipid and TAG before and after parturition. After parturition, i.e. in the biopsy harvested ~2-3 h post-LPS challenge on d 7, we observed a greater (Diet × Time $P < 0.05$) concentration of lipid and TAG in the overfed group and this difference persisted until 14 d for total lipid and until 30 d for TAG concentration.

Metabolic and Oxidative Stress Indices in Blood

Figure 8 shows blood serum concentration of the metabolic indicators NEFA, BHBA, glucose, insulin, creatinine, ROM, TAG, and urea during the early postpartal period. After LPS challenge, concentration of NEFA decreased ($P < 0.05$) in the control group but it increased slightly or remained unchanged in overfed cows resulting in a Diet × Time response ($P < 0.05$) during d 10 to 21 post-partum. Concentration of BHBA decreased (Time $P < 0.05$) after LPS challenge through d 14 regardless of diet, and then increased to pre-challenge concentrations by d 21.

Glucose concentration remained constant from 2 to 7 DIM after which a significant (Time $P < 0.05$) increase regardless of diet was observed by 10 through 21 DIM (Figure 8). In contrast, overfed cows had greater (Diet × Time $P = 0.09$) blood insulin due to differences at d 2 postpartum. Temporal blood insulin concentration seemed to reflect changes in blood glucose with a marked increase (Time $P < 0.05$) from 7 to 10 DIM.

Overall concentration of ROM was greater for cows in the overfed group (Diet $P < 0.06$) than the control due primarily to greater values at 10 and 21 DIM. Although control cows had greater (Diet × Time $P < 0.05$) creatinine concentration at 2 d postpartum, regardless of diet creatinine decreased over time ($P < 0.05$) in both groups to a nadir at 10 DIM.

The concentration of TAG decreased (Time $P < 0.05$) gradually from 2 to 21 d after parturition in both groups. In addition, cows in the control group tended ($P = 0.11$) to have overall higher concentrations of urea. Its concentration decreased (Time $P < 0.05$) in both groups from 7 to 14 d followed by a return to basal concentrations by d 21.

Liver Function and Inflammation Indices in Blood

Figure 10 shows concentrations of cholesterol, albumin, ceruloplasmin, haptoglobin, glutamic oxaloacetic transaminase (Gottipati et al.), and bilirubin. Cholesterol concentration was greater (Diet × Time $P < 0.05$) in the overfed group at 10 and 14 DIM when compared to controls but the trend for both groups was for a gradual increase through 21 DIM. Albumin concentration was greater at 2 and 21 DIM in the control group resulting in a significant interaction ($P < 0.05$). Regardless of dry period diet, ceruloplasmin concentration increased gradually (Time $P < 0.05$) from 2 to 10 DIM; subsequently, the concentration was constant until 21 DIM. Furthermore, the overfed group had greater (Diet $P = 0.08$) ceruloplasmin concentrations during the 21 d period. Concentration of haptoglobin increased (Time $P < 0.05$) sharply between 7 and 10 DIM then decreased by d 14 to 21 to values below those observed at 2 and 7 DIM. Concentration of angiopoietin-like 4 (ANGPTL4), which has been recently identified as an acute-phase protein in rodents (Lu et al., 2010), was greater overall (diet P = 0.06) in cows fed controlled-energy. Concentrations of both GOT and bilirubin decreased (Time $P < 0.05$) gradually by 10 to 21 DIM regardless of diet. Regardless of dry period diet, vitamin A, E and β-carotene concentration increased (Time $P < 0.05$) over time (Figure 11) and overall vitamin E concentration was greater ($P = 0.09$) in the overfed group due mainly to differences at 10, 14 and 21 DIM.

Growth Hormone (GH) and Insulin-like Growth Factor-1 (IGF-1)

Figure 13 shows the concentrations of GH and IGF-1 along with mRNA expression of two genes associated with GH signaling in liver. There was an interaction effect (Diet × Time $P = 0.07$) for GH due primarily to greater blood concentrations at 0, 2, and 30 d relative to calving. In contrast, we observed a significant interaction (Diet × Time $P < 0.05$) for the concentration of IGF-1 due to the response prepartum in overfed cows which had greater concentrations through calving. The temporal changes (Time $P < 0.05$) in concentration of GH and IGF-1 were typical of this physiological stage, i.e. gradual increase in GH from late prepartum through early lactation coupled with a gradual decrease in IGF-1.

Neutrophil Chemotaxis and Phagocytosis

Neutrophil chemotaxis evaluated with human C5a or IL-8 did not differ between treatments (Figure 12). However, there was an overall time effect ($P < 0.05$) for chemotaxis assessed with IL-8. This temporal response was characterized by a gradual increase in chemotaxis from 7 through 120 DIM. Total neutrophil phagocytosis capacity (Figure 12) was lower (Diet × Time $P < 0.06$) at 14 and 120 DIM in the overfed group versus the control. Despite a rebound in phagocytosis capacity between 14 to 60 DIM in overfed cows (Diet × Time $P < 0.05$), phagocytosis again decreased markedly between 60 and 120 DIM. Overall, the temporal response observed in overfed cows compared with the steady increase in phagocytosis capacity in control cows led to lower overall phagocytosis due to prepartal energy overfeeding.

Hepatic Gene Expression

Table 15 and Figure 14 and 15 show relative mRNA expression of genes related with metabolism, stress and inflammation, and control of gene transcription. We observed upregulation over time ($P < 0.01$; Table 15) in *DGAT1* and *FAAH* both of which are involved in

metabolism of lipids. Among the stress and inflammation-related genes, *PERK* expression increased over time ($P < 0.01$; Table 15) with no differences among prepartal diet. Another gene associated with cellular stress is *XBP1* and was downregulated over time ($P < 0.01$; Table 15) with no differences among prepartal diet. Among transcription regulators evaluated, *NFKB1, CREB3L3* and *PPARD* behaved in a similar fashion with a decrease in mRNA expression after d 7 (Time $P < 0.01$) with no differences observed due to diet.

Figure 14 shows relative mRNA expression of key genes related with fatty acid oxidation, ketogenesis, and growth hormone (**GH**) signaling in liver. Our data revealed significant upregulation (Diet × Time $P < 0.05$) between 7 and 14 DIM in the expression of *ACOX1*, *HMGCS2,* and *PPARA* due to feeding control with a corresponding decrease in *CPT1A*, *HMGCS2*, *STAT5B,* and *SOCS2* in overfed cows. In addition, the increase in *PPARA* by 14 DIM with controls resulted in greater expression (Diet × Time $P < 0.05$) than overfed cows; whereas, the decrease in *STAT5B* and *SOCS2* by 14 DIM in overfed cows resulted in lower expression (Diet × Time $P < 0.05$) than controls.

The only genes affected by prepartal diet were *SOD2* and *MYD88*, with overfed cows having greater expression ($P < 0.05$; Figure 15). Postpartum, the expression pattern of *SOD2, TNF*, *MYD88*, *TLR4*, and *NFKB1* was similar ($P > 0.10$) between treatments; downregulation was observed after LPS infusion from d 7 to d 14 after which the expression level was maintained relatively stable through d 30 ($P < 0.01$). In the case of *NR3C1* expression was downregulated (Diet × Time $P < 0.10$) between 7 and 14 d in both overfed and control cows but expression decreased further by 30 DIM in overfed cows; whereas, cows fed control had stable expression through 30 DIM.

DISCUSSION

Short-term Adaptations to LPS

The 12-h blood metabolite analysis provided some evidence of acute effects of LPS on adipose lipolysis, ketogenesis, and liver function (i.e., bilirubin clearance). Similar results for NEFA and BHBA were observed in cows infused i.v. with LPS during mid-lactation (Waldron et al., 2003); however, in non-lactating heifers there was an actual increase in NEFA and glycerol coupled with decreased BHBA (Steiger et al., 1999). In non-ruminants and ruminants (Steiger et al., 1999) bacterial LPS elicits inflammation (e.g. increased blood TNF) leading to elevated circulating levels of NEFA resulting from enhanced adipose lipolysis (Zu et al., 2009). These data (Steiger et al., 1999; Waldron et al., 2003) seem to suggest that ketogenesis (at least in the short-term) may have been impaired by LPS-induced inflammation, an effect that has been clearly demonstrated in non-ruminants (Khovidhunkit et al., 2004). Systemic hypertriglyceridemia also is a consequence of an inflammatory response caused by LPS in non-lactating animals (Feingold et al., 1992). This effect has been attributed to impairment in clearance of triglyceride-rich lipoproteins from the circulation, enhanced hepatic VLDL production, and/or reduced activity of tissue (e.g. adipose, heart, muscle) lipoprotein lipase (Kaufmann et al., 1976). In our study, mammary utilization of TAG fatty acids and NEFA may have played a role in the rapid return to basal concentrations of both metabolites.

At least in non-ruminants, the increase in bilirubin concentration (an index of liver function; Bionaz et al., 2007) as a consequence of inflammation appears to be caused by the proinflammatory cytokine IL-1β, which inhibits the nuclear activator NFKB that regulates the mRNA expression of key hepatic enzymes involved in bilirubin clearance (Assenat et al., 2004). The results from our study are somewhat comparable with those reported by Bionaz et al. (2007)

where plasma bilirubin was highest (10.1 vs. 6.2 ± 0.6 μmol/L) in cows classified retrospectively as having low liver function. The discussion below will focus on longer-term adaptations to LPS challenge as it relates to prepartal consumption of dietary energy.

Performance Effects due to Prepartal Energy and LPS

Our performance data (Table 9, Figure 6) are similar to those reported in previous experiments (Douglas et al., 2006; Janovick and Drackley, 2010) in cows fed to meet or exceed prepartal energy requirements (ca. 100 to 150% of NRC requirements). Although data reported by Janovick and Drackley (2010) showed that overfed cows tended ($P = 0.10$) to produce more milk over the first 8 wk of lactation, the tendency was negated when yields were adjusted for previous mature equivalent yield. Together, our performance data during the first ~6 wk postpartum showed that there was no benefit of prepartal overconsumption of energy and demonstrated evidence of more severe NEB early postpartum.

Liver Lipid Composition

The observed concentration of total lipid in liver we observed post-LPS challenge in overfed cows was similar to cows classified as having fatty liver (~8% to ~11% total lipid) during the first ~3 wk postpartum (Ametaj et al., 2005). Control LPS-challenged cows had similar total liver lipid as healthy/controls in the study of Ametaj et al. (2005). There are several mechanisms that can be proposed to explain the differences to LPS between diets. First, overfeeding during the dry period leads to overconditioning (i.e. excessive adipose tissue deposition) and greater BCS prior to calving and a more marked reduction in appetite around calving (Hayirli et al., 2002). As a consequence, prepartal overconditioned high-producing dairy cows often go into a more severe NEB than cows that have a normal appetite (Hayirli et al., 2002; Rukkwamsuk et al., 1999), which is in line with what we observed (Table 9). Under such

conditions, it is likely that the capacity of the liver to maintain the balance between export of the TAG in VLDL with hepatic TAG synthesis is inadequate, thus, resulting in TAG accumulation (Drackley et al., 2005). Our observations that overfeeding energy prepartum led to greater accumulation of TAG in liver soon after LPS challenge (and in the longer-term) is in line with blood metabolic indicators of liver lipid metabolism, i.e. overfed cows had greater blood NEFA between 10 and 21 DIM, numerically-greater TAG between 10 and 14 DIM, but had a similar decrease in blood BHBA between 7 and 14 DIM (Figure 10). Together, those data are suggestive of greater impairment of liver lipid metabolism due to LPS.

Although previous studies have examined blood markers of inflammation in response to endotoxin or *E. coli* mastitis early postpartum (Hoeben et al., 2000), we are unaware of studies with early postpartal cows that have examined any link between liver lipid composition in response to LPS or endotoxin challenge and prepartal feeding management. However, there is evidence of increased plasma NEFA concentrations following LPS infusion (Steiger et al., 1999; Waldron et al., 2003) or TNF (Kushibiki et al., 2003), and of liver TAG accumulation following s.c. TNF in mid-lactation cows (Bradford et al., 2009). Our data revealed that prepartal energy overfeeding coupled with an inflammatory challenge early postpartum elicited a sustained lipolytic response, hence, favoring higher levels of NEFA in the circulation and resulting in persistent TAG accumulation during early lactation.

Markers of Inflammation and Oxidative Stress in Blood

Overall, the blood data on inflammatory and oxidative stress markers were suggestive of a chronic alteration in liver fatty acid oxidation and ketogenesis in the prepartal energy-overfed cows when exposed to an inflammatory challenge. The dramatic increase in hepatic mRNA expression of TNF at 7 vs. -14 DIM with both diets (Figure 15) likely reflected an acute response

to LPS, and agrees with previous data showing greater liver TNF mRNA expression in response to s.c. injection of bovine TNF (Bradford et al., 2009). Although there were no differences in blood NEFA, s.c. injection of TNF in mid-lactation cows for 7 d resulted in greater liver TAG concentration (Bradford et al., 2009). The TNF response in the overfed cows vs. controls might have remained elevated during the subsequent d post-LPS challenge, thus, favoring adipose tissue lipolysis (Kushibiki et al., 2003) and partly explaining the higher NEFA concentrations at 10 and 14 d (Figure 10).

Inflammatory responses to LPS in our cows seemed to differ from those observed by Trevisi et al. (2009) after sustained IFNα challenge around parturition where, despite a difference in BHBA concentrations, the increase in NEFA in treated and control cows was similar. Together the data seem to indicate that the nature of the inflammatory challenge (e.g., LPS, IFN-α, or pathogen challenge) in peripartal cows could dictate the type of physiological response by the animal leading to impaired ketogenesis in cows managed to overconsume energy prepartum, i.e. diets promoting overconditioning.

Excess accumulation of ROM can cause cell and tissue injury and lead to oxidative stress (Sordillo and Aitken, 2009). Cows with higher prepartal BCS, an indirect measure of body fatness, and elevated levels of NEFA at calving are in more precarious oxidative status [e.g., lower superoxide dismutase (**SOD**) activity in blood] rendering them more sensitive to oxidative stress (Bernabucci et al., 2005). A more recent study also showed that cows in more severe NEB during early lactation had higher blood ROM levels and inadequate availability of biological antioxidants, thus, were under increased oxidative stress (Pedernera et al., 2009). Our data appeared consistent with the above studies in the sense that overfed cows were in more positive energy balance prepartum, more NEB before the LPS challenge (Table 9), but ROM

concentrations between treatments did not differ until after LPS challenge when overfed cows also had a greater increase in concentration of NEFA.

Liver Function and Inflammation

Because of the direct link between liver synthesis of cholesterol, haptoglobin, and ceruloplasmin and their correlation with inflammation, the concentrations of these molecules in blood have been routinely used as indicators of liver function (Bertoni et al., 2008; Bionaz et al., 2007; Trevisi et al., 2009). Trevisi et al (2009) reported lower blood cholesterol in peripartal cows receiving IFN-α than controls. After LPS challenge, the greater temporal concentration of cholesterol and ceruloplasmin in overfed cows was suggestive of a more vigorous and sustained acute-phase response to LPS potentially as a counter regulatory mechanism to reach a normal set-point. This idea is supported by other data (e.g, ROM, NEFA, liver TAG, ceruloplasmin) showing that overfeeding energy prepartum clearly placed a bigger toll on metabolism which was exacerbated once the animal was faced with an inflammatory challenge. It is noteworthy that the temporal profile of ROM resembled that of ceruloplasmin (Figure 11), a response not observed in the inflammatory challenge study of Trevisi et al. (2009), which also could be taken as indication of some degree of impairment of liver function.

In the above context, it is well-established that inflammation protects animals against infection and tissue injury, but also can have deleterious consequences if it becomes deregulated (Sordillo et al., 2009). Our blood concentrations of cholesterol, ceruloplasmin, and albumin regardless of diet were within a non-pathological range (Bertoni et al., 2008). It was noteworthy, however, that albumin concentration was greater in control vs. overfed cows prior to LPS and also at 21 DIM which would be suggestive of higher liver function in those cows (Ametaj et al., 2005; Bertoni et al., 2008; Cavestany et al., 2005). Overall, the sustained increased in blood

cholesterol over time is a typical response (Bionaz et al., 2007; Cavestany et al., 2005) and has been previously related with an improvement in energy balance status (Bertoni et al., 2008).

Despite the apparent signs of normal liver function regardless of diet, the actual concentration of haptoglobin at 2 to 10 d in both groups was similar to those observed in postpartal cows with signs of inflammation and low liver function at 7 DIM (Trevisi et al., 2009). However, our data on liver TAG, cholesterol, and haptoglobin do not support a correlation between these parameters as reported by Ametaj et al. (2005) using early postpartal cows classified as healthy or with fatty liver, i.e. negative correlations between total liver lipid and cholesterol but positive correlations between total liver lipid and haptoglobin. Our data on liver TAG and NEFA post-LPS with overfed cows do support a positive relationship between both parameters as observed by Ametaj et al. (2005). Clearly, additional mechanisms at the level of liver or whole-animal likely play a role in the overall adaptations of the postpartal cow to an inflammatory challenge. Some of those are discussed in the gene expression section.

A previous study found lower temporal vitamin A concentration in response to inflammatory challenge with IFN-α around calving (Trevisi et al., 2009), which supported data demonstrating a negative relationship between inflammation and liver function around calving (Bertoni et al., 2008). The concentrations we observed (assessed as retinol) regardless of diet during the first 14 DIM were below the range associated with optimal liver function in early postpartal cows (Sordillo and Aitken, 2009) but they were within the range reported in the study of Trevisi et al. (2009). The overall increase in vitamin A concentration between 7 and 14 DIM suggested that LPS challenge did not impair the hepatic response despite the fact that overfed cows (as noted above) were under a more apparent stressful state.

Sordillo and Aitken (2009) provided a detailed review of the role of vitamin E during inflammation and immune response. There is evidence that both vitamin E and Selenium status of cows could affect the extent of oxidative stress experienced by the cow, hence, have an impact on the functional capability of peripartal blood neutrophils (Hogan et al., 1992; Sordillo et al., 2009). Neutrophil phagocytosis, bacterial killing, and oxidative metabolism are enhanced when cows have adequate levels of both vitamin E and Selenium (Sordillo and Aitken, 2009). Concentrations of vitamin E observed in this study between 2 and 7 DIM were above those reported by Bionaz et al. (2007) in cows grouped according to baseline paraoxonase activity. In fact, overall concentrations observed in our study by 21 DIM were nearly doubled those reported by Bionaz et al. (2007), suggesting that cows in our study likely were in adequate vitamin E status.

The temporal increase in vitamin E in overfed cows likely was related with the increase in ROM as a counter regulatory mechanism to stop or control their production (Sklan, 1983) induced by LPS. Furthermore, the temporal pattern of vitamin E post-LPS in overfed cows also resembled that of cholesterol and seemed to be in agreement with data showing a positive relationship between vitamin E and serum cholesterol concentration in chickens (Sklan, 1983). The rate of incorporation of acetate into hepatic cholesterol is >2-fold in chicken than cow (Emmanuel and Robblee, 1984), and both supplemental vitamin E and A increased bovine hepatic cholesterol and TAG synthesis (Pullen et al., 1990). Lower rates of cholesterogenesis partly explain the reduced capacity for VLDL export in ruminant liver (Pullen et al., 1990) but more importantly our data on liver TAG coupled with blood cholesterol and vitamin E after LPS in overfed cows appeared to highlight a mechanistic link, i.e. a potential adaptation by liver to handle excess NEFA and accumulation of TAG. Thus, despite the greater metabolic load facing

overfed cows there were counter regulatory mechanisms in place to respond to inflammation. Whether such responses would occur in all cows may depend on the overall health status of the animal, i.e. whether cows are free from metritis, mastitis, or other diseases typical of the postpartal period.

Neutrophil Function

Commonly, neutrophil function (e.g., phagocytosis, superoxide anion generation, chemotaxis) declines gradually as parturition approaches and lowest levels are often reached soon after parturition through 15 d postpartum (Gilbert et al., 1993; Kimura et al., 2002; Moya et al., 2008). Subsequently, neutrophil function increases through at least 6 wk postpartum (Sordillo et al., 2009). Contrary to previous studies mentioned above we found that feeding controlled-energy prepartum resulted in a stable response in total neutrophil phagocytosis postpartum through the first 30 DIM. Furthermore, in control cows the stable response remained through the LPS challenge; whereas, a clear decrease in phagocytic capacity was observed in the overfed cows (Figure 13). Despite the greater concentration of vitamin E post-LPS (Figure 12), the lower phagocytosis in overfed cows could have been related with greater blood NEFA and ROM (not BHBA), in particular, because of the well-established negative effects of such molecules on immune cell response during the peripartal period (Sordillo et al., 2009). These findings offer further support for controlling energy intake during the dry period, i.e. it could be more advantageous to the cow in terms of maintaining a more active immune environment during the first 30 DIM when they likely are most susceptible to mastitis pathogens.

Metabolic Hepatic Gene Expression

In non-ruminants, the nuclear receptor *PPARA* is considered the master regulator of hepatic lipid metabolism during fasting or undernutrition (Kersten et al., 1999). Activation of

PPARA promotes uptake, utilization, and catabolism of fatty acids via coordinated upregulation of *CPT1A*, *ACOX1*, and *HMGCS2* among other genes (Loor et al., 2005; Selberg et al., 2005). There also is evidence that PPARA activation can regulate inflammation partly by neutralizing and promoting the degradation of Leukotriene B_4 in both PMN and macrophages (Pyper et al., 2010). Initial data with transition dairy cows provided indirect evidence that circulating NEFA might be ligands for PPARA potentially resulting in the upregulation and downstream activation of genes with key functions in fatty acid oxidation, ketogenesis, and gluconeogenesis (Loor et al., 2005; Selberg et al., 2005). Although a recent study with dairy calves reported no change in expression of *PPARA* when injected with the PPAR ligand clofibrate (Litherland et al., 2010), this ligand increased or tended to increase expression of the PPARA target genes *ACADVL*, *ACOX1*, and *CPT1A*. Furthermore, work with bovine kidney cells recently showed that LCFA, e.g. palmitate, stearate, and eicosapentanoate, also might activate PPARA-regulated pathways (Bionaz et al., 2011). Therefore, the relative change in expression of PPAR target genes could be taken as indicators of treatment effects on the activity of this nuclear receptor.

The lack of increase in expression of *PPARA* between 7 and 14 DIM (i.e. post-LPS; Figure 12) in overfed vs. control cows might be explained in part by a derangement in the mechanisms that needed to be in place for the PPARA response to take place, e.g. downregulation of RXR or other co-activators (Pyper et al., 2010). Furthermore, it could be possible that GH signaling in liver is mechanistically linked with PPARA as in rodents (Ljungberg et al., 2007). The fact that both *STAT5B* and *SOCS2* decreased markedly after LPS only in overfed cows was suggestive of an exacerbated uncoupling of the GH/IGF-1 axis (Lucy, 2008) in response to inflammation. The NEFA and ROM response between 7 and 14 DIM in overfed cows clearly suggested an altered cellular state induced by LPS in energy-overfed cows.

The decrease in *HMGCS2* after LPS in overfed cows also suggested that inflammation in bovine could lead to impaired ketogenesis as in rodents (Khovidhunkit et al., 2004; Prieur et al., 2009).

Despite the inflammatory challenge, control cows clearly responded in a way that might have been expected under normal physiological conditions. The response in *PPARA*, *ACOX1*, and *HMGCS2* after the LPS-challenge in control cows followed the same trend (Figure 14) and appeared to be inversely associated (at least through d 14) with concentrations of liver TAG and blood NEFA and BHBA (Figure 8 and 10). Such response may have been associated with greater rates of LCFA oxidation as shown in dairy calves dosed with clofibrate (Litherland et al., 2010). In dairy cows grouped retrospectively based on concentrations of BHBA, correlations between BHBA and *HMGCS2* were significant regardless of grouping at 4 wk in lactation (van Dorland et al., 2009). Interestingly, in that study the group with higher BHBA concentration also had a positive correlation between *HMGCS2* and *CPT1A* as our data with control cows seemed to confirm.

Taken together, these data seem to suggest that energy-overfeeding despite increasing availability of NEFA to liver resulted in other alterations (e.g. inflammation) that dampened PPAR signaling. In fact, the decrease in *CPT1A* after LPS in overfed cows is similar to previous data from mid-lactation cows injected with TNF (Bradford et al., 2009), providing evidence that inflammation has the potential to inhibit fatty acid oxidation and indirectly result in TAG accumulation partly through increases in *CD36* and *AGPAT* mRNA expression (Khovidhunkit et al., 2004). There was no evidence of long-term effect of diet or inflammation on *MTTP* expression (Table 15) but the decrease in expression of *DGAT1* between 7 and 14 DIM regardless of diet could be taken as an indication of a reduction in VLDL synthesis. The ER-bound *DGAT1* enzyme, rather than the cytosolic *DGAT2*, is thought to be associated with

channeling of fatty acids towards the ER lumen for TAG synthesis and assembly of mature VLDL (Lavoie and Gauthier, 2006). The decrease in blood TAG between 7 and 14 DIM (Figure 10), particularly in control cows, seems to support this idea.

Our contrasting responses between groups in regards to BHBA, NEFA, *CPT1A*, *HMGCS2*, *PPARA*, and liver TAG point at additional mechanisms in control cows that prevented hepatic accumulation of TAG despite the apparent decrease in ketogenesis, i.e. lower BHBA from 7 through 14 DIM. In that regard, it should be noted that an important effect of PPARA on systemic lipid metabolism is the positive effect it has on peripheral VLDL utilization, a process driven via upregulation of hepatic *APOA5* and its activation of lipoprotein lipase in peripheral tissues (Kraja et al., 2010). Although we did not measure *APOA5*, a positive effect of *PPARA* upregulation on this apoprotein may have been associated with the gradual decrease in NEFA and TAG that was observed in control cows. Whether the LPL effect was at the level of mammary or adipose is difficult to determine; however, the fact that insulin increased after LPS regardless of diet may have been associated with a response in adipose and/or skeletal muscle.

Hepatic Expression of Inflammation- and Stress-related Genes

Although there was no control data to compare with, the striking upregulation of *TNF*, *NFKB1*, and *SOD2*when comparing data at -14 vs. 7 DIM, i.e. 2.5-3 h post-LPS challenge, probably reflected the acute response to inflammation. The same could be envisaged for *ANGPTL4* which has recently been shown to respond as a positive acute-phase protein in mice challenged with LPS (Lu et al., 2010). *TNF* is a well-known pro-inflammatory cytokine and its hepatic expression was upregulated at ~3 h post infusion of LPS in mid-lactation dairy cows (Vels et al., 2009). Previous work with peripartal cows showed that activity of SOD in erythrocytes was higher prepartum than postpartum but blood indices of oxidative stress [e.g.

ROM, thiobarbituric acid-reactive substances (TBARS)] were greater postpartum than prepartum, i.e. oxidative stress is a characteristic of the peripartal period (Bernabucci et al., 2005).

The relatively similar pattern of expression we observed for *NFKB1* and *MYD88* (along with *TNF*, *TLR4*, and *IRAK1*) was suggestive of the existence of TLR4-mediated signaling likely due to direct binding of LPS, i.e. this molecule seems to be a hepatic TLR4 agonists as it was proposed in bovine macrophages (Ibeagha-Awemu et al., 2008). IRAK1 is a key mediator of TLR/MYD88 signaling during bacterial infections, which leads to robust inflammatory target gene expression in part via NFKB1 activation (Taraktsoglou et al., 2011). We previously found an upregulation of *IRAK1* in mammary tissue and blood neutrophils from dairy cows receiving an intramammary challenge with *S. uberis* (Moyes et al., 2010a; Moyes et al., 2010b). The greater *TLR4* expression at 30 DIM in cows fed control is puzzling but may underscore the importance of the TLR/MYD88 pathway in liver for establishing a successful immune response. Furthermore, it also could imply that cows in more negative energy balance due to overfeeding energy that undergo an immune challenge would have a compromised TLR response.

In regard to immune response, the marked downregulation of *STAT5B* and its regulator *SOCS2* (Nicholson and Hilton, 1998) between 7 and 14 DIM in overfed cows supports the idea of a compromised response to inflammation, e.g. *SOCS2* negatively regulates cytokine signaling (Udy et al., 1997). More importantly, downregulation of *STAT5B* may have had direct negative effects on metabolism by dampening GH signaling (Lin et al., 1996) and IGF-1 production. The fact that STAT5B also is activated by cytokines and growth factors in dairy cows leading to $CD4^{+}$T cell differentiation during inflammation (He et al., 2011) suggests that overfeeding

energy prepartum has wide-ranging negative effects on liver metabolism and immune response after calving.

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Table 8. Ingredients and chemical composition of experimental diets.

	Prepartal energy level		
	Control	Overfed	Lactation
Ingredients			
Wheat straw	41.9	-	-
Corn silage	29.3	50.3	29.9
Alfalfa silage	10.0	18.0	14.8
Soybean meal	9.64	3.54	2.39
Ground shelled corn	3.59	13.9	-
Alfalfa hay	3.35	6.06	5.55
Magnesium sulfate	0.64	0.63	-
Magnesium oxide	0.42	0.43	0.13
Vitamin E	0.27	0.24	-
Mineral and vitamin mix[1]	0.18	0.18	0.22
Magnesium chloride	0.17	0.35	0.00
Urea	0.17	-	0.13
Salt	0.15	0.24	0.13
Sodium phosphate	0.13	-	-
Vitamin A	0.01	0.01	-
Vitamin D	0.01	0.01	-
Whole cottonseed	-	5.03	5.55
Calcium carbonate	-	0.9	0.56
Corn ground	-	-	20.3
Wet brewer's grain	-	-	12.9
Soybean hulls	-	-	5.55
Sodium bicarbonate	-	-	0.83
Dicalcium phosphate	-	-	0.54
Vitamin H	-	-	0.28
Chemical composition			
DM, %	51.9	50.0	60.5
$NE_{L,}$ Mcal/kg DM	1.34	1.62	1.69
CP, % DM	12.0	15.0	17.4
AP, % DM	11.2	14.3	11.9
ADICP, % DM	0.70	0.73	5.53
NDF, % DM	53.4	36.6	34.1
ADF, % DM	36.6	25.7	21.8
Ca, % DM	0.67	0.73	0.80
P, % DM	0.24	0.31	0.43
Mg, % DM	0.50	0.57	0.33
K, % DM	1.45	1.28	1.16
S %DM	0.21	0.25	0.21
Na % DM	0.07	0.09	0.29
Fe, ppm	305	339	203
Zn , ppm	66.6	80.0	65.8
Cu, ppm	13.0	14.6	10.9
Mn, ppm	72.0	70.3	67.0

[1]Mineral and vitamin mix: zinc = 60 ppm, copper = 15 ppm, manganese = 60 ppm, selenium 0.3 ppm, iodine = 0.6 ppm, iron = 50 ppm, and cobalt = 0.2 ppm. Rumensin: 360mg/day in the lactation diet.

Table 9. The effect of intramammary LPS challenge at 7 d postpartum on dry matter intake, milk production, body weight (BW), body condition score (BCS) and energy balance in cows (n = 9/treatment) fed a control diet (1.34 Mcal/kg DM) or a moderate-energy diet (overfed; 1.62 Mcal/kg DM) during the entire dry period.

	Prepartum diet			P value		
	Overfed	Control	SEM[1]	Diet	Time	Diet × time
DMI						
-4 to -1 wk, % BW	1.68	1.59	0.16	0.72	--	--
7 to 14, % BW	2.20	2.74	0.19	0.05	0.001	0.89
7 to 41, % BW	2.90	3.45	0.17	0.02	0.001	0.34
-4 to -1 wk, kg/d	13.8	11.7	1.1	0.17	--	--
7 to 14, kg/d	16.1	17.5	1.2	0.40	0.001	0.62
7 to 41, kg/d	20.0	20.8	1.0	0.60	0.001	0.13
Milk yield						
7 to 14, kg/d	35.1	34.2	2.4	0.79	0.03	0.24
7 to 41, kg/d	41.9	40.7	2.3	0.71	0.001	0.54
wk 1, % Fat	3.35	3.73	0.18	0.16	--	--
wk 2 to 6, % Fat	3.80	3.80	0.13	0.98	0.13	0.82
wk 1, % Protein	3.06	2.97	0.05	0.23	--	--
wk 2 to 6, % Protein	2.98	3.03	0.07	0.58	0.21	0.24
wk 1, % Lactose	4.75	4.90	0.08	0.22	--	--
wk 2 to 6, % Lactose	4.80	4.94	0.04	0.01	0.19	0.15
3.5% FCM yield[2]						
7 to 41, kg/d	42.6	42.3	3.1	0.92	0.001	0.35
Apparent efficiency[4]						
7 to 41, kg/d	2.2	2.1	0.2	0.43	0.04	0.92
Body Weight						
wk -4 to -1, kg	834	749	28	0.04	--	--
wk 1, kg	747	666	24	0.03	--	--
wk 1 and 2, kg	723	646	23	0.02	0.001	0.68
wk 1 to 6, kg	683	625	20	0.03	0.001	0.32
Body Condition Score						
wk -4 to -1	3.47	3.12	0.10	0.03	--	--
wk 1	2.98	2.74	0.16	0.30	--	--
wk 1 and 2	2.87	2.73	0.13	0.45	0.27	0.38
wk 1 to 6	2.73	2.63	0.13	0.57	0.002	0.69
Calf birth weight, kg	49.3	47.5	1.8	0.47	--	--
Energy balance						
wk -4 to -1, Mcal/d	5.8	0.2	1.8	0.03	--	--
wk -4 to -1, % req[3]	136	102	11	0.05	--	--
wk 1, Mcal/d	-11.8	-6.8	1.3	0.04	--	--
wk 1, % req	65.3	77.7	3.9	0.04	--	--
wk 2, Mcal/d	-6.5	-2.9	1.9	0.21	--	--
wk 2,% req	82.8	91.5	5.0	0.24	--	--
wk 2 to 6, Mcal/d	-7.1	-4.2	1.8	0.28	0.04	0.99
wk 2 to 6, % req	84.2	90.8	4.2	0.28	0.10	0.95

[1]Largest SEM is shown.
[2] 3.5 % FCM, kg = Milk yield, kg × [(0.4324) + (16.218 × fat % × 0.01)].
3% of requirements
[4]Defined as 3.5% FCM (kg) divided by DMI (kg).

Table 10. Description of genes selected for qPCR.

Symbol	Description	GenBank accession
MYD88	myeloid differentiation primary response gene (88)	NM_001014382.2
CREB3L3	cAMP responsive element binding protein 3-like 3	NM_001034432.1
NR3C1	nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	XM_612999.4
TLR4	toll-like receptor 4	NM_174198.6
TNF	tumor necrosis factor	NM_173966.2
FAAH	fatty acid amide hydrolase	NM_001099102.1
HMGCS2	3-hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial)	NM_001045883.1
ACOX1	acyl-CoA oxidase 1, palmitoyl	NM_001035289.2
CPT1A	carnitine palmitoyltransferase 1A (liver)	FJ415874.1
ANGPTL4	angiopoietin-like 4	NM_001046043.2
FGF21	fibroblast growth factor 21	XM_002695200.1
PPARA	peroxisome proliferator-activated receptor alpha	NM_001034036.1
PPARD	peroxisome proliferator-activated receptor delta	NM_001083636.1
XBP1	X-box binding protein pseudogene 1	XM_001255847.2
DGAT1	diacylglycerol O-acyltransferase homolog 1	NM_174693.2
PERK	eukaryotic translation initiation factor 2-alpha kinase 3	NM_001098086.1
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NM_001076409.1
SOD2	superoxide dismutase 2, mitochondrial	NM_201527.2
IRAK1	interleukin-1 receptor-associated kinase 1	NM_001040555.1
MTTP	microsomal triglyceride transfer protein	NM_001101834.1

Table 11. Gene ID, GenBank accession number, hybridization position, sequence and amplicon size of primers for ***Bos taurus*** used to analyze gene expression by qPCR. Similar information for remaining genes was reported previously (2, 3).

Gene ID	Accession #	Gene	Primers[1]	Primers (5'-3')[2]	bp[3]
444881	NM_001014382.2	*MYD88*	F.367 R. 471	GGAGGACTGCCAAAAGTATATTCTG GCCATGTCATTTATCCGAGTTATG	105
513010	NM_001034432.1	*CREB3L3*	F.1606 R.1705	TGGAGATGCACAGATACACAGCTA AGATTTCTCAGACTTTGTGGCCTTA	100
281946	XM_612999.4	*NR3C1*	F.395 R.494	AAGCACCCCCAGTAGAGAAGAA CACAGTAGCTCCTCCCCTTAGG	100
281536	NM_174198.6	*TLR4*	F.103 R.201	GCTGTTTGACCAGTCTGATTGC GGGCTGAAGTAACAACAAGAGGAA	99
280943	NM_173966.2	*TNF*	F.174 R.287	CCAGAGGGAAGAGCAGTCCC TCGGCTACAACGTGGGCTAC	114
540007	NM_001099102.1	*FAAH*	F.1332 R.1436	TTCCTGCCAAGCAACATACCT CACGAAATCACCTTTGAAGTTCTG	105
503684	NM_001045883.1	*HMGCS2*	F.837 R.936	TTACGGGCCCTGGACAAAT GCACATCATCGAGAGTGAAAGG	100
513996	NM_001035289.2	*ACOX1*	F.180 R.279	ACCCAGACTTCCAGCATGAGA TTCCTCATCTTCTGCACCATGA	100
506812	FJ415874.1	*CPT1A*	F.141 R.240	TCGCGATGGACTTGCTGTATA CGGTCCAGTTTGCGTCTGTA	100
509963	NM_001046043.2	*ANGPTL4*	F.28 R.136	AGGAAGAGGCTGCCCAAGAT CCCTCTCTCCCTCTTCAAACAG	109
281992	NM_001034036.1	*PPARA*	F.729 R.830	CATAACGCGATTCGTTTTGGA CGCGGTTTCGGAATCTTCT	102
353106	NM_001083636.1	*PPARD*	F.460 R.559	TGTGGCAGCCTCAATATGGA GACGGAAGAAGCCCTTGCA	100
541236	XM_001255847.2	*XBP1*	F.618 R.697	GAGAGCGAAGCCAATGTGGTA ACTGTGAATTCAGGGTGATCTTTCT	80
282609	NM_174693.2	*DGAT1*	F.210 R.314	ACCGCCTGCAGGATTCC ATAACCGTGCGTTGCTTAAGATC	105
535820	NM_001098086.1	*PERK*	F.3160 R.3262	ATATGAGCCCGGAACAGATTCAT AGTGCCGAACGGGTATAGTAATTC	101
282609	NM_001076409.1	*NFKB1*	F.172 R.266	TTCAACCGGAGATGCCACTAC ACACACGTAACGGAAACGAAATC	95
281496	NM_201527.2	*SOD2*	F.620 R.714	TGTGGGAGCATGCTTATTACCTT TGCAGTTACATTCTCCCAGTTGA	101
533953	NM_001040555.1	*IRAK1*	F.950 R.1052	CCTCAGCGACTGGACATCCT GGACGTTGGAACTCTTGACATCT	103
280868	NM_001101834.1	*MTTP*	F.617 R.716	ACCAGGCTCATCAAGACAAAGTG GTGACACCCAAGACCTGATGTG	100

[1] Primer direction (F – forward; R – reverse) and hybridization position on the sequence.
[2] Exon-exon junctions are underlined.
[3] Amplicon size in base pair (bp).

Table 12. Sequencing results of PCR products from primers of genes designed for this experiment. Best hits using BLASTN (http://www.ncbi.nlm.nih.gov) are shown.

Gene	Sequence
MYD88	GACGCGGAGCATCGTAGAGGCCTTACGGTGGACTCTATAGACAGGC AGCATAACTCGGATAAATGGACATGGGCAACAC
CREB3L3	GCCAGGATCGGGTCCACGTAAACGGATTCTCAGACAAAGGACCATT AAGGGCCACAAAGTCTGAGAAATTCTACA
NR3C1	CCGTACGGTGTCTGTGTCAGAGAGGGAATGTGATGGACTTCTATAAA ACCCCTAAGGGGGAGGGAGCTACTGTGGAGTGCCC
TLR4	GCATCCCTCACCGTTATGGTCAGGTGAATTCCTGGGATAAGGCCAGG CTTCCTCTTGTTGGTTACTTCAGCCAGAAA
TNF	TCACTCTCCGGGGCAGCTCCGGTGGTGGGACTCGTATGCCAATGCCC TCATGGAA
FAAH	GCGGACGGGCAGGGGTGGCGTTTCAGTGACGGGTGCACGACCTTCCT ACAGAACTTCAAAGGTGATTTCGTGGATTCAAA
HMGCS2	CACTAGCCAGATCGAGAACAGGTGAAGCAAGCTGGCATCGAATCGG CCTTTCACTCTCGATGAATGTGCAAAACCC
ACOX1	ATCCTCGTATCCGCGTTCAGGGTGCGTTTAAGAAGAGTGCCATCATG GTGCAGAAGATGAGGAAATCCCC
CPT1A	GGACTATGAAGGTAAACCAGGCCCGGGACGCCCTTCGTACAGGCCT CTCGCTCCAGCTGGCTCATTACAAGGGACCA
ANGPTL4	GCCCATCAGCATCCTCAACCGTGAAGCGGCCAGTATTTCCACTCCAT TTCCAAGGGAAGA
PPARA	CGAGATCTGAAGCAAATTGAGGCAGAAATCCTTACGTGTGAGCATG ACCTAGAAGATTCCGAAACCGCGA
PPARD	GCATGGGGACGGCGTCGGGCTCACTACGGCGTTCACGCTTGTGAGGG ATGCAAGGGCTTCTTCCGTCCACAAA
XBP1	GGGATGTAGGACACTTTTCAGCCCTCAGAGAAAGATCACCCTGAATT CACAGGTAAG
DGAT1	CTAGGCTTCCACTACCGGTGCATCCTGAATTGGTGTGTGGGTGGATG CTGATCCTTAAGCAACGCACGGGTTATAATA
PERK	GATCAGCATCTCTCATAGTGACATCTTTTCTTTGGGCCTCATTCTGTT TGAATTACTATACCCGTTCGGCACCTCGAA
NFKB1	CGATATCTTCGTGTCAAGCAAAAGTATTCGCAACACTGGAAGCACGA ATGACAGATGCCTGTATACGGGGCATCAGAAGGCCGTA
SOD2	GCATGTTTGGCCGATTATCTGAGGCCATTTTGGAATGTGATCAACTG GGAGAATGTAACTGCAATAC
IRAK1	GCTAGCGGGCATCTAGTTCTTACATCAAGGAATAGCCCCAGCCCTCA TCCATGGAGAATGTCAAGAAGTTCCAACGCTCCAAAAAGGG
MTTP	GCACTGCCCCCTCTATTTTTCAGTTAATCCAAAGCCCTTTAATTTTTG GTCACTTTGTCTTGATGAGCCCTGAGGAATATCAAA

Table 13. Sequencing results of genes using BLASTN from NCBI against nucleotide collection (nr / nt) with total score.

Gene	Best hits	Score
MYD88	Bos taurus myeloid differentiation primary response gene (88) (MYD88), mRNA	73.4
CREB3L3	Bos taurus cAMP responsive element binding protein 3-like 3 (CREB3L3), mRNA	64.4
NR3C1	PREDICTED: Bos taurus nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor) (NR3C1), mRNA	69.8
TLR4	Bos taurus toll-like receptor 4 (TLR4), mRNA	64.4
TNF	Bos taurus tumor necrosis factor (TNF), mRNA	93.3
FAAH	Bos taurus fatty acid amide hydrolase (FAAH), mRNA	105
HMGCS2	Bos taurus 3-hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial) (HMGCS2), nuclear gene encoding mitochondrial protein, mRNA	80.6
ACOX1	Bos taurus acyl-CoA oxidase 1, palmitoyl (ACOX1), mRNA	68
CPT1A	PREDICTED: Bos taurus carnitine palmitoyltransferase 1A liver-like (CPT1A), mRNA	71.6
ANGPTL4	Bos taurus angiopoietin-like 4 (ANGPTL4), mRNA	48.2
PPARA	Bos taurus peroxisome proliferator-activated receptor alpha (PPARA), mRNA	98.7
PPARD	Bos taurus peroxisome proliferator-activated receptor delta, mRNA	98.7
XBP1	Bos taurus X-box binding protein pseudogene 1 (XBPP1), mRNA	64.4
DGAT1	Bos taurus diacylglycerol O-acyltransferase homolog 1 (mouse) (DGAT1), mRNA	75.2
PERK	Bos taurus eukaryotic translation initiation factor 2-alpha kinase 3 (EIF2AK3), mRNA	102
NFKB1	Bos taurus nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (NFKB1), mRNA	89.7
SOD2	Bos taurus superoxide dismutase 2, mitochondrial (SOD2), nuclear gene encoding mitochondrial protein, mRNA	82.4
IRAK1	Bos taurus interleukin-1 receptor-associated kinase 1 (IRAK1), mRNA	77
MTTP	Bos taurus microsomal triglyceride transfer protein (MTTP), mRNA	75.2

Table 14. qPCR performance among the genes measured in Liver Tissue

Gene	Median Ct	Median ΔCt	Slope	(R^2)	Efficiency	%Relative mRNA Abundance
MYD88	23.484	3.096	-3.025	0.999	2.141	0.876
CREB3L3	21.131	0.855	-3.21	0.996	2.049	5.007
NR3C1	22.230	1.839	-3.196	0.995	2.056	2.456
TLR4	32.903	12.659	-2.698	0.925	2.348	0.0002
TNF	27.493	7.156	-3.083	0.992	2.113	0.044
FAAH	21.967	1.771	-3.232	0.992	2.039	2.618
HMGCS2	18.215	-2.144	-3.05	0.995	2.127	46.633
ACOX1	19.873	-0.507	-3.321	0.998	2.00	13.14
CPT1A	21.255	0.998	-3.118	0.998	2.093	4.424
ANGPTL4	23.605	3.228	-3.209	0.998	2.049	0.913
PPARA	21.262	0.892	-3.063	0.998	2.121	4.728
PPARD	25.626	5.249	-3.399	0.992	1.969	0.264
XBP1	21.979	1.611	-2.882	0.992	2.223	2.553
DGAT1	23.799	3.407	-3.036	0.996	2.135	0.698
PERK	24.166	3.804	-3.125	0.994	2.089	0.561
NFKB1	23.547	3.162	-3.233	0.992	2.039	0.972
SOD2	20.389	0.034	-3.124	0.998	2.092	9.017
IRAK1	24.095	3.726	-3.133	0.993	2.085	0.598
MTTP	21.197	0.841	-2.561	0.998	2.457	4.3414

Table 15. The effect of intramammary LPS challenge at 7 d postpartum on hepatic relative mRNA expression (log-scale) in cows (n = 6/treatment) fed a control diet (1.34 Mcal/kg DM) or a moderate-energy diet (overfed; 1.62 Mcal/kg DM) during the entire dry period.

		Day relative to parturition					P value		
Genes	Diet	-14	7	14	30	SEM[1]	Diet	Time	Diet × Time
Metabolism									
DGAT1	Overfed	-0.15^{b}	0.14^{a}	0.01^{b}	0.03^{ab}	0.20	0.94	0.01	0.38
	Control	-0.21^{b}	0.27^{a}	-0.03^{b}	0.07^{ab}				
FAAH	Overfed	0.39^{a}	-0.02^{b}	0.07^{b}	0.37^{a}	0.09	0.44	0.01	0.43
	Control	0.25^{a}	-0.11^{b}	0.09^{b}	0.24^{a}				
MTTP	Overfed	0.19^{a}	-0.11^{b}	-0.07^{b}	-0.12^{b}	0.11	0.86	0.01	0.32
	Control	0.12^{a}	0.04^{b}	-0.03^{b}	-0.16^{b}				
Stress and inflammation									
PERK	Overfed	0.49^{b}	0.32^{c}	0.69^{a}	0.73^{a}	0.30	0.71	0.01	0.04
	Control	0.75^{ab}	0.55^{c}	0.62^{bc}	0.89^{a}				
TLR4	Overfed	0.88^{ab}	1.20^{a}	0.58^{b}	0.56*^{b}	0.25	0.16	0.04	0.03
	Control	0.93^{b}	1.31^{ab}	1.02^{b}	1.60*^{a}				
XBP1	Overfed	-0.75^{c}	0.10^{a}	-0.26^{b}	-0.41^{b}	0.36	0.98	0.01	0.01
	Control	-0.46^{b}	0.44^{a}	-0.62^{b}	-0.64^{b}				
Inflammation and metabolic transcription regulators									
NFKB1	Overfed	-0.39^{c}	0.68^{a}	-0.56^{b}	-0.45^{bc}	0.12	0.91	0.01	0.99
	Control	-0.39^{c}	0.67^{a}	-0.61^{b}	-0.43^{bc}				
CREB3L3	Overfed	-0.16^{b}	0.24^{a}	-0.10^{b}	0.21^{a}	0.15	0.43	0.01	0.06
	Control	-0.07^{bc}	0.15^{a}	-0.44^{c}	-0.05^{ab}				
PPARD	Overfed	-0.16^{b}	0.24^{a}	-0.10^{b}	0.21^{a}	0.15	0.43	0.01	0.06
	Control	-0.07^{b}	0.15^{a}	-0.44^{c}	-0.05^{ab}				

[1]Largest SEM is shown.

$^{a\text{-}c}$ Differences between days (time $P < 0.05$ or diet × time effects $P < 0.10$).

*Denote significant interactions (diet × time effects $P < 0.10$) at a given day.

Figure 6. Daily DMI and milk production in cows (n = 9/treatment) fed a control diet (1.34 Mcal/kg DM) or a moderate-energy diet (overfed; 1.62 Mcal/kg DM) during the entire dry period.

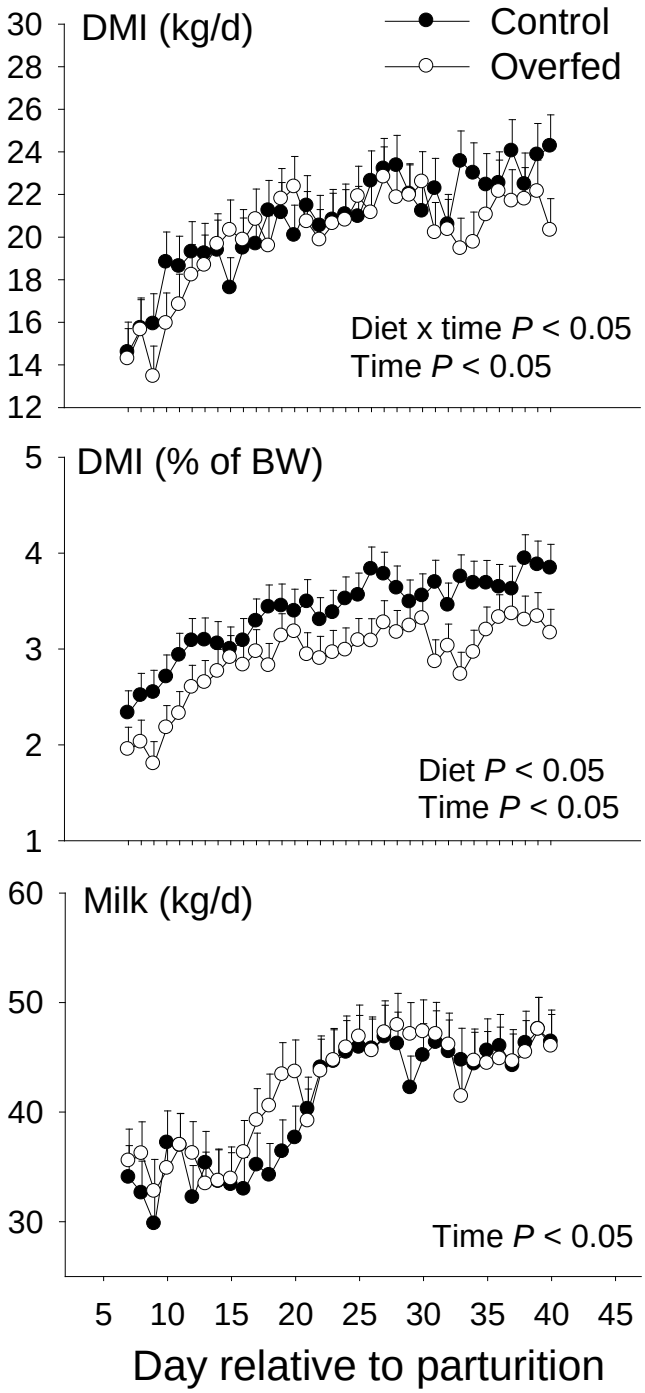


Figure 7. Liver lipid and triglyceride in cows (n = 9/treatment) fed a control diet (1.34 Mcal/kg DM) or a moderate-energy diet (overfed; 1.62 Mcal/kg DM) during the entire dry period. Sample at d 7 was collected 2.5 h after LPS Challenge. [a-b] Differences between days (time or within diet × time effects); *Diet × time at a given day.

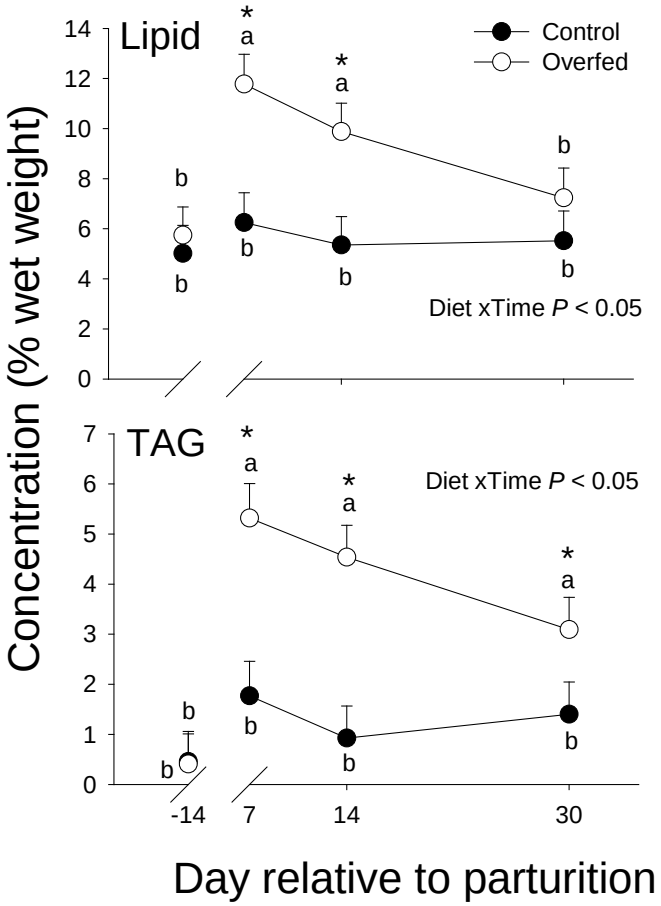


Figure 8. Blood concentration of metabolic indicators in cows (n = 9/treatment) fed a control diet (1.34 Mcal/kg DM) or a moderate-energy diet (overfed; 1.62 Mcal/kg DM) during the entire dry period. Sample at d 7 was collected before LPS Challenge. [a-c] Differences between days (time or within diet × time effects); *Diet × time at a given day.

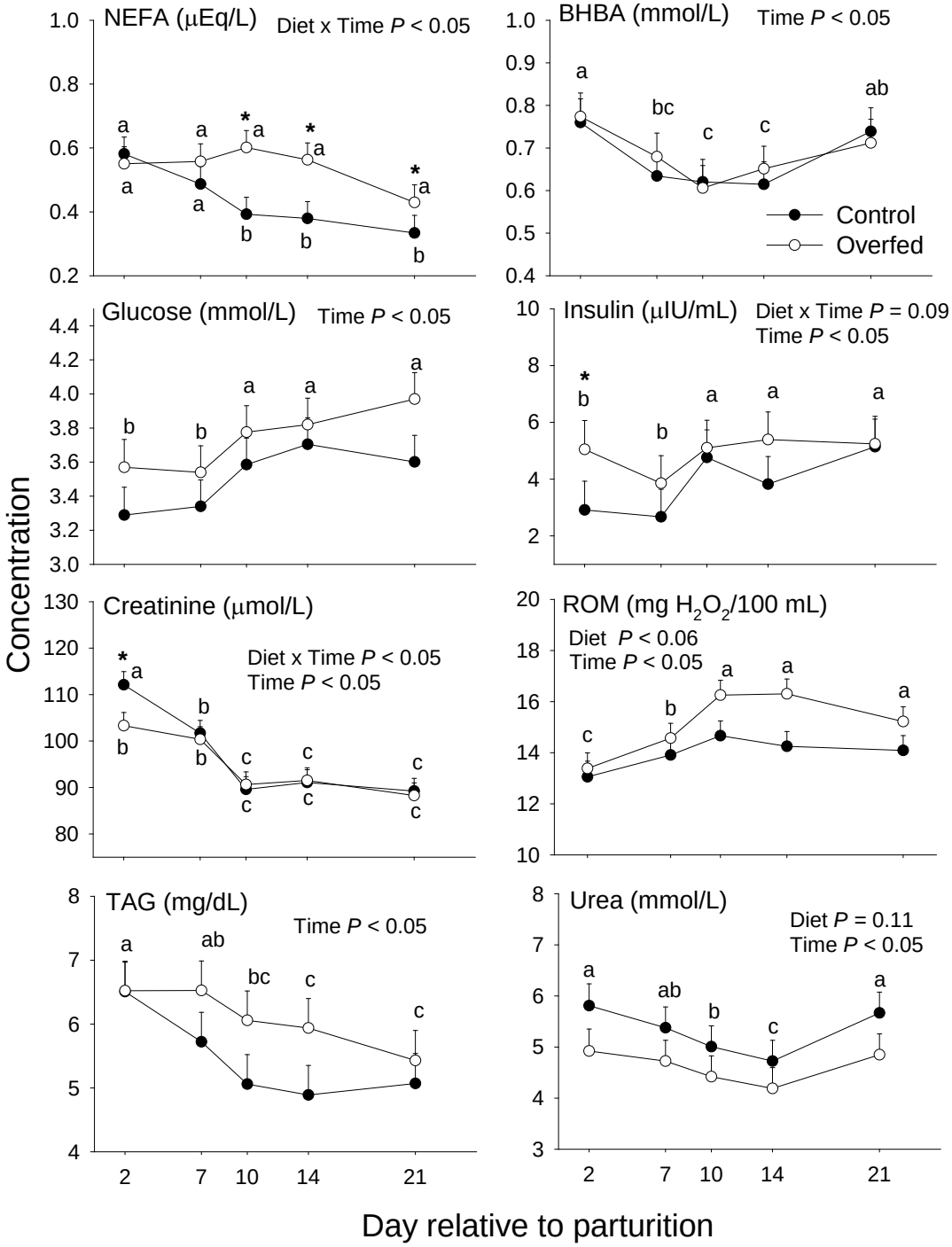


Figure 9. Blood concentration of indexes of inflammation and liver function in cows (n = 9/treatment) fed a control diet (1.34 Mcal/kg DM) or a moderate-energy diet (overfed; 1.62 Mcal/kg DM) during the entire dry period. Sample at d 7 was collected before LPS Challenge. [a-d] Differences between days (time or within diet × time effects); *Diet × time at a given day.

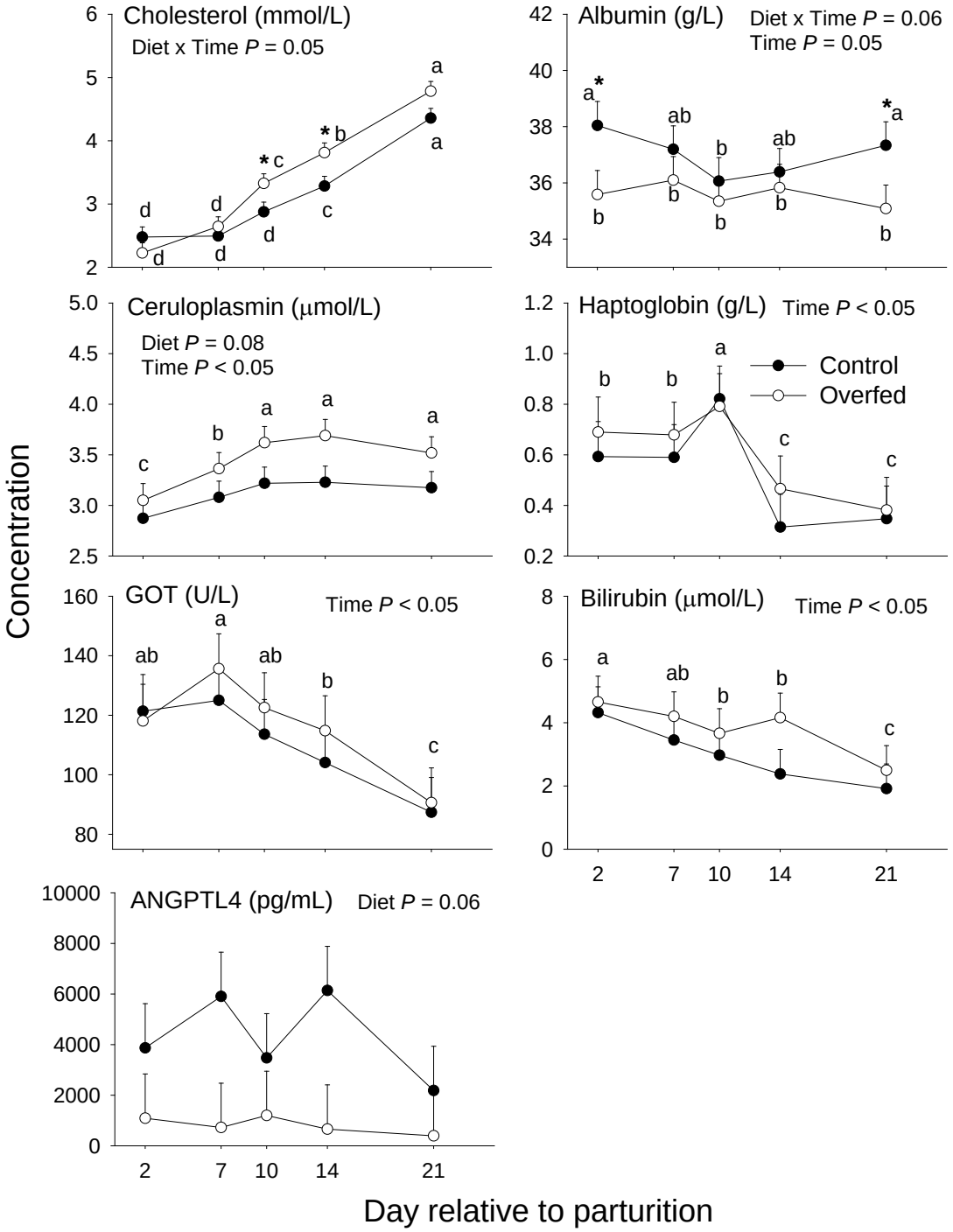


Figure 10. Blood concentration of selected vitamins in cows (n = 9/treatment) fed a control diet (1.34 Mcal/kg DM) or a moderate-energy diet (overfed; 1.62 Mcal/kg DM) during the entire dry period. Sample at d 7 was collected before LPS Challenge. [a-c] Differences between days (time or within diet × time effects); *Diet × time at a given day.

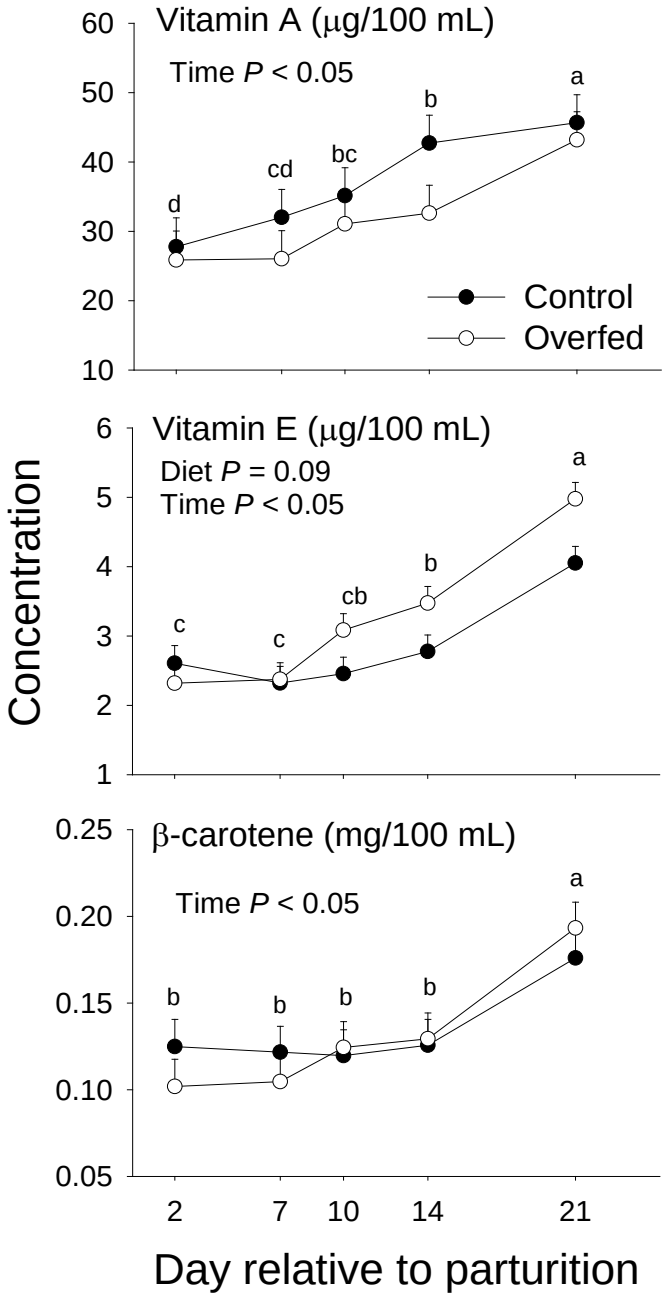


Figure 11. Neutrophil chemotaxis assessed with C5a (panel A) and human IL-8 (panel B), and phagocytosis in cows (n =9/treatment) fed a control diet (1.34 Mcal/kg DM) or a moderate-energy diet (overfed; 1.62 Mcal/kg DM) during the entire dry period. Sample at d 7 was collected before LPS Challenge. [a-c] Differences between days (time or diet × time); *Diet × time at a given day.

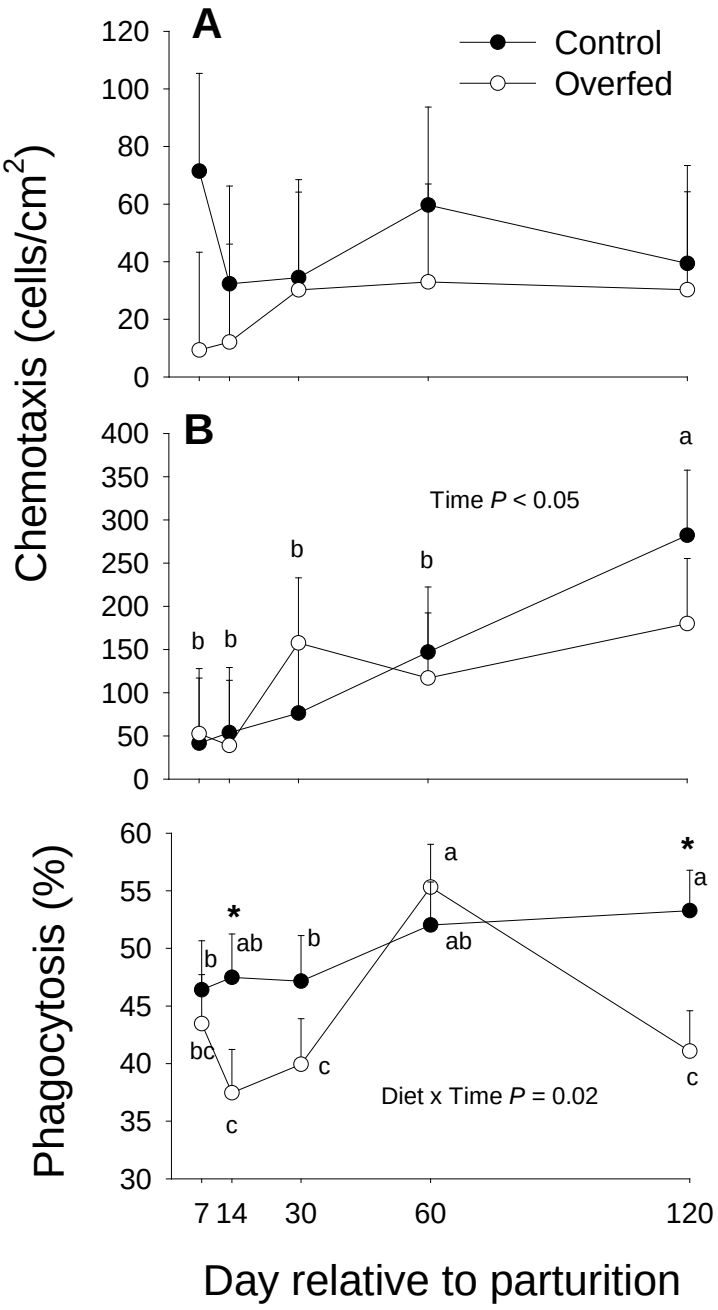


Figure 12. Expression pattern of genes associated with lipid metabolism (*CPT1A*, *ACOX1*, *HMGCS2*, *PPARA*) and GH signaling (*STAT5B, SOCS2*) in liver from cows fed a control diet (1.34 Mcal/kg DM; N = 6) or a moderate-energy diet (overfed, n = 6; 1.62 Mcal/kg DM) during the entire dry period and receiving an intramammary LPS challenge at 7 d postpartum. [a-c] Differences between days (time or within diet × time effects); *denote significant interactions (diet × time effects) at a given day.

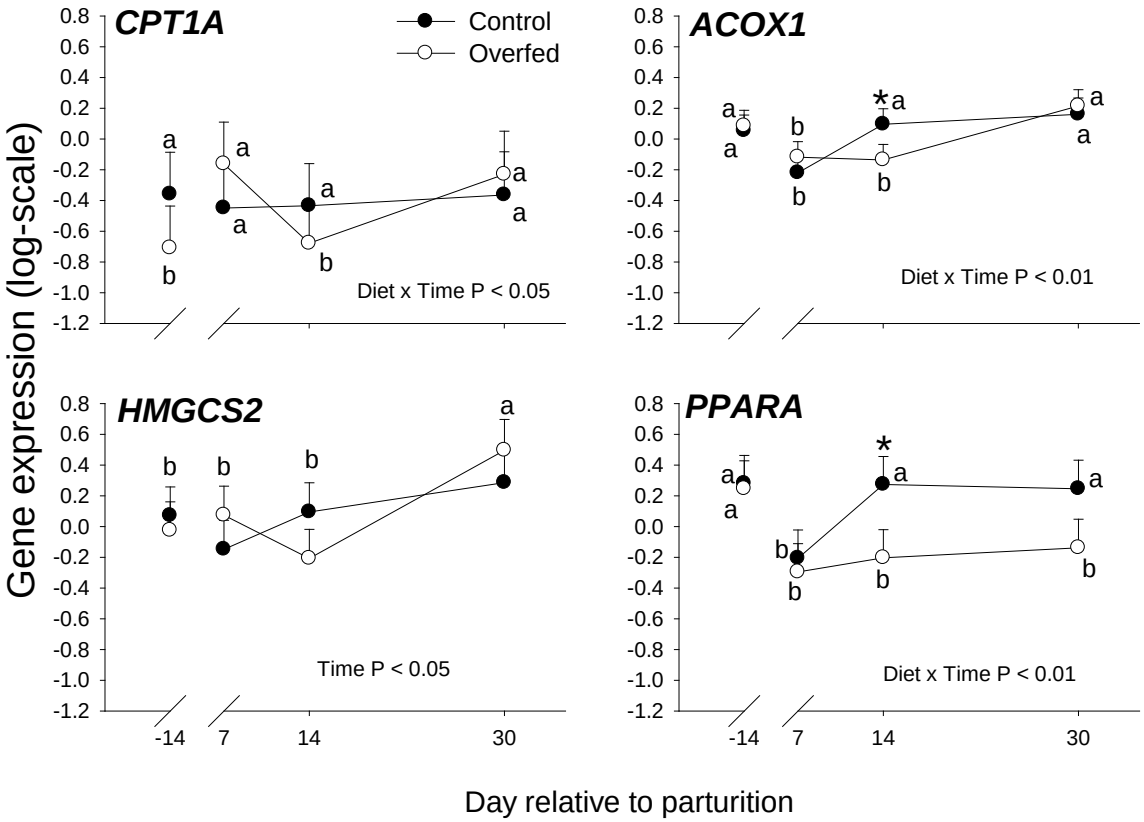


Figure 13. Blood serum concentration of growth hormone (GH) and insulin-like growth factor 1 (IGF-1), and expression of GH signaling-related genes (*STAT5B, SOCS2*) in liver from cows fed a control diet (1.34 Mcal/kg DM; n = 6) or a moderate-energy diet (overfed, n = 6; 1.62 Mcal/kg DM) during the entire dry period and receiving an intramammary LPS challenge at 7 d postpartum. [a-c] Differences between days (time or within diet × time effects). *denote significant interactions (diet × time effects) at a given day.

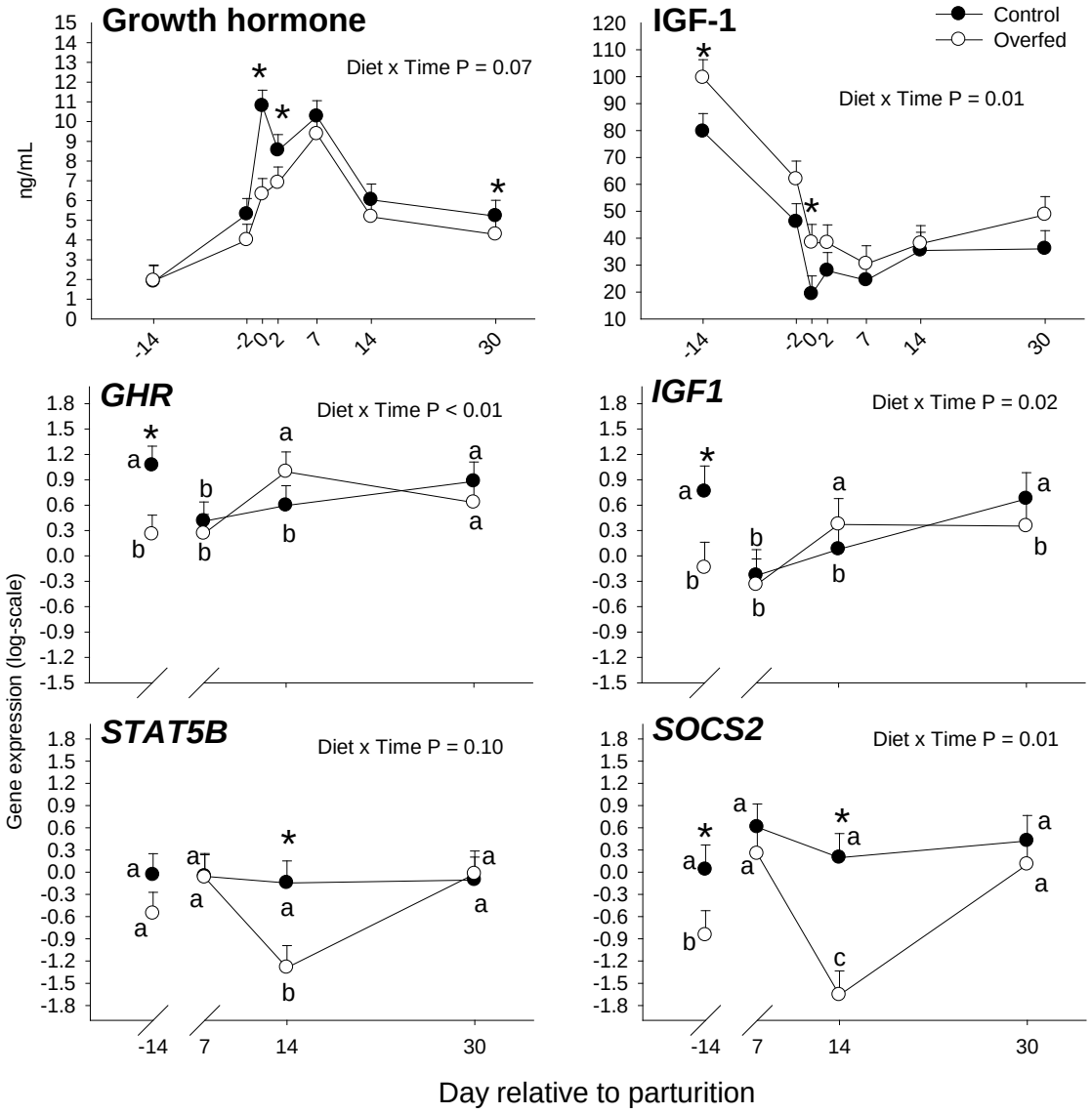


Figure 14. Expression pattern of genes associated with stress (*SOD2*, *NR3C1*) and inflammation (*TNF*, *ANGPTL4*, *IRAK1*, *MYD88*) in liver from cows fed a control diet (1.34 Mcal/kg DM; n = 6) or a moderate-energy diet (overfed, n = 6; 1.62 Mcal/kg DM) during the entire dry period and receiving an intramammary LPS challenge at 7 d postpartum. [a-c] Differences between days (time or within diet × time effects). *denote significant interactions (diet × time effects) at a given day.

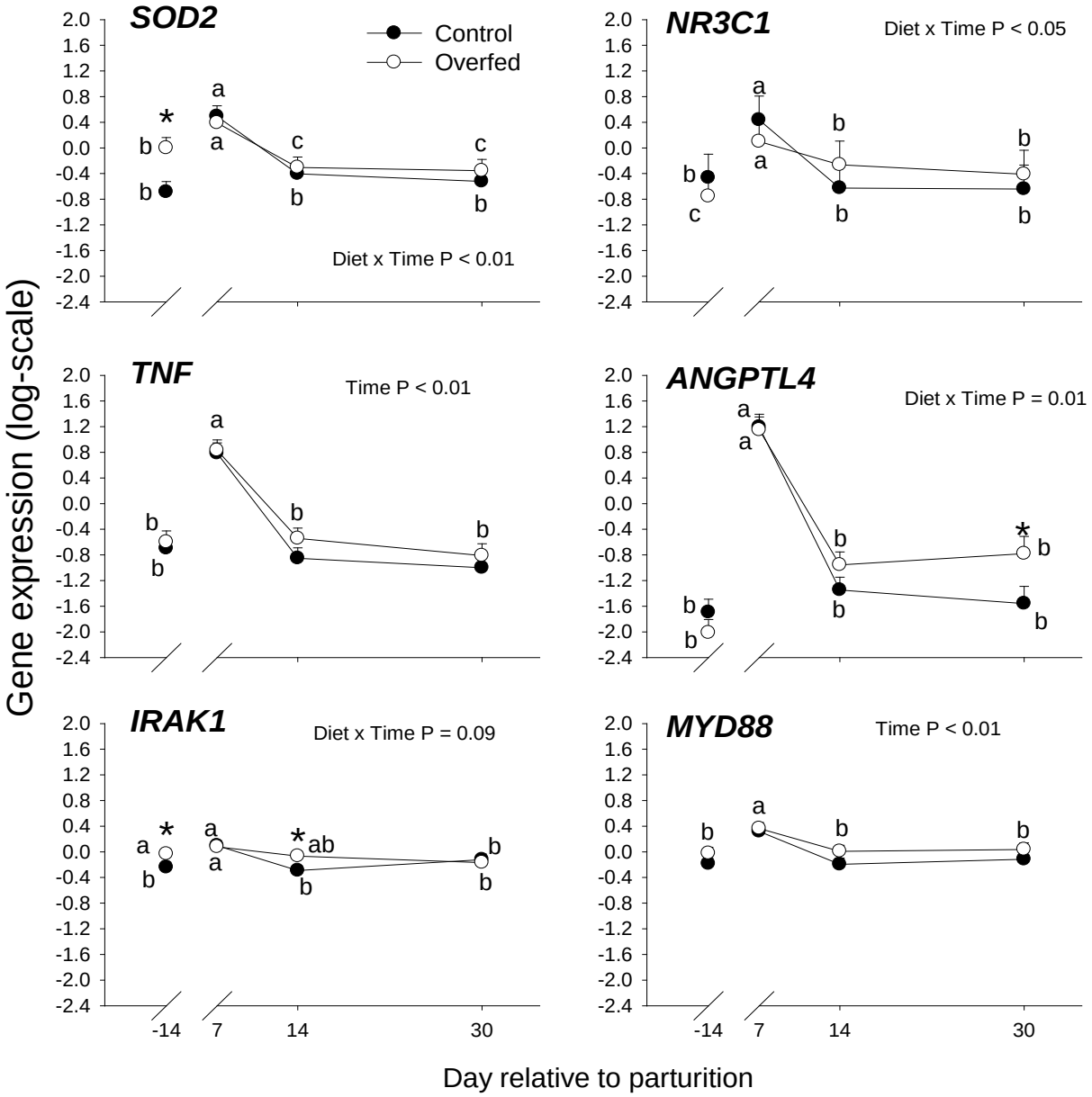
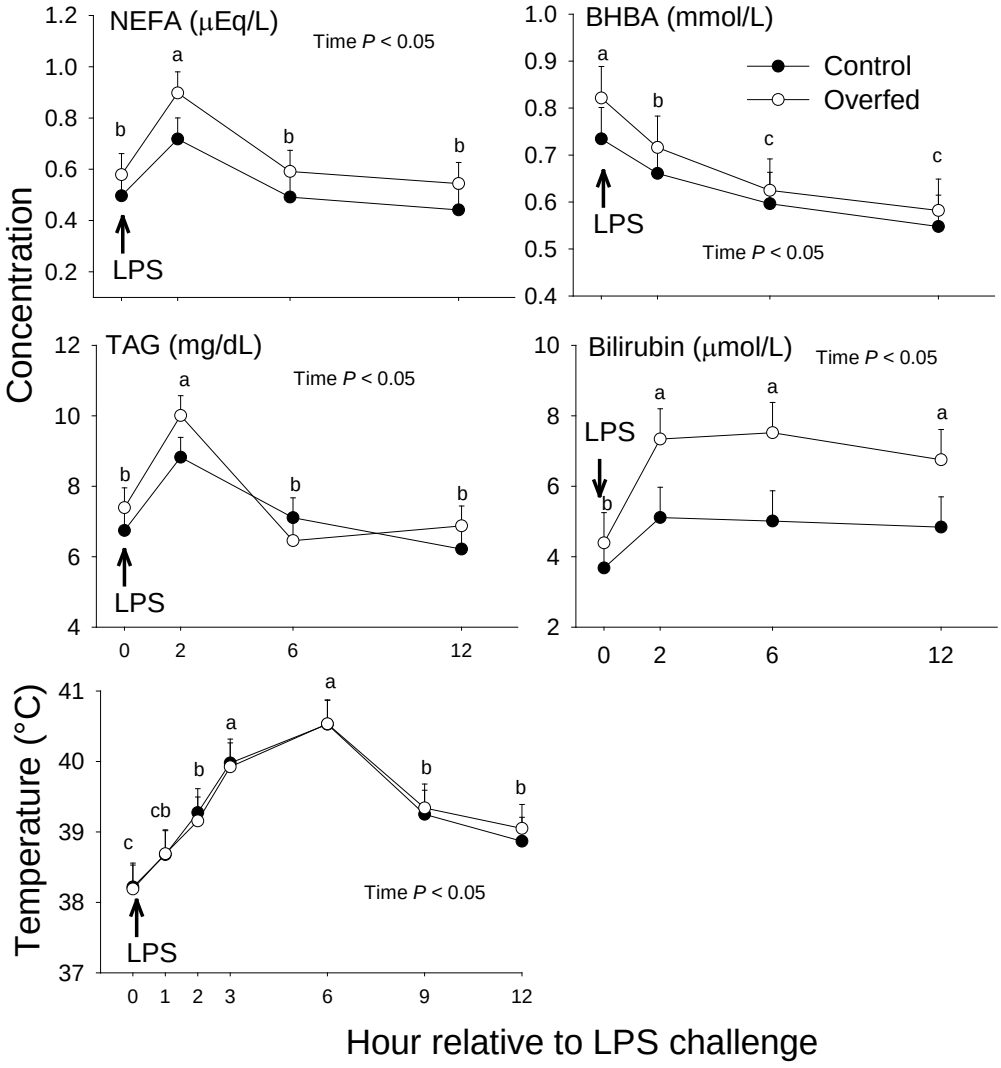


Figure 15. Blood concentration of non-esterified fatty acids (NEFA), hydroxybutyric acid (BHBA), bilirubin and rectal temperature after intramammary LPS-challenge at 7 d postpartum in cows fed a control diet (1.34 Mcal/kg DM) or overfed energy (1.62 Mcal/kg DM) during the entire dry period. [a-c] Differences between days (time or diet × time effects); *denote significant interactions (diet × time effects) at a given day.



CHAPTER 4:

Liver and Mammary Gland Transcript Profiles Affected by Prepartum Dietary Energy and Early-Lactation *E. coli* Lipopolysaccharide Challenge in Dairy Cattle

D.E. Graugnard*†, S.L. Rodriguez–Zas†, R.E. Everts†, H.A. Lewin†, and J.J. Loor*†§.

*Mammalian NutriPhysioGenomics, †Department of Animal Sciences and §Division of Nutritional Sciences, University of Illinois, Urbana, IL 61801

INTRODUCTION

Peripartal cows experience some degree of negative energy balance (NEB) and immunosuppression around parturition (Goff, 2006). Neutrophil trafficking, phagocytosis, and killing capacity are impaired at calving (Kehrli et al., 1989). Lymphocyte numbers decrease around parturition as a function of reduced proliferation (Kehrli et al., 1989). The metabolic challenges associated with the onset of lactation are factors capable of affecting immune function not only in neutrophils but also liver and mammary (Goff, 2006). The NEB associated with parturition leads to extensive mobilization of fatty acids stored in adipose tissue, thus, causing marked elevations in blood non-esterified fatty acids (**NEFA**) and hydroxybutyrate (**BHBA**) concentrations (Drackley et al., 2001).

Prepartal level of dietary energy can potentially affect adipose tissue deposition and, thus, the amount of NEFA released into blood (Janovick and Drackley, 2010) and available for metabolism in liver and mammary gland (Drackley et al., 2005). From a health standpoint, clinical mastitis is most likely to occur during the first mo of lactation (Oviedo-Boyso et al., 2007) and in many cases results from an infection established during the dry period or during early lactation (Goff and Horst, 1997). Once a pathogen is detected by the receptors in the epithelial cells of the mammary tissue the acute phase response begins and activates the immune system to eliminate the pathogen. This defense mechanism includes anatomical, cellular, and soluble factors that act in coordination and are crucial to the modulation of the mammary resistance and susceptibility to infection (Oviedo-Boyso et al., 2007).

The liver is a central organ during an inflammatory response in the organism. It is responsible for determining the level of essential metabolites during the critical stages of stress. In addition, the liver synthesizes the necessary components for immediate defense at the site of

tissue damage (Baumann and Gauldie, 1994). Current dry period feeding practices can lead to elevated intakes of energy, which can increase fat deposition in the viscera and upon parturition lead to compromised liver metabolism (Beever, 2006, Drackley et al., 2005). Our general hypothesis was that overfeeding dietary energy during the dry period, accompanied by the metabolic challenges associated with the onset of lactation would render the cow's immune function less responsive early postpartum when the likelihood of a mammary infection is higher . Transcript profiling using microarrays is an excellent technique to decipher complex gene networks underlying differences in physiological state that can help understanding relationships with the liver and mammary gland with other tissues. In addition it has been demonstrated that many of these adaptations can be influenced by nutritional management strategies during the dry period (Loor et al., 2005, Loor et al., 2006, Loor et al., 2007).

OBJECTIVES

The main objectives of this study were to determine gene expression patterns in dairy cattle liver and mammary tissues:

1) In response to an early-lactation *E. coli* lipopolysaccharide intra-mammary challenge (LPS vs. non-LPS) in cows overfed during the dry period.

2) In response to different prepartal dietary energy levels (1.34 vs. 1.62 Mcal/kg DM) and an early-lactation E. *coli* lipopolysaccharide intra-mammary challenge.

MATERIALS AND METHODS

All procedures involving animals received approval from the University of Illinois Institutional Animal Care and Use Committee (protocol # 06145).

Animals and Diets

A detailed description of experimental design and data collection was previously described in Chapters 2 and 3. Briefly 28 Holstein cows entering their second or greater lactation were enrolled in the study. Cows were assigned to a control diet (n =14, controlled energy, high fiber), which was fed for *ad libitum* intake to provide at least 100% of calculated NE_L (1.34 Mcal/kg diet DM), or fed a diet providing ~159% calculated NE_L requirements (n=14, overfed diet, 1.62 Mcal/kg DM) during the entire 45-d dry period. Diets were fed as TMR once daily (0600 h) using an individual gate feeding system (American Calan, Northwood, NH, USA). Cows were housed in a ventilated enclosed barn during the dry period and had access to sand-bedded free stalls until 5 d before expected calving date, when they were moved to an individual maternity pen bedded with straw. After parturition, all cows were moved to a tie-stall barn and were fed a common lactation diet (NE_L = 1.69 Mcal/kg DM) as TMR once daily (0600 h) and milked twice daily (0400 and 1600 h). Diets were mixed in a Keenan Klassik 140 mixer wagon (Richard Keenan & Co., Ltd., Borris, County Carlow, Ireland) equipped with knives and serrated paddles; straw in large square bales was chopped directly by the mixer without preprocessing.

Lipopolysacharide Challenge

At ~7 DIM, 7 cows on each of the two experimental diets were assigned to receive an intra-mammary *E. coli* lipopolysacharide (LPS) challenge (200 μg, strain 0111:B4, cat. # L2630,

Sigma Aldrich, St. Louis, MO) and 7 cows in the overfed diet only, served as non-LPS controls. Prior to LPS challenge (~2 days), foremilk samples from all quarters of each cow were cultured and confirmed to be bacteriologically negative. LPS was dissolved in 20 mL of 0.09% sterile physiological saline (Hospira, Lake Forest, IL). Immediately after milking (0530 h), one rear mammary quarter was disinfected with cotton wool pre-soaked in 70% ethanol and the LPS was infused via a sterile disposable syringe fitted with a sterile teat cannula using the full insertion infusion method. The quarter was thoroughly massaged.

Liver and Mammary Biopsies

Biopsies of liver were sampled via puncture biopsy (Dann et al., 2006) from cows under local anesthesia. Samples were collected during d 7 relative to parturition. The procedure was performed at approximately 0730 h (2 h after LPS or non-LPS) to avoid excessive infiltration of PMN, i.e. as a means to avoid excessive confounding on tissue gene expression.Biopsies of mammary tissue were collected simultaneously with the liver biopsies from a rear quarter in all the cows (infused with LPS or selected randomly in the case of the non-LPS group). Cows were restrained in a squeeze chute to minimize movement and were sedated using an intravenous administration of xylazine HCl (35 µg/kg BW; Phoenix Pharmaceuticals, St. Joseph, MO). The hair around the tip of the tail was clipped and the tail was tied to prevent contamination of the surgical site. The biopsy site was carefully selected to avoid subcutaneous blood vessels as well as the cisternal region. An area of skin (10 cm^2) on the rear quarter was clipped closely, washed, and sterilized with iodine surgical scrub. For local anesthesia, lidocaine HCl (5 mL; Phoenix Pharmaceuticals, St. Joseph, MO) was administered subcutaneously. The biopsy site was then washed an additional 3X as described above. After washing, a 3 cm incision was made through

the skin and underlying fascia to the point where the mammary gland capsule was visible. The biopsy trocar was attached to a high-speed (16 Volts) cordless drill. The trocar consisted of a stainless steel cannula of 90 mm in length with a 6-mm diameter containing a retractable blade at the cutting edge of the cannula to sever the core of the tissue once it was cut. Once the tissue was collected, pressure was applied to the incision area until bleeding ceased. The skin incision was closed with Michel wound clips (11 mm; Down Surgical, Mississauga, ON, Canada) and a coating of Prodine (Phoenix Pharmaceutical, Inc., St. Joseph, MO) iodine ointment was applied to the surgical site. Liver and mammary tissues (~ 1g) were frozen immediately in liquid nitrogen and stored until isolation of RNA. Cows were monitored during two weeks after biopsy during the milking in order to evacuate blood clots and ensure proper healing of the incision (cleaning and iodine ointment applications were performed when necessary).

RNA Isolation

RNA was isolated from mammary tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA). Mammary tissue was thawed and immediately homogenized in TRIzol reagent with 1 µL of linear acrylamide (Ambion, Inc., Austin, TX) using a Polytron power homogenizer at maximum speed. Upon centrifugation, total RNA was separated with chloroform followed by acid phenol:chloroform (Ambion, Inc., Austin, TX). Total RNA was then precipitated with isopropanol, and the RNA pellet was cleaned with 75% ethanol prior to reconstitution in RNA storage buffer (Ambion, Inc., Austin, TX) for storage at -80°C. RNA integrity and quality was confirmed if OD_{260mm}/OD_{280mm} absorption ratio was > 1.7 (NanoDrop ND-1000, NanoDrop Technologies, Rockland, DE).

Microarrays

A bovine oligonucleotide (70-mers) microarray with >13,000 annotated sequences developed at the University of Illinois (Loor et al., 2007) was used for transcript profiling. Details on the development, annotation, and use of this microarray have been reported previously (Loor et al., 2007). Methods for microarray hybridization and scanning were as reported by Loor et al. (2007). Briefly, slides were hydrated, dried, and placed in a UV Stratalinker® 1800 (Stratagene, La Joya, CA) for ~5 min. Slides were washed with 0.2% SDS solution, rinsed with MilliQ® (Millipore) H_2O, and placed in warm prehybridization soln for 45 min at 42 ^oC. The same amount of Cy3- or Cy5-labelled cDNA from mammary and a reference standard RNA pool (made of different bovine tissues) were co-hybridized using a dye-swap design (i.e., two microarrays per sample). Slides were incubated for 48 h at 45 ^oC prior to scanning. Criteria for evaluation of slide quality included: identification of number of spots with a minimum median signal intensity of 3 SD above background; keeping slides with a minimum of 20,000 spots with minimum median signal intensity of 3 SD above background in both Cy3 and Cy5 channels; and keeping slides with a minimum mean intensity of 400 relative fluorescent units in both Cy3 and Cy5 channels across the entire slide.

Data Analyses

GeneSpring GX (Agilent Technologies) was used for data visualization and preliminary data mining. Subsequently data from microarrays (84 slides) was normalized for dye and microarray effects (i.e., Lowess normalization and microarray centering) and used for statistical analysis. Data were analyzed using the Proc MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). Fixed effects were diets (control, overfed) treatment (LPS, non-LPS) and dye (Cy3, Cy5).

Random effects included cow and microarray. Raw *P* values were adjusted using Benjamini and Hochberg's false discovery rate (FDR) (Reiner et al., 2003). Differences in relative expression due to diet or treatment were considered significant at an FDR-adjusted $P \leq 0.38$ or at $P \leq 0.02$ (Table 16).

Dynamic Impact Approach

This novel bioinformatics approach has been described in part (Loor et al., 2011) and in more detail by Bionaz, Periasamy, Rodriguez-Zas, Everts, Lewin, Hurley, and Loor (currently under review at PLoS One). The Dynamic Impact Approach (DIA) is based on a calculated impact and the direction of the impact (i.e., induce/increase or inhibited/decrease) of DEG on the biological terms (e.g., pathways, functions, and other terms). The DIA was implemented using MS Excel and calculations run automatically the biological terms obtained from different data bases. In this analysis the entire microarray data set with associated statistical *P*-values and fold changes was imported into the Database for Annotation, Visualization and Integrated Discovery (DAVID v6.7) and Kyoto Encyclopedia of Genes and Genomes (KEGG) software in order to obtain significant biological processes, molecular functions and pathways that were further analyzed with the DIA approach.

The rational of the method lies on the fact that in cells the transcriptome expression is non-random. From this assumption the change in flux of a metabolic or a signaling pathway is determined by the change in number and amount of proteins involved in the pathway. If a large number of proteins involved in a pathway are significantly affected by the treatment or physiological phase we can expect to have a large impact of such treatment on the pathway. However, the final flux is not just determined by the number of protein that change by the

treatment but also by the direction of the change (i.e., down-regulated or up-regulated). For instance a pathway where all proteins are significantly affected by a specific treatment can be highly impacted, but the direction of the flux will be determined (considering all the proteins having the same effect on the pathway) by the number of up-regulated vs. the number of down-regulated proteins. When the ratio of up-regulated/down-regulated = 1, the flux can be considered overall unchanged, despite the fact that the treatment had a large impact on the pathway (Morandini, 2009). In addition, also the magnitude of change of proteins content determines the impact on metabolic or signaling flux. For instance, if two treatments affect expression of the same proteins with the same direction of change (e.g., up-regulated) but one treatment change the amount of proteins in average twice as much compared to the other treatment we will expect to have ca. twice as much flux in the former treatment compared to the latter. When considering the fact that we use few samples to infer the effect of treatments or physiological phases for the universal population we need to account also for the significance of the change of protein expression. For instance if on the previous example the number of proteins and the magnitude of change (and direction of change) of the proteins were equal between the two treatment but in the first treatment the average significance is 10-fold lower than the second treatment we will expect that in the universal population the flux of the pathway will be 10-fold more significantly affected in the second than in the first treatment.

Considering all the above points we can summarize that a specific treatment impacts a pathway (or any biological term) in proportion of the number of proteins involved in such pathway significantly affected by the treatment, the average magnitude of the effect on proteins expression, and the average significance of the proteins affected. For the microarray analysis we measure only gene expression and not protein expression, but we assumed that the expression of

genes is proportional to expression of proteins. This is a gross, but unavoidable, assumption because the correlation between mRNA and protein is highly variable between types of mRNA and estimated to be less than 0.5 (Gygi et al., 1999, Schwanhausser et al., 2011). Based on this rationale the impact and the direction of the impact are calculated as:

Impact *= [Proportion of DEG in the pathway (corrected by the number of genes in the pathway present in the array or background)] × [average log2 fold change of the DEG] × [average of – log P-value of the DEG]*

Direction of the Impact *= Impact of up-regulated DEG – Impact of down-regulated DEG*

RESULTS AND DISCUSSION

As explained in the materials and methods section in this study we did not use the non-LPS group fed a control diet during the dry period due to removal of cows because of health problems. However, the effect of prepartal dietary energy supplementation was assessed in the group of cows that received LPS (Overfed energy diet vs. Control diet, all cows receiving LPS challenge) and the effect of LPS was evaluated solely in cows overfed energy during the dry period (LPS vs. Non-LPS. The analysis was performed in liver and mammary tissues resulting in 4 comparisons. After the correction of *P*- value and FDR (Table 16) we uncovered 676 and 51 DEG in liver and mammary tissue, respectively, due to prepartal diet. In addition, 758 and 859 DEG in liver and mammary tissue, respectively, were found due to LPS in cows overfed energy prepartum.

Chromosome Evaluation

The determination of impact of DEG on chromosomes can be useful to identify regions of chromosomes with larger groups of affected genes. This information can help in genetic selection and identification of genes in quantitative trait loci (**QTL**). The impact and the direction of the impact of the DEG on bovine chromosome are reported in Table 18. Overall, the most impacted chromosomes in this comparison were BTA12, BTA14, and BTA22. BTA12 has been associated with milk production and fertility traits (Olsen et al., 2011). BTA14 has been associated with traits of economic importance and some of the genes (*CRH,CYP11B1, DGAT1, FABP* and *TG*) responsible for those traits have also been identified (Wibowo et al., 2008). BTA14 also has been associated with a trait related to ovulation rate (Gonda et al., 2004). BTA22 has been associated with traits related with mechanisms of defense and the immune system (Band et al., 2000). The direction of the flux of BTA12 and BTA14 in the liver dataset indicated inhibition during LPS challenge in the overfed group compared to the control. In an opposite direction, the flux in those chromosomes seemed to be increased or activated in LPS challenged cows compared to controls (in liver and mammary gland tissues). BTA22 flux direction was inhibited in the liver for both effects (Prepartal diet and LPS challenge) in the overfed group. BTA22 was activated in the mammary gland of LPS treated cows, as expected, compared to the controls.

Prepartum Dietary Energy Effect in Liver

Table 17 shows the canonical KEGG pathways. Overall, the direction of the flux was inhibited in most of the pathways in the overfed group compared to the control. The most impacted pathway was “translation” with a consistent decrease in flux. Also lipid and energy

metabolism were among the impacted canonical pathways. Lipid metabolism had an increasing in flux in the overfed group. This last category is quite extensive, and comprises fatty acid oxidation. This finding is in agreement with higher rates of mobilization resulting from overfeeding during the dry period (Drackley, 1999, Drackley et al., 2005, Zammit, 1984) . Table 19 shows the results of the top KEGG subcategories of pathways in liver in the overfed vs. control comparison. The direction of flux for the majority of the impacted functions was inhibition.

Table 20 shows molecular functions provided by DAVID in the comparison of diet effect in liver. The results of this comparison revealed that the most impacted functions had a decreasein flux in the overfed group. The most impacted function was phospholipid-hydroperoxide glutathione peroxidase activity. This enzyme provides significant protection against singlet oxygen generated lipid peroxidation via removal of lipid hydroperoxides and suggest that lipid hydroperoxides are major mediators in this cell injury process (Wang et al., 2001). Other impacted functions were “RNA primary transcript binding” and CD4 receptor binding. Table 21 shows DAVID biological processes. The overall flux direction of the impacted biological processes was inhibition, which followed a similar pattern to the direction of the KEGG analysis. Among the processes relevant to the context of prepartal energy feeding, connective tissue replacement during inflammation response was highly impacted with a lower flux in the overfed group. Also in this comparison we observed positive regulation of centrosome duplication and positive regulation of tyrosine phosphorylation of STAT protein as two pathways that were activated in the overfed group. The STAT (Signal Transducer and Activator of Transcription) group of proteins regulates many aspects of growth, survival and differentiation of cells. (Khatib et al., 2009). STAT3 for instance is a transcription factor that regulates the

expression of a variety of genes in response to cell stimuli, and thus plays a key role in many cellular processes such as cell growth and apoptosis (Yuan et al., 2004).

Prepartum Dietary Energy Effect in the Mammary Gland

A total of 51 DEG genes resulting from the prepartal diet effect in mammary gland satisfied the *P*-value restriction but did not meet the FDR established (0.38). Interestingly, in the context evaluated the response observed in the mammary gland suggested a state of self preservation. Performance and metabolic indices results related to energy balance affected by prepartal diet (Chapters 1and 3), clearly indicated that during the transition period dramatic changes are occurring in terms of energy mobilization. However these changes and adaptations impact the metabolically active tissues in different ways. For instance the adipose tissue hydrolyzes triglycerides in order to provide energy to meet the increasing requirements during the onset of lactation (Horst et al., 2005). The liver oxidizes and accumulates NEFA providing intermediate energy compounds for the mammary gland and other tissues. Finally the mammary gland initiates the synthesis of milk and this event instinctively becomes a priority (Drackley, 1999). Physiologically, meeting the energy requirements for milk production becomes a priority as well. This behavior compels the adipose and liver tissues to make adaptations in terms of energy utilization that are reflected at the gene level explaining the greater amount of DEG in the liver (676 genes) compared to mammary gland (51). The mammary gland seems to be programmed to produce milk, regardless of the source of the necessary intermediate molecules, to warranty the survival of the newborn and in this way preserve the specie. This mechanism, could explain in part the few changes observed in the mammary gland as a consequence of the prepartal dietary treatment.

Lipopolysaccharide Effect in Liver

Table 17 shows KEGG canonical pathway results. In the liver the comparison of LPS vs. Non LPS seemed to be overall activated. In this comparison we observed some pathways with relatively high impact that included metabolism of other amino acids and biosynthesis of other secondary metabolites. In the LPS challenged group the direction of flux of "metabolism of other amino acids" and "biosynthesis of other secondary metabolites" was inhibition. Table 22 showed the top KEGG pathways sub-categories based on relative impact. Among the most impacted sub-categories were fatty acid elongation in mitochondria, p53 signaling pathway, pyruvate metabolism, apoptosis and PPAR signaling. In the LPS challenged group the direction of the flux in the p53 signaling pathway was activation. The p53 activation is induced by a number of stress signals, including oxidative stress. The p53 protein is employed as a transcriptional activator of p53-regulated genes. This results in three major outputs including cell cycle arrest, cellular senescence or apoptosis. In dairy cows this pathway has been related to be activated in response to negative energy balance (Morris et al., 2009).

Table 23 shows the top molecular functions from DAVID based on relative impact. Among the molecular functions with high impact and relevance were calcium-dependent cysteine-type inhibitor activity and functions related with thyroid hormone activation. However, a priori, the top molecular functions observed in this comparison do not seem to have great relevance to the effect of LPS challenge in the liver.

Table 24 shows the top biological processes from DAVID. Among the most impacted biological processes was monocyte chemotaxis. This process is of great importance for immune cells during inflammation or immune response. In the LPS challenged group the flux direction of monocyte phagocytosis was activation, thus, providing evidence of the liver response to the LPS.

Other relevant biological process were positive regulation of tyrosine phosphorylation of STAT3 protein with an activated flux in the LPS infused group. Phosphorylation of STAT3 has been related with immune cells and there is evidence of STAT3 upregulation in the presence of LPS (Chen et al., 2011) .

Lipopolysaccharide Effect in the Mammary Gland

The most impacted KEGG pathway was immune system with a flux direction indicating activation in the LPS challenged group (Table 17). Table 25 shows the KEGG pathways sub-categories; overall, most of the categories in this table had an activated flux direction due to the LPS challenge. Among the relevant pathways sub-categories that were highly impacted and also with an activated flux direction in the LPS treated group were apoptosis and chemokine signaling pathway (Table 25).

Among the most impacted molecular functions from DAVID in the LPS challenged group were nuclear localization sequence binding, CXCR chemokine receptor binding, fibroblast growth factor 2 binding and fibrinogen binding (Table 26). Most of the molecular functions were activated in the mammary gland. Overall, most of the biological processes were with an increased flux in the LPS group as well (Table 27). The most relevant and impacted processes included negative regulation of NF-kappaB, negative regulation of antigen processing and presentation, monocyte chemotaxis and toll-like receptor 4 signaling pathway.

Overall, the response observed in the mammary gland in the comparison assessing the effect of LPS revealed a great level of activation in the different analysis. The mammary gland during the transition period seemed to be extremely responsive to inflammation at the gene level.

CONCLUSION

This analysis provided specific functions, processes and pathways that can facilitate the comprehension of the underlying mechanism between metabolic status during the transition period and the risk of mastitis during early lactation. Liver tissue analysis revealed an evident increase in lipid metabolism that was as a consequence of prepartal overfeeding. In response to LPS different pathways associated with immune cells signaling were impacted. Results from the mammary tissue revealed activation associated with inflammation and the immune system in cows challenged with LPS early postpartum.

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Table 16. Overall microarray differentially expressed genes in liver and mammary tissues restricted by *P*-value and Benjamini and Hochberg's false discovery rate.

		Cutoffs	
Effect	DEG[1]	*P*- value	FDR[2]
Overfed vs. Control			
Liver	676	0.02	0.38
Mammary	51	0.02	1.00
LPS vs. Non-LPS			
Liver	758	0.02	0.35
Mammary	859	0.02	0.31

[1]Differentially expressed genes
[2]False discovery rate

Table 17. Impact of DEG during early lactation (7 d) in canonical pathways from KEGG. All the cows in the comparison Overfed vs. Control received an LPS challenge. All the cows on the LPS vs. Non-LPS comparison were fed 1.62 Mcal/kg DM of energy during the dry period (Overfed energy diet).

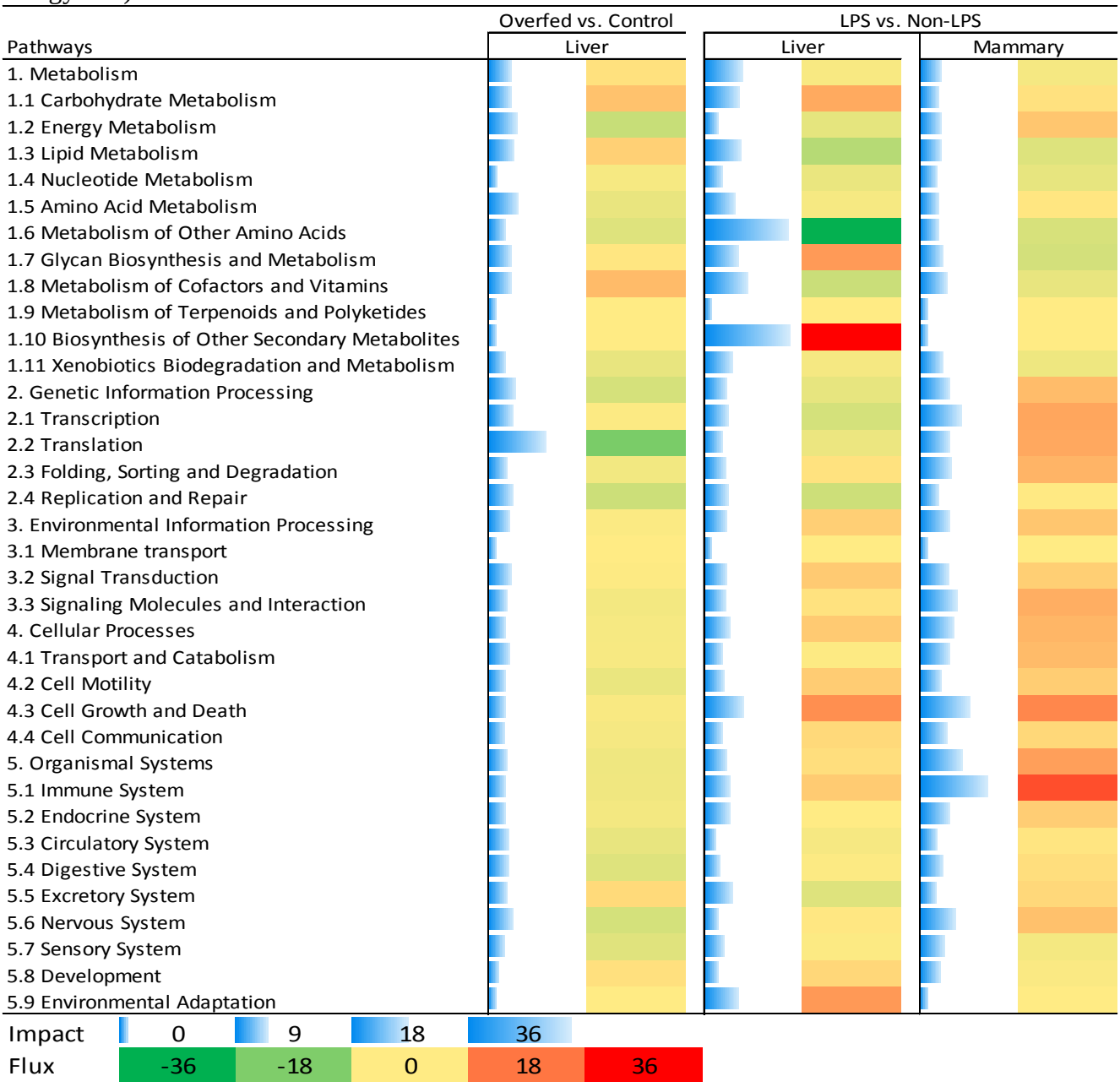


Table 18. Impact of DEG during early lactation (7 d) in bovine chromosomes. All the cows in the comparison Overfed vs. Control received an LPS challenge. All the cows on the LPS vs. Non-LPS comparison were fed 1.62 Mcal/kg DM of energy during the dry period (Overfed energy diet).

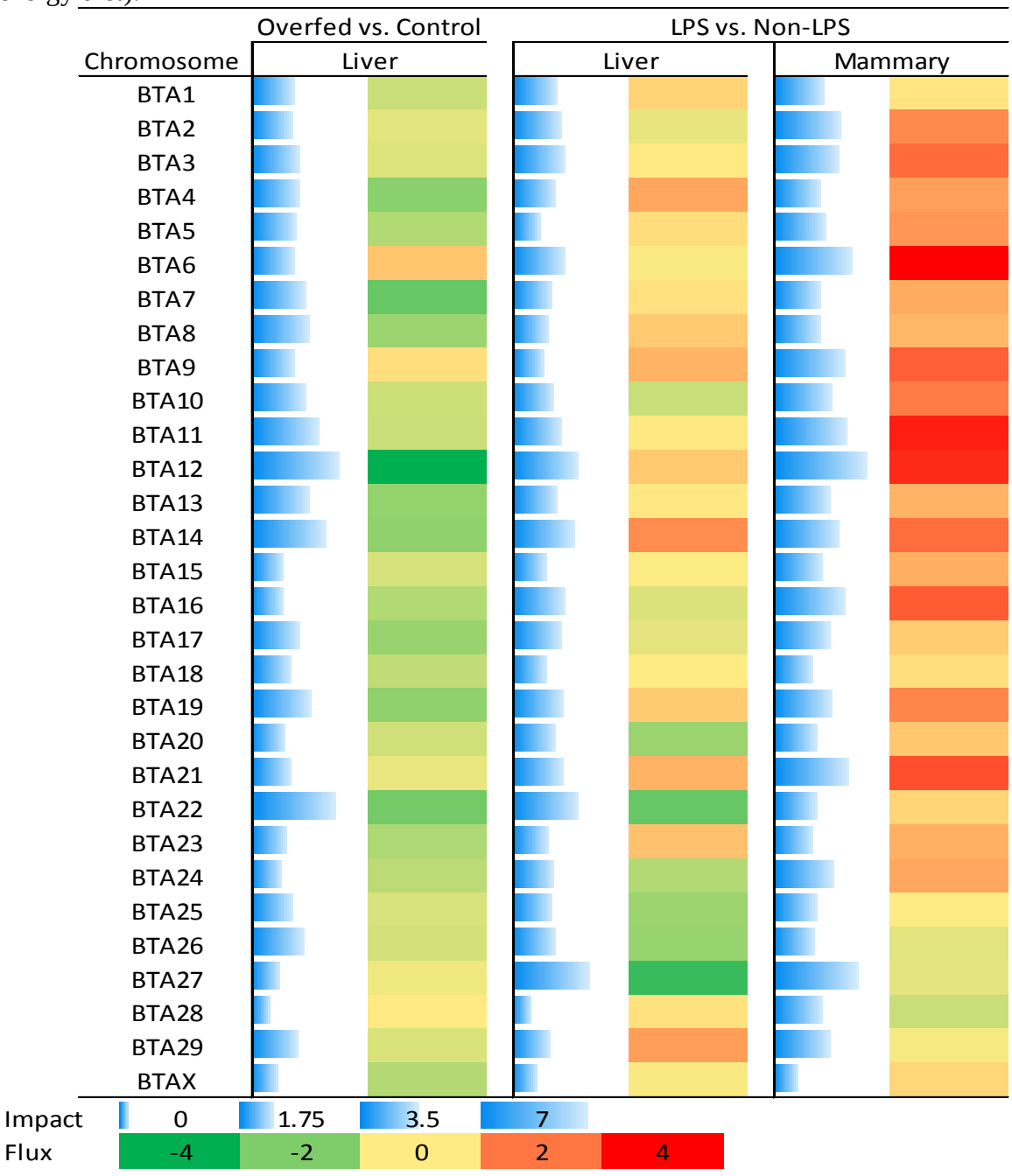


Table 19. Sub-categories from KEGG impact of DEG during early lactation (7 d) in liver. All the cows received an LPS challenge.

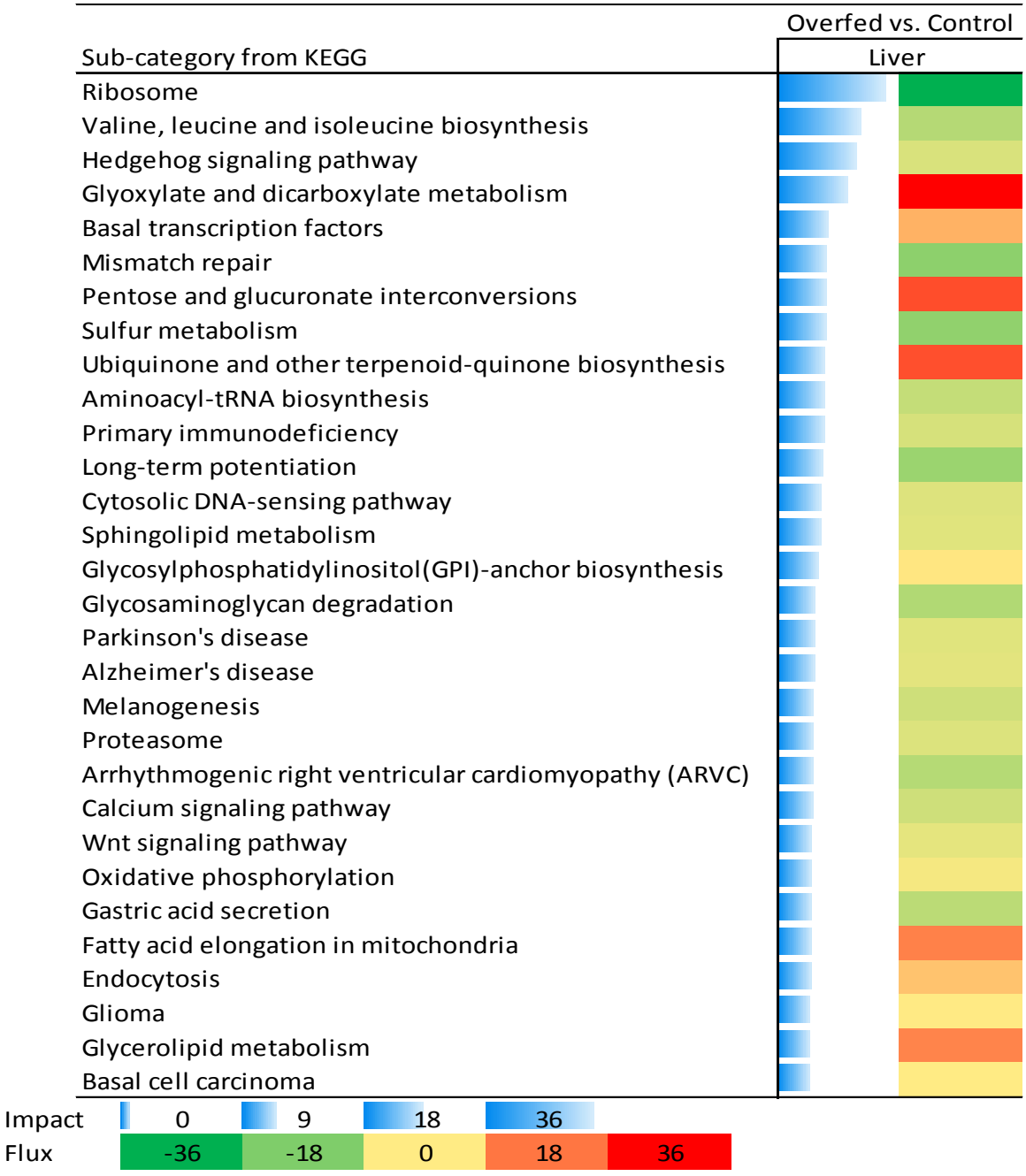


Table 20. Molecular functions from DAVID impact of DEG during early lactation (7 d) in liver. All the cows received an LPS challenge.

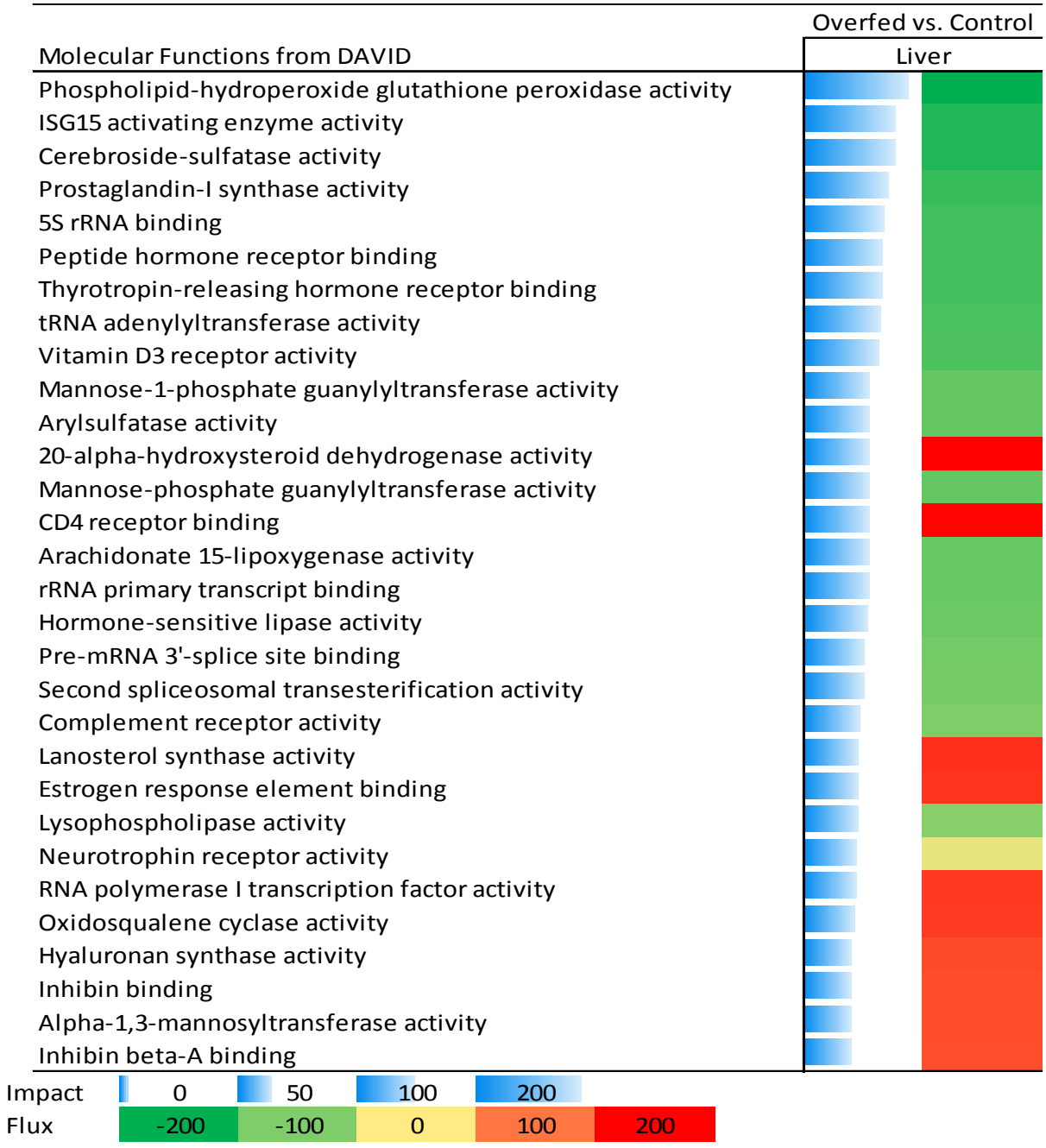


Table 21. Biological processes from DAVID impact of DEG during early lactation (7 d) in liver. All the cows received an LPS challenge.

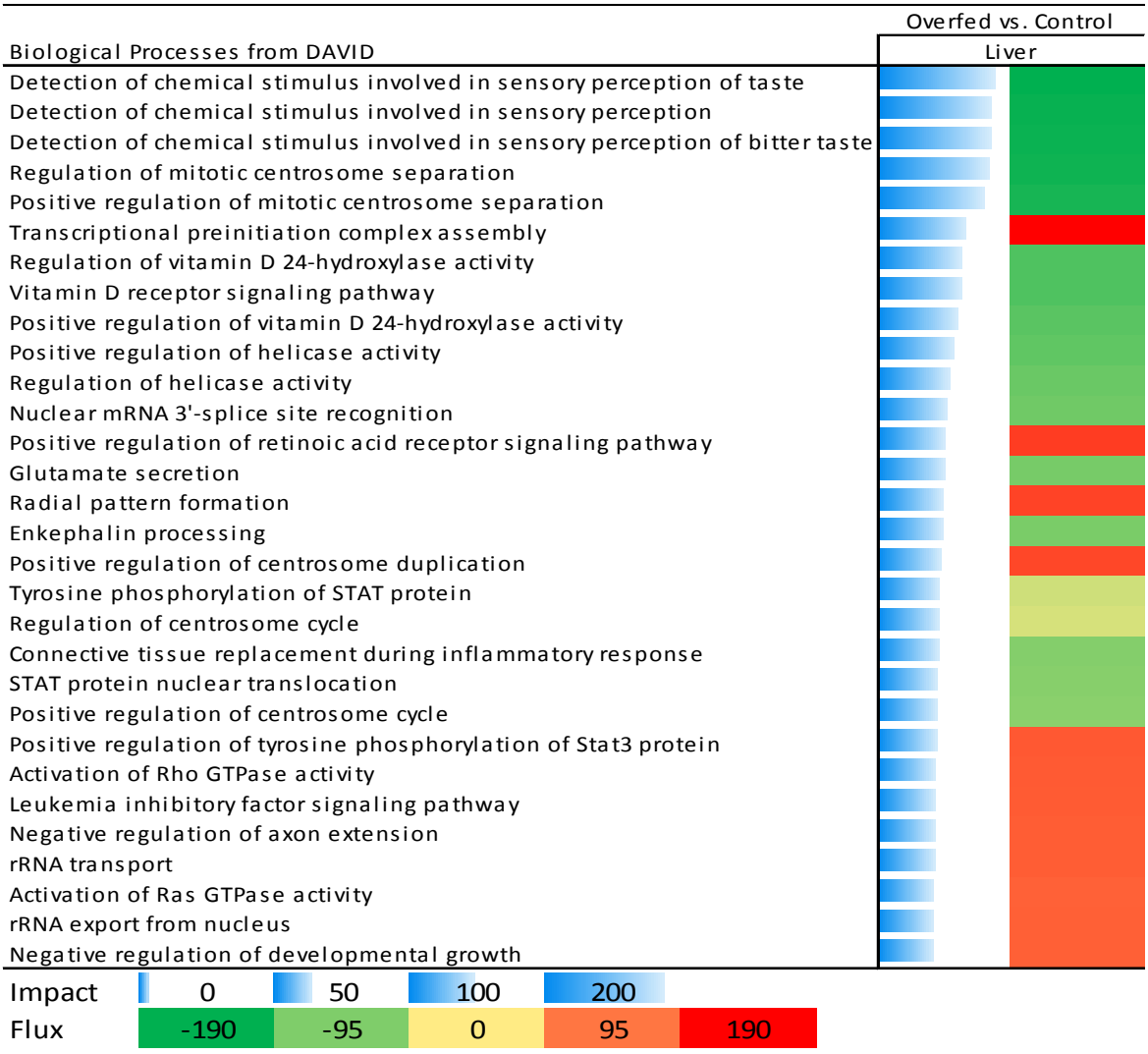


Table 22. Sub-categories from KEGG impact of DEG during early lactation (7 d) in liver. All the cows were fed 1.62 Mcal/kg DM of energy during the dry period (Overfed energy diet).

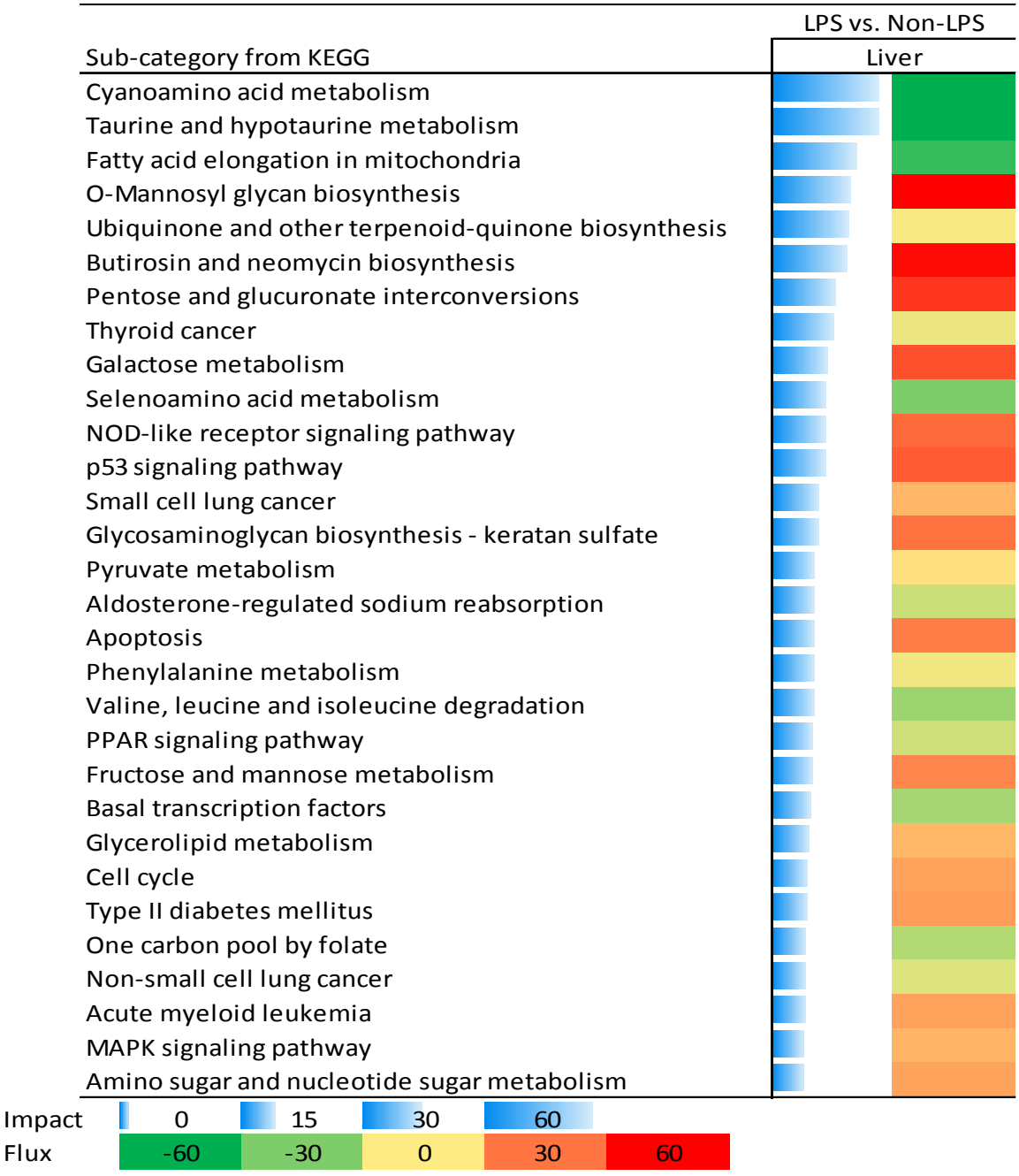


Table 23. Molecular functions from DAVID impact of DEG during early lactation (7 d) in liver. All the cows were fed 1.62 Mcal/kg DM of energy during the dry period (Overfed energy diet).

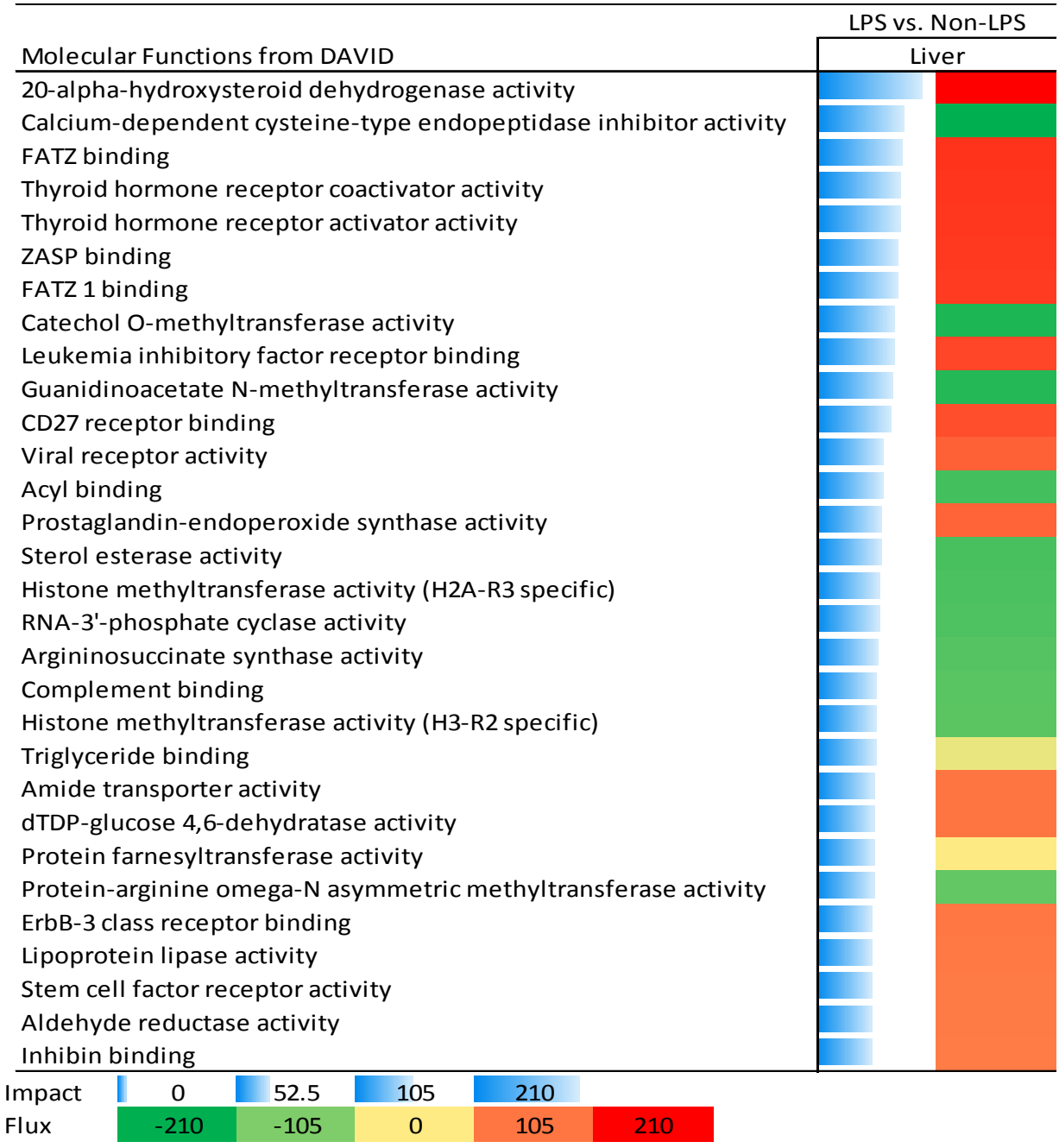


Table 24. Biological processes from DAVID impact of DEG during early lactation (7 d) in liver. All the cows were fed 1.62 Mcal/kg DM of energy during the dry period (Overfed energy diet).

Table 25. Sub-categories from KEGG impact of DEG during early lactation (7 d) in mammary gland. All the cows were fed 1.62 Mcal/kg DM of energy during the dry period (Overfed energy diet).

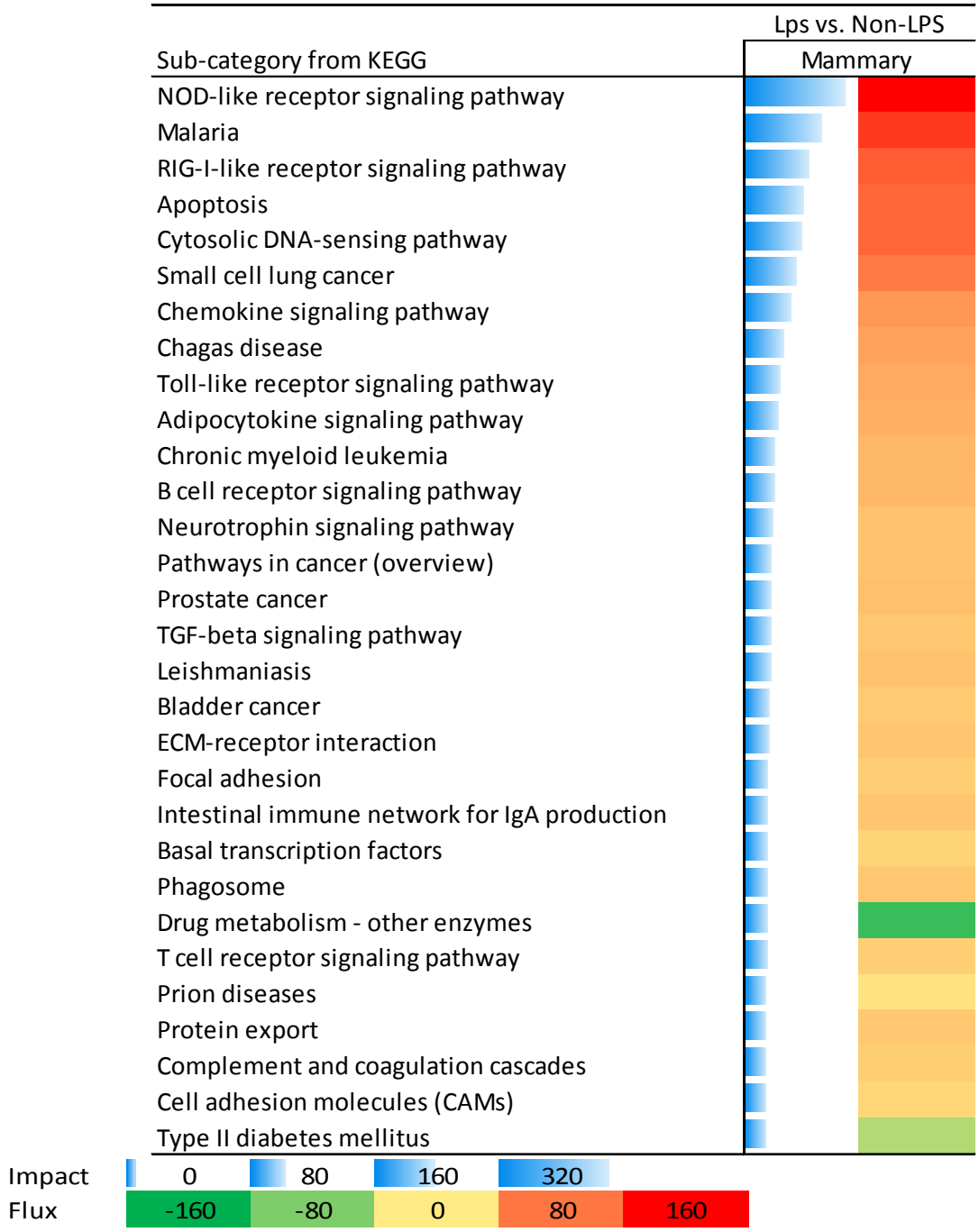


Table 26. Molecular functions from DAVID impact of DEG during early lactation (7 d) in mammary gland. All the cows were fed 1.62 Mcal/kg DM of energy during the dry period (Overfed energy diet).

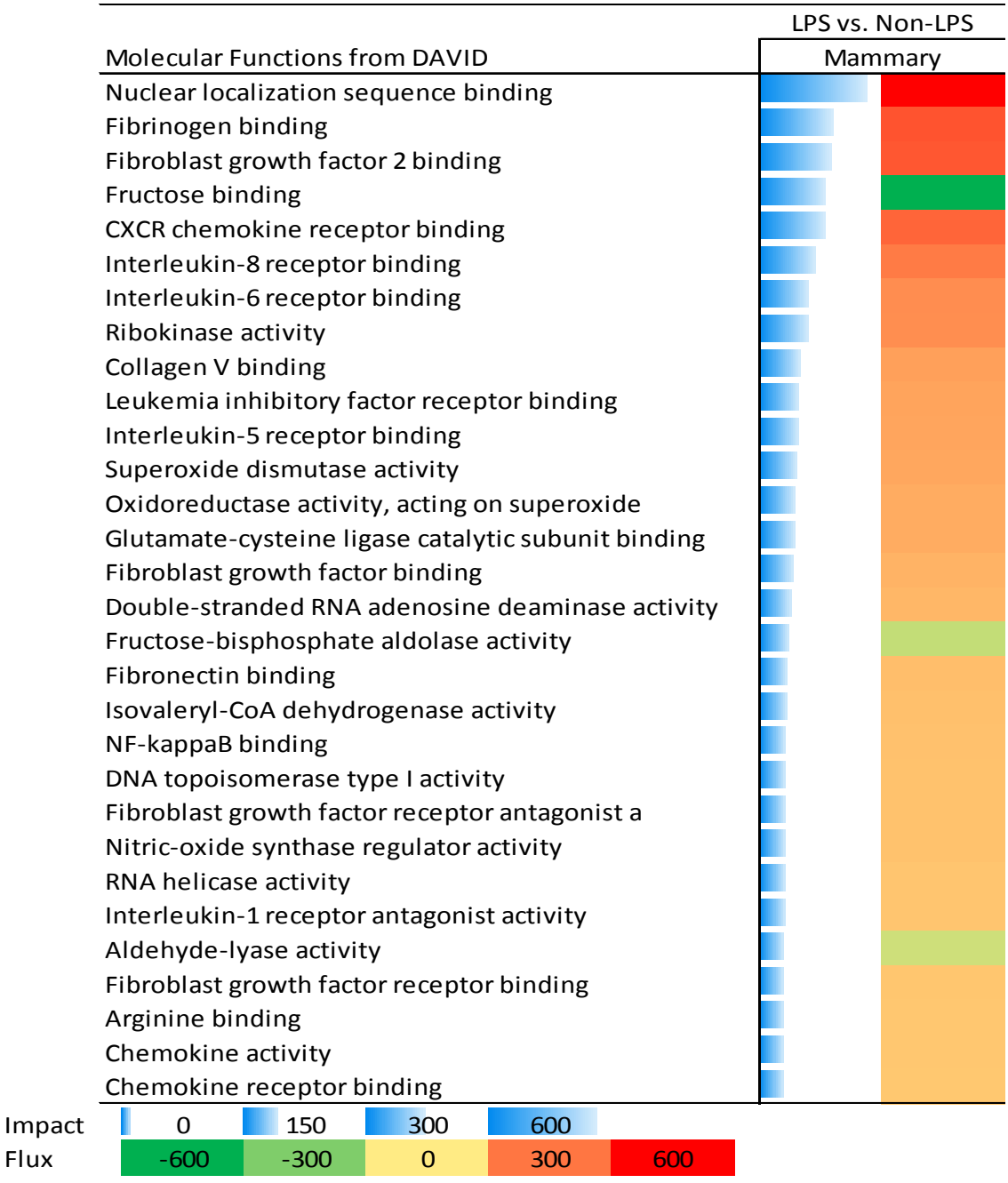
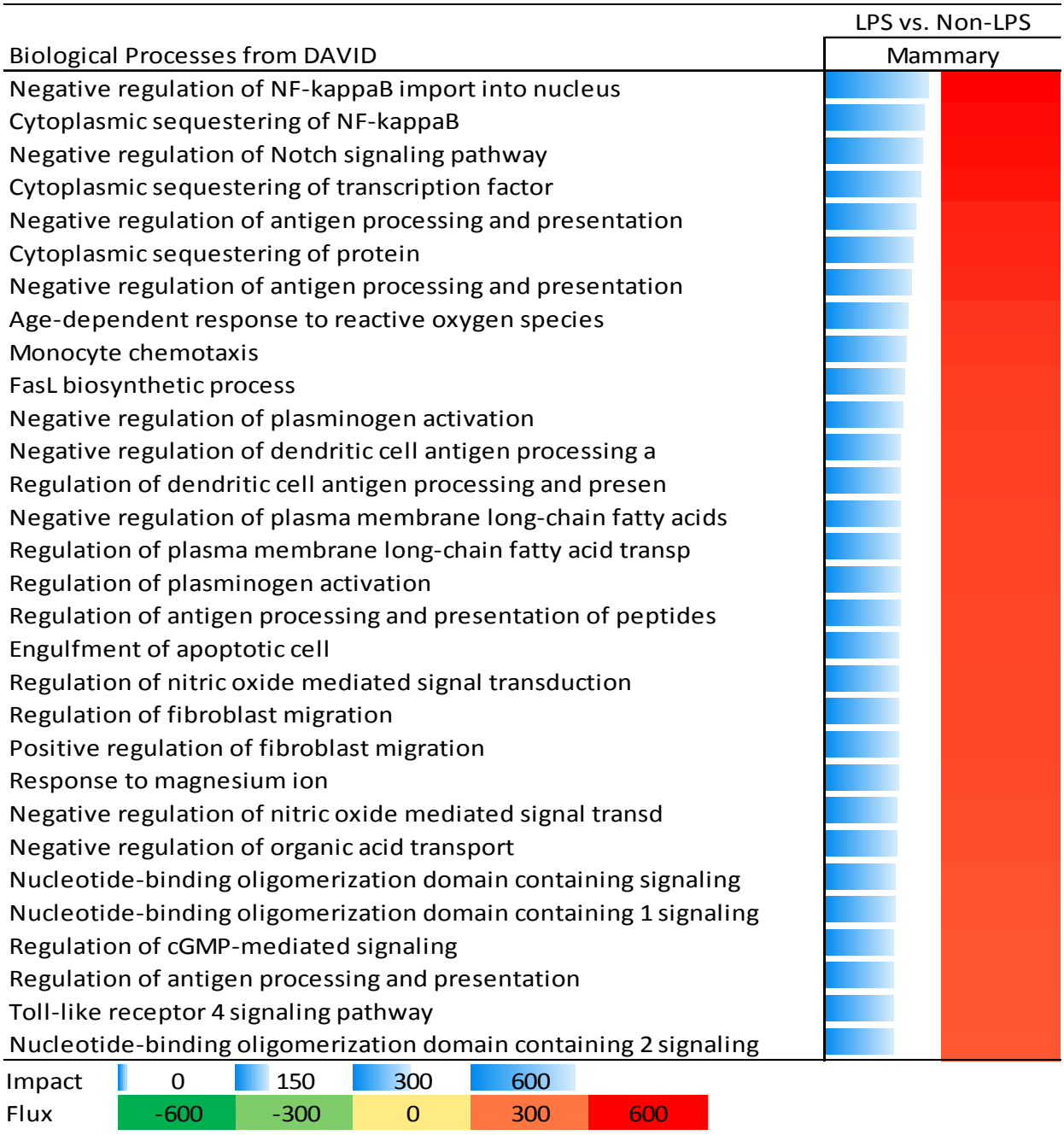


Table 27. Biological processes from DAVID impact of DEG during early lactation (7 d) in mammary gland. All the cows were fed 1.62 Mcal/kg DM of energy during the dry period (Overfed energy diet).



CHAPTER 5:

Peripartal Bovine Blood Neutrophil Metabolic, Antioxidant and Inflammatory Gene Networks Affected by Prepartal Level of Dietary Energy

D.E. Graugnard*† and J.J. Loor*†§.

*Mammalian NutriPhysioGenomics, †Department of Animal Sciences and §Division of Nutritional Sciences, University of Illinois, Urbana, IL 61801

INTRODUCTION

During the transition period the increase in nutrient demand, the drastic changes in endocrine status and the decrease in DMI during late gestation influence metabolism rendering dairy cows in a state of immunosuppression that leads to increased susceptibility to mammary infections (Mallard et al., 1998) and metabolic disorders (Drackley, 1999). Clinical mastitis is most likely to occur during the first mo of lactation. Once a pathogen is detected by the receptors in the epithelial cells of the mammary gland the acute phase response begins and the immune system is activated to eliminate the pathogen (Oviedo-Boyso et al., 2007).

After initiation of the inflammatory response, blood neutrophils or polymorphonuclear leukocytes (**PMN**) become the predominant cell type observed during an infection. Neutrophils constitute up to 70% of the circulating white blood cells (Goldsby et al., 2000). During the transition period, the level of energy consumption prepartum may be a determinant factor in allowing cows to resolve an inflammatory situation. There is evidence that dairy cows can easily over consume energy during the dry period (Dann et al., 2006). However, during early lactation there is a period of negative energy balance where the cow relies on adipose tissue mobilization that can cause metabolic disorders like ketosis and fatty liver (Drackley, 1999). In addition, in vitro studies showed evidence of reduction in PMN viability due to high levels of NEFA and that could impair the immune response to pathogens (Scalia et al., 2006). Our group is interested in the use gene expression technology on PMN cells to uncover relationships between the intensity of lipid mobilization, and bovine immune cell function.

OBJECTIVE

The main objective of this study was to determine the gene expression patterns related to inflammation and lipid metabolism in blood PMN from peripartal dairy cows in response to different prepartum dietary energy level (1.34 vs. 1.62 Mcal/kg DM).

MATERIALS AND METHODS

All procedures involving animals received approval from the University of Illinois Institutional Animal Care and Use Committee (protocol # 06145).

Animals and Diets

Ten 10 Holstein cows entering their second or greater lactation were enrolled in the study. Cows were assigned (n=5/diet) to a control or overfed diet, which were fed *ad libitum* intake to provide at least 100% of calculated NE_L (1.34 Mcal/kg diet DM) or ~159% calculated NE_L requirements (1.62 Mcal/kg DM) respectively during the entire 45-d dry period. Diets were fed as TMR once daily (0600 h) using an individual gate feeding system (American Calan, Northwood, NH, USA). Cows were housed in a ventilated enclosed barn during the dry period and had access to sand-bedded free stalls until 5 d before expected calving date, when they were moved to an individual maternity pen bedded with straw. After parturition, all cows were moved to a tie-stall barn and were fed a common lactation diet (NE_L = 1.69 Mcal/kg DM) as TMR once daily (0600 h) and milked twice daily (0400 and 1600 h). Diets were mixed in a Keenan Klassik 140 mixer wagon (Richard Keenan & Co., Ltd., Borris, County Carlow, Ireland) equipped with knives and serrated paddles; straw in large square bales was chopped directly by the mixer without preprocessing.

Neutrophil Isolation

Samples of of blood (~120 mL) were collected at ~0700 h from the coccygeal vein or artery in vacutainer tubes containing acid citrate dextrose (**ACD** Solution A; Fisher Scientific) at -14, 7 and 14 d relative to parturition. After blood collection, tubes were placed on ice (~30 min) until isolation (Auchtung et al., 2004, Moyes et al., 2009, Salak et al., 1993). Samples were centrifuged at 600 × *g* for 15 min at 4 °C. The buffy coat and approximately one-fourth of red blood cells were removed and discarded. The remaining sample was poured into a 50 mL tube. Twenty milliliters of deionized water at 4 °C were added to lyse red blood cells followed by addition of 5 mL 5X PBS at 4 °C to restore an iso-osmotic environment. Samples were centrifuged at 200 × *g* for 10 min at 4°C. Three subsequent washings using 1X PBS at 4 °C were performed with samples centrifuged at 500 × *g* for 3 min at 4 °C. Neutrophils were immediately homogenized in 2 mL of TRIzol reagent (Invitrogen, Carlsbad, CA) with 1 µL of linear acrylamide (Ambion, Inc., Austin, TX) using a Polytron power homogenizer at maximum speed. The suspension was then transferred equally into two RNA-free microcentrifuge tubes (2mL; Fisher Scientific, Pittsburgh, PA) and stored at -80°C until further analysis.

RNA Isolation

The suspension of RNA and TRIzol reagent was thawed and upon centrifugation, total RNA was separated with chloroform followed by acid phenol:chloroform (Ambion, Inc., Austin, TX). Total RNA was then precipitated with isopropanol, and the RNA pellet was cleaned with 75% ethanol prior to reconstitution in RNA storage buffer (Ambion, Inc., Austin, TX) for storage at -80°C. RNA integrity and quality was confirmed by OD_{260mm}/OD_{280mm} absorption ratio (NanoDrop ND-1000, NanoDrop Technologies, Rockland, DE).

Quantitative Polymerase Chain Reaction Analysis (qPCR)

RNA isolated from neutrophils was used for qPCR analysis. Complementary DNA was synthesized using 100 ng RNA, 1 μg dT18 (Operon Biotechnologies, AL), 1 μL 10 mmol/L dNTP mix (Invitrogen Corp., CA), 1 μL random primers (Invitrogen Corp., CA), and 10 μL DNase/RNase free water. The mixture was incubated at 65 °C for 5 min and kept on ice for 3 min. A total of 6 μL of master mix composed of 5.5 μL 5X Recation Buffer, 0.25 μL (50 U) of RevertAid™ Reverse Transcriptase (Fermentas Inc., MD), and 0.25 μL of RNase Inhibitor (10 U, Promega, WI) was added. The reaction was performed in an Eppendorf Mastercycler® Gradient using the following temperature program: 25 °C for 5 min, 42 °C for 120 min and 70 °C for 15 min. cDNA was then diluted 1:3 with DNase/RNase free water.

Quantitative PCR was performed using 4 μL diluted cDNA combined with 6 μL of a mixture composed of 5 μL 1 × SYBR Green master mix (Applied Biosystems, CA), 0.4 μL each of 10 μM forward and reverse primers, and 0.2 μL DNase/RNase free water in a MicroAmp™ Optical 384-Well Reaction Plate (Applied Biosystems, CA). Each sample was run in triplicate and a 6 point relative standard curve plus the non-template control (Kozniewska et al.) were used (User Bulletin #2, Applied Biosystems, CA). The reactions were performed in an ABI Prism 7900 HT SDS instrument (Applied Biosystems, CA) using the following conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C (denaturation) and 1 min at 60 °C (annealing + extension). The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures to 95 °C for 15 s plus 65 °C for 15 s. Data were calculated with the 7900 HT Sequence Detection Systems Software (version 2.2.1, Applied Biosystems, CA). The final data were normalized using the geometric mean of the three most stable genes (*GAPDH*,

UXT and *RPS9*)among the ones tested as internal controls, as reported previously (Bionaz and Loor, 2007).

Primers were designed using Primer Express 2.0 with minimum amplicon size of 80 bp (when possible amplicons of 100-150 bp were chosen) and limited 3' G+C (Applied Biosystems, CA). When possible, primer sets were designed to fall across exon–exon junctions. Primers were aligned against publicly available databases using BLASTN at NCBI (Nucleotide BLAST, 2008) and UCSC's Cow (*Bos taurus*) Genome Browser Gateway. Prior to qPCR primers were tested in a 20 μL PCR reaction using the same protocol described for qPCR except for the final dissociation protocol. For primer testing we used a universal reference cDNA (RNA mixture from 5 different bovine tissues) to ensure identification of desired genes. Five μL of the PCR product were run in a 2% agarose gel stained with ethidium bromide (2 μL). Only those primers that did not present primer-dimers and a single band at the expected size in the gel, and had the right amplification product (verified by sequencing) were used for qPCR. The accuracy of a primer pairs also was evaluated by the presence of a unique peak during the dissociation step at the end of qPCR.

Statistical Analysis

Data were analyzed using the MIXED procedure in SAS (SAS Institute, Inc., Cary, NC, USA). Fixed effects in the model included diet, time, and diet × time. Random effect was cow within diet. A repeated measures statement using an autoregressive covariate structure was implemented. Statistical differences were declared significant at $P \leq 0.10$.

RESULTS AND DISCUSSION

It is well known that the immune status of dairy cows early after parturition is of importance during the transition period because there is a significant risk for development of new mastitis infections in the udder that may result in cases of clinical mastitis (Hogan et al., 1989). Dairy cows during the transition period normally experience a marked decrease in DMI some days before parturition, which in turn limits the consumption of dietary energy and has a negative impact on the energy balance equilibrium (Bertics et al., 1992). At the same time, nutrient demands for initiation of milk synthesis are increased, which aggravates the energy balance status (Grummer, 1995). After parturition, as milk production increases the energy needed for milk production also increases resulting in a state of negative energy balance. In results presented previously (Chapter 1) we observed a greater DMI prepartum in overfed cows and as designed, energy requirements were exceeded during the prepartal period resulting in significantly higher energy balance. However, both groups were in negative energy balance after calving with a larger drop observed in the overfed group causing dramatic changes in metabolism and potentially affecting the immune status. Negative energy balance has been associations with dramatic changes in metabolites around parturition, characterized by increased NEFA and BHBA but low glucose, and increased susceptibility to mastitis (Godden et al., 2003, Janosi et al., 2003, Nyman et al., 2008)

The mechanisms by which changes in metabolism affect neutrophils are unclear; however, in vitro and in vivo studies have reported that higher concentrations of BHBA or NEFA representative of cows during NEB resulted in altered neutrophil activity including chemotaxis (Suriyasathaporn et al., 1999), viability (Scalia et al., 2006), respiratory burst (Hoeben et al., 1997), and phagocytosis (Grinberg et al., 2008) when compared to cells under

normal concentrations. In this study, we present PMN gene expression patterns that might help understanding how the changes in whole-cow metabolism affect the immune cells.

Cytokines and Genes Associated with Inflammation

Cytokines are essential proteins secreted by the nervous and immune system that are used extensively during intracellular communication (Cannon, 2000). Figure 16 shows gene expression of the cytokines evaluated (*IL6, IL1B* and *IL10*) that revealed important patterns related to inflammation that can be associated with the dramatic metabolic changes occurring during the transition period. The marked increase from -14 through d 7 and 14 in cows fed control led to a diet × time ($P < 0.05$) effect for *IL6* due to. Thus, expression of IL6 at 7 and 14 d was 2-fold greater in controls vs. overfed cows. *IL6* has anti-inflammatory capability through inhibition of *IL1B* and TNF-α production (Bannerman et al., 2008). Of these two, we found greater (diet × time $P < 0.01$) expression of *IL1B* at -14 d and 7 d in the overfed group followed by similar expression between groups at 14 d. IL1B is considered a cytokine that mediates the inflammatory response.

Similar to IL1B, there was a diet × time ($P < 0.01$) effect for *IL10* expression due to greater values in overfed cows at d -14 and 7; that was followed by similar expression between groups at 14 d. *IL10* is known to carry multiple functions in immunoregulation and inflammation, especially related with anti-inflammation signaling (Eskdale et al., 1997). Thus, the mRNA expression data in neutrophils in this study suggested suggested a higher degree of inflammation in the days prior and around parturition in cows overfed energy during the dry period. Neutrophils represent a key target for *IL10* and it has been demonstrated that this

cytokine is of extreme importance in controlling the degree and duration of the inflammatory reactions (Bazzoni et al., 2010).

Other cytokines measured included *TNF, CCL2* and *CCL5* (Table 29). Expression of *CCL2* was undetectable in most samples, thus, data were not analyzed statistically. *TNF* is known to be involved in neutrophil apoptosis which contributes to the resolution of an inflammatory response (Salamone et al., 2001). No overall changes were observed in this study due to time or diet, but the marked decrease in expression between 7 and 14 d in overfed cows led to a significant diet × time effect. It could be speculated that in PMN the expression of TNF around calving might not be as important as other cytokines.

The chemokine *CCL5* is involved in recruiting immune cells to the site of inflammation. Eexpression of *CCL5* decreased over time (time $P < 0.01$) and was affected by the prepartal dietary treatment (diet $P < 0.05$). *CCL5* was greater in the overfed group compared to the control a response that might be explained by the poorer immune status of those animals that would promote sustained chemokine signaling. Overall, the expression of the PMN cytokines revealed a more pronounced state of chronic inflammation in cows overfed energy during the dry period. Similar results have been observed in non-ruminats where excess of energy promotes cytokine production, proinflammatory signaling and oxidative stress (Peng et al., 2011)

The PMN expresses important genes associated with the protection of the host organism and the termination of an inflammatory response. For instance, *SELL* plays an important role in slowing immune cell trafficking through the circulation by promoting adhesion and subsequent pathogen elimination (Mommsen et al., 2011). In humans, there is evidence of upregulation of *SELL* in neutrophils after surgical trauma, and TNF-α has been suggested to be the regulator (Mommsen et al., 2011). In this study, the expression of SELL was greater (diet × time $P < 0.01$)

at d -14 to 7 in the overfed group, after which expression was similar at 14 d. The pattern of *SELL* was similar to *IL10* and also IL1B suggesting a more chronic state of inflammation during the transition period in the overfed group.

The expression and activity of *SOD2* is associated with defending cells against oxidative stress (Al-Gubory et al., 2010). Our results revealed greater expression (diet × time $P < 0.01$) at -14 and 7 d in the overfed group followed by similar expression between groups at 14 d. This expression pattern goes along with the pattern observed for *IL10* and IL1B and supporting the existence of a higher level of stress and inflammation as a consequence of a more pronounced negative energy balance state. Our data support previous findings of higher expression of *SOD2* during times of high generation of hydrogen peroxide as those characterizing PMN phagocytosis (Olsson et al., 2011).

In table 29 we present results of *NR3C1,* this gene encodes the glucocorticoid receptor that functions as a transcription factor upregulating the expression of anti-inflammatory proteins or repressing the expression of pro-inflammatory proteins (Lu et al., 2006). Expression of this gene did not change in overfed cows; however, in control cows expression decreased markedly between -14 and 7 d and then increased to peak expression by 14 d (diet × time $P < 0.03$). Those responses might partly explain the increase in expression of *IL6, IL1B* and *IL10* between 7 and 14 d in control cows.

Transcription Factors and Nuclear Receptors

Peroxisome proliferators activated receptors (PPAR's) are well known ligand-activated transcription factors that belong to the nuclear hormone receptor superfamily. The three PPAR isoforms (α, γ and δ) have been found to affect multiple aspects of lipid metabolism

simultaneously(Li and Glass, 2004). PPAR's are also known to act by altering the transcription of many target genes (Michalik et al., 2006); the majority of these genes are known to play a central role in energy metabolism including fatty acid oxidation (Heim et al., 2002, Wan et al., 2010). The activating ligands for PPAR's are determined by the ligand concentration and cell type (Crisafulli and Cuzzocrea, 2009). Non esterified fatty acids are known to be common endogenous ligands that bind all three PPAR's (Bensinger and Tontonoz, 2008).

Our results (Table 28 and Figure 17) showed that *PPARA* was the most abundant of the three isoforms. Figure 17 shows gene expression of the PPAR's and co-regulators that revealed relevant differences to prepartum dietaryenergy. *PPARA, PPARG* and *PPARD* had similar expression patterns with both treatments. While *PPARD* had a clear response to diet (diet $P < 0.01$) due to greater overall expression in response to overfeeding energy, there was an interaction (diet × time $P < 0.01$) for *PPARG* expression due to greater expression in the overfed group primarily at -14 d. Interestingly, the postpartal responses were different with a decrease in PPARG expression between 7 and 14 d in overfed cows but an increase in control cows. Thus, despite the greater prepartal PPARG and PPARD in overfed cows, the response for both genes in control cows at 14 d seemed to suggest the existence of a different regulatory mechanism.

Overfeeding during the dry period has been shown to result in a greater rates of adipose tissue mobilization compared to feeding diets that meet the energy requirements during the dry period (Dann et al., 2006, Drackley, 1999, Janovick and Drackley, 2010). However, in non-ruminants the intermediate products of lipid mobilization seem to activate and cause a greater expression of PPAR's (Mochizuki et al., 2006). In this case, neutrophils from overfed animals were exposed to greater concentrations of NEFA that could have served as activating ligands, thus, helping to explain the greater PPAR gene expression. Previous data revealed that 16:0

activates PPARγ bovine mammary cells (Kadegowda et al., 2009). More recently, it was shown that both 16:0 and 18:0 also uprgeulated expression of several PPARα in bovine kidney cells (Bionaz et al., 2001).

Activated PPAR play a role during the inflammatory processes by inhibiting proinflammatory transcription factor signaling pathways in vascular and inflammatory cells and, consequently, curtail the activation of inflammatory genes (Moraes et al., 2006). PPAR activation also decreases immune cell recruitment by inhibiting the release of chemokines (Lee et al., 2000, Murao et al., 1999). Whether activation of PPAR in bovine PMN has an anti-inflammatory role is unknown; however, recent data showed that PPARα activation (via 16:0 and 18:0) in bovine kidney cells upregulated expression of IL6 and several acute-phase proteins (e.g. SAA3, ANGPTL4, SPP1) suggesting that these nuclear receptors may have an active role in the PMN during the inflammation that characterizes the transition period.

PPAR regulate gene expression by binding with RXR (Retinoid X Receptor) as a heterodimeric partner that attach to DNA promoter regions to induce transcription of target genes (Palmer et al., 1995). Figure 17 shows the pattern of *RXRA* which was similar to the PPAR's, i.e. greater (diet × time $P < 0.01$) expression in the overfed group during all the time points. The PPAR: RXR heterodimer exists in both an active and inactive state. When inactive, it is bound to corepressors such as *NCOR1* (Nuclear Receptor Corepressor 1) that is known to disrupt and restrict *PPARA* and *PPARG* binding and signaling (Battaglia et al., 2010). *NCOR1* (Figure 17) had a greater (diet × time $P < 0.05$) expression in the neutrophils of the control group, which seemed to support the more inactive state of the PPAR in those cows as a result of a more favorable energy balance status compared to the overfed group. *NCOR2* plays a similar function as *NCOR1* and has been associated with different transcription target genes including the thyroid

hormone receptors (Jonas et al., 2007). However, in this study *NCOR2* expression did not change over time or due to diet.

Other transcription factors evaluated were *STAT3*, *NFKB1* and *MED1* (Table 29). These are involved in different functions such as inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis (Escoubet-Lozach et al., 2002, Li and Nabel, 1997, Silva, 2004). *MED1* had greater expression in cows fed control (diet $P < 0.05$). Both *NFKB1* and *STAT3* expression increased from -14 and 7 d and was greater (diet × time $P < 0.02$) at d 7 in overfed cows than controls. Those data support the notion of greater inflammatory response in PMN of cows overfed energy prepartum.

Neutrophil Signaling Influenced by Lipid Metabolism

Leukotrienes are signaling molecules resulting from the metabolism of lipids that have great influence in immune cell function (Granstrom, 1983, Ramos et al., 1991). Leukotrienes may also act upon PPAR signaling and their targets influencing the inflammatory response (Samuelsson, 1983, Woszczek et al., 2003). Different proteins are involved in leukotrienes biosynthesis. Figure 17 shows the expression pattern of the phospholipase enzyme *PLA2G4A*, which is capable of hydrolyzing membrane phospholipid fatty acids (Holinstat et al., 2011). *ALOX5* and *ALOX5AP* encode enzymes involved in catalyzing different steps in leukotriene biosynthesis from fatty acids, thereby playing a role in the inflammatory processes (Stephensen et al., 2011). Both *LTA4H* and *LTC4S* participate in the formation of leukotrienes from arachidonic acid. These compounds play numerous roles in inflammation, immunological functions and maintaining biological homeostasis (Evans et al., 1986, Kasirga et al., 1999).

In our study, there was a diet × time ($P < 0.05$) interaction for *PLA2G4A* expression. Expression was greater at -14 in overfed cows and it increased markedly by 7 d with both diets but to a greater extent in cows overfed energy. Thereafter, expression increased in cows fed control but decreased in cows overfed energy to values lower than prepartum. These expression patterns were similar to those of most inflammatory and anti-inflammatory cytokines, SELL, PPAR, and SOD2 suggesting a mechanistic relationship between generation of eicosanoids after calving and inflammatory phenomena.

Regardless of treatment, *ALOX5* and *LTA4H* expression decreased from -14 to 7 d and expression was maintained until 14 d (time $P < 0.01$); whereas, *ALOX5AP* and *LTC4S* expression increased from -14 to 7 d regardless of treatment and remained unchanged until 14 d (time $P < 0.01$). The peak observed for *PLA2G4A* at 7 d might have been caused by incoming NEFA triggering a response potentially mediated via PPAR and leading to activation of this enzyme to generate eicosanoids. That would have triggered the necessary pro-inflammatory response in PMN to adjust cellular metabolism to the change in energy balance. The increase in *PLA2G4A, ALOX5AP*, and *LTC4S* strongly suggest that eicosanoid and leukotriene synthesis increased in PMN after calving and to a greater extent in overfed cows. These data underscore an important role for leukotriene biosynthesis and function in the PMN adaptations to calving.

Another important factor in the metabolism of lipids involves insulin signaling. For instance *INSR* (Table 29), the receptor for insulin that mediates different metabolic functions related to this hormone was greater (diet $P < 0.05$) in the overfed group, and expression decreased after calving regardless of diet (time $P < 0.06$). Despite that response, expression of *AKT1* (Figure 16) was lower (diet × time $P < 0.01$) preapartum in the overfed group and increased by d 7; whereas, no change during -14 and 7 d was observed with the control diet. The

protein encoded by this gene regulates multiple signaling pathways essential for cell functioning including glucose metabolism (Zaraza et al., 2010). The role of *AKT1* during inflammation has been studied in *AKT1*-deficient mice and results showed markedly reduced edema. However, reduced inflammation has been associated with a dramatic decrease in neutrophil and monocyte infiltration (Di Lorenzo et al., 2009).

CONCLUSION

Overall, the gene expression data from neutrophils revealed that cows overfed during the dry period were more susceptible to stress and chronic inflammation during the transition period (*SELL, IL10* and *SOD2*) and that cows fed to meet the energy requirements showed more favorable indices of inflammation during the transition period (*AKT1* and *IL6*). Our data also showed evidence that overfed cows had greater expression of transcription factors involved in metabolism of lipids (*PPARA, PPARD, PPAG* and *RXRA*) indicating that immune cells might be predisposed to use endogenous ligands available in circulation (NEFA) for the nuclear receptors.

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Figure 16. The effect of prepartum diet on neutrophil mRNA expression (log-scale) of indices of signaling and inflammation in cows fed a control diet (1.34 Mcal/kg DM) or overfed diet (1.62 Mcal/kg DM) during the entire dry period.

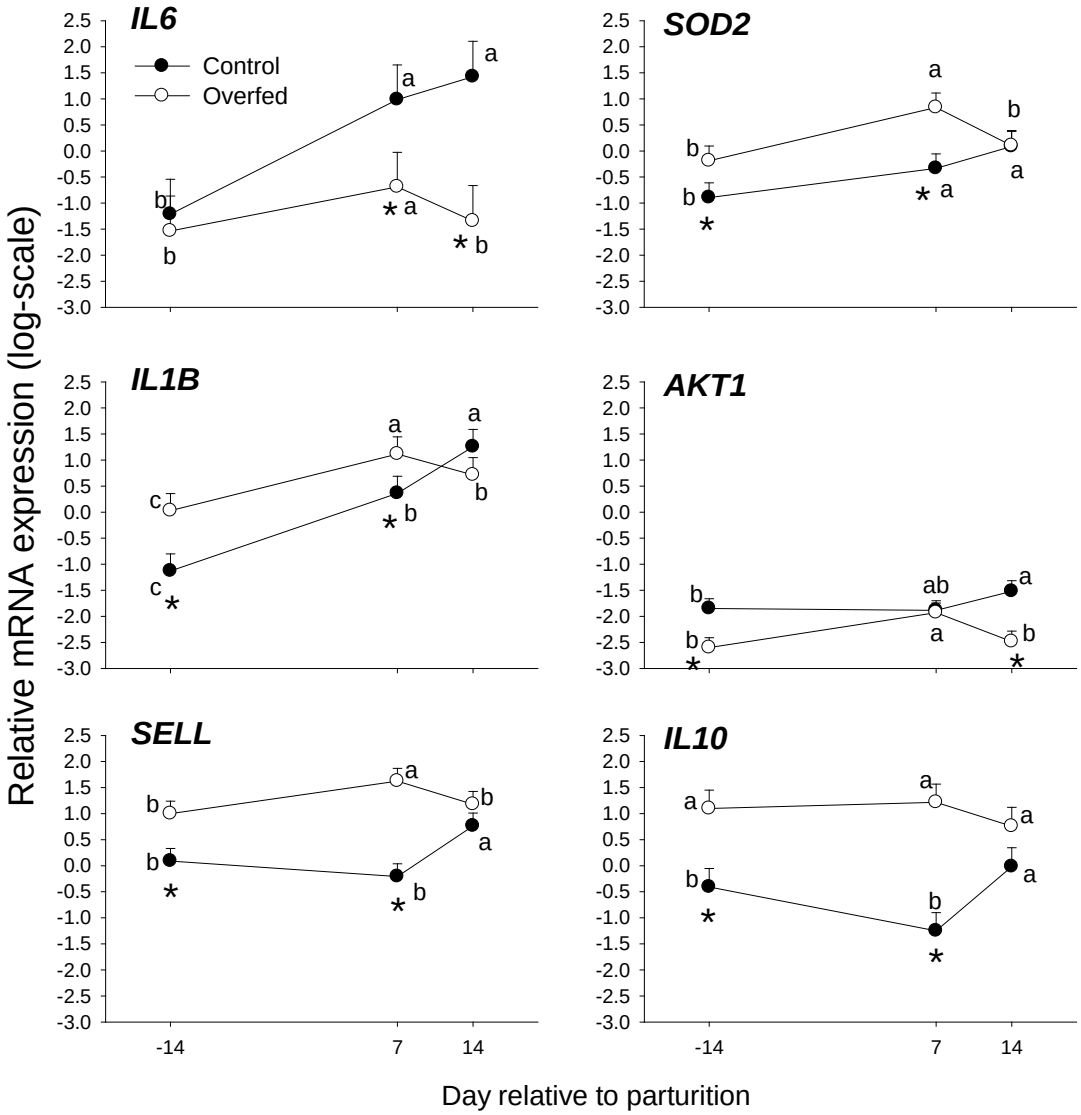

Figure 17. The effect of prepartum diet on neutrophil mRNA expression (log-scale) of genes involved in lipid metabolism in cows fed a control diet (1.34 Mcal/kg DM) or overfed diet (1.62 Mcal/kg DM) during the entire dry period.

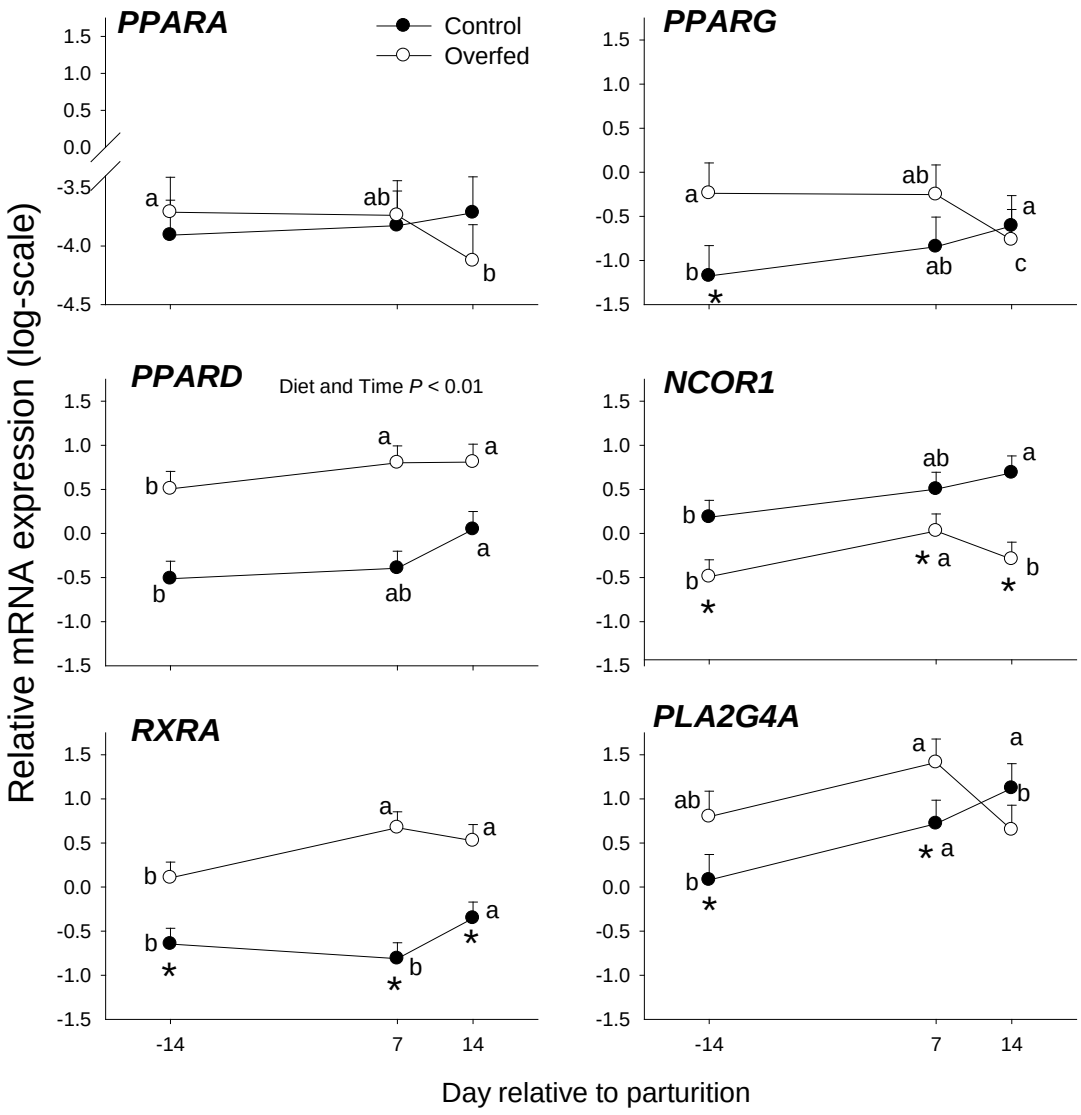

Table 28. Gene symbol and description of genes evaluated from bovine PMN.

Symbol	Description
AKT1	v-akt murine thymoma viral oncogene homolog 1
ALOX5	arachidonate 5-lipoxygenase
ALOX5AP	arachidonate 5-lipoxygenase-activating protein
CCL5	chemokine (C-C motif) ligand 5
IL10	interleukin 10
IL1B	interleukin 1, beta
IL6	interleukin 6 (interferon, beta 2)
INSR	Insulin receptor
LTA4H	leukotriene A4 hydrolase
LTC4S	leukotriene C4 synthase
MED1	mediator complex subunit 1
NCOR1	nuclear receptor corepressor 1
NCOR2	nuclear receptor corepressor 2
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NR3C1	nuclear receptor subfamily 3, group C, member 1
PLA2G4A	phospholipase A2, group IVA
PPARA	peroxisome proliferator-activated receptor alpha
PPARD	peroxisome proliferator-activated receptor delta
PPARG	peroxisome proliferator-activated receptor gamma
RXRA	retinoid X receptor, alpha
SELL	selectin L
SOD2	superoxide dismutase 2
STAT3	signal transducer and activator of transcription 3
TNF	tumor necrosis factor

Table 29. The effect of prepartum diet on neutrophil mRNA expression (log-scale) in cows fed a control diet (1.34 Mcal/kg DM) or overfed diet (1.62 Mcal/kg DM) during the entire dry period.

		Day relative to parturition				*P* value		
Gene	Diet	-14	7	14	SEM[1]	Time	Diet	Diet × Time
TNF	Overfed	0.95[a]	0.86[a]	0.20[b]*	0.30	0.12	0.19	0.06
	Control	1.21	1.14	1.24*				
MED1	Overfed	-0.50	-0.23	-0.51	0.23	0.73	0.01	0.27
	Control	0.47	0.36	0.43				
NFKB1	Overfed	0.35[b]	0.80[a]*	0.56[ab]	0.15	0.06	0.77	0.01
	Control	0.53[ab]	0.39[b]*	0.61[a]				
NR3C1	Overfed	0.21	0.48	0.14	0.24	0.98	0.97	0.03
	Control	0.34[ab]	0.03[b]	0.43[a]				
ALOX5	Overfed	1.12	-0.80	-0.76	0.45	0.01	0.66	0.43
	Control	0.35	-0.79	-0.68				
ALOX5AP	Overfed	0.81	1.61	1.53	0.25	0.01	0.14	0.13
	Control	-0.04	1.15	1.40				
STAT3	Overfed	-0.19[b]	0.47[a]*	-0.26[b]	0.16	0.04	0.08	0.01
	Control	-0.45[b]	-0.56[b]*	0.03[a]				
CCL5	Overfed	0.83	-0.33	-0.30	0.43	0.01	0.02	0.87
	Control	-0.65	-1.81	-1.62				
INSR	Overfed	-1.06	-1.27	-1.05	0.14	0.01	0.06	0.15
	Control	-1.31	-1.52	-1.56				
NCOR2	Overfed	-0.34	-0.05	-0.33	0.13	0.11	0.12	0.42
	Control	-0.09	0.04	0.05				
LTA4H	Overfed	-0.46	-1.21	-0.82	0.22	0.01	0.44	0.19
	Control	-0.82	-1.44	-1.41				
LTC4S	Overfed	-2.89	-1.87	-3.50	0.81	0.01	0.66	0.23
	Control	-2.84	-1.80	-2.17				

[1]Largest SEM is shown.
[a-e] Differences between days (time $P < 0.05$ or diet × time effects $P < 0.05$).
*Denote significant interactions (diet × time effects $P < 0.05$) at a given day.

Table 30. qPCR performance among the genes measured in PMN.

Gene	Median Ct	Median ΔCt	Slope	(R^2)	Efficiency	Abundance[1]
AKT1	24.581	4.955	-3.82	0.97	1.827	1.944
ALOX5	24.310	4.753	-2.64	0.98	2.392	1.865
ALOX5AP	20.710	0.915	-3.38	0.98	1.976	0.359
CCL2	30.846	10.903	-3.11	0.93	2.097	4.278
CCL5	24.687	4.637	-3.48	0.96	1.938	1.820
IL10	21.158	1.210	-3.18	0.91	2.063	0.475
IL1B	26.438	6.569	-3.10	0.91	2.102	2.578
IL6	31.999	11.923	-2.12	0.90	2.963	4.679
INSR	24.215	4.518	-3.56	0.97	1.909	1.773
LTA4H	24.098	4.483	-3.54	0.92	1.916	1.759
LTC4S	30.357	10.507	-3.02	0.93	2.144	4.123
MED1	22.883	3.328	-3.30	0.90	2.009	1.306
NCOR1	20.924	1.264	-3.40	0.84	1.968	0.496
NCOR2	24.340	4.649	-3.46	0.90	1.945	1.824
NFKB1	22.932	3.253	-3.28	0.94	2.018	1.277
NR3C1	24.036	4.315	-3.24	0.87	2.035	1.693
PLA2G4A	22.762	3.128	-2.91	0.95	2.206	1.227
PPARA	28.363	8.751	-3.37	0.91	1.980	3.434
PPARD	24.821	4.888	-3.57	0.98	1.906	1.918
PPARG	24.970	5.163	-3.16	0.85	2.072	2.026
RXRA	19.685	0.439	-3.40	0.92	1.968	0.172
SELL	20.482	0.815	-3.28	0.95	2.018	0.320
SOD2	20.349	0.711	-3.41	0.92	1.965	0.279
STAT3	21.319	1.590	-3.25	0.90	2.031	0.624
TNF	27.059	7.433	-2.27	0.95	2.758	2.917

[1] % of mRNA abundance relative to an internal control gene (UXT).

Table 31. GenBank accession number, hybridization position, sequence and amplicon size of new primers designed for *Bos taurus* used to analyze gene expression by qPCR. Similar information for remaining genes was reported previously.

Accession #	**Gene**	**Primers**[1]	**Primers (5'-3')**[2]	**bp**[3]
NM_001075864.1	*PLA2G4A*	F. 1134 R.1238	CTCCATGTCAAACCCGATGTC GTCAGGCGCCATAAAAGTACCA	105
NM_001034280.1	*LTA4H*	F.1042 R.1141	ACATTTGTGGACGACTGTTTGGT TGGGTCTCCCCAAAAGTCTTT	100
NM_001046098.2	*LTC4S*	F.217 R.316	CATCTACCGAGCCCAAGTGAA CAGTGCTGCCGCACCTT	100

[1] Primer direction (F – forward; R – reverse) and hybridization position on the sequence.
[2] Exon-exon junctions are underlined.
[3] Amplicon size in base pair (bp).

Table 32. Sequencing results of PCR products from primers of genes designed for this experiment. Best hits using BLASTN (http://www.ncbi.nlm.nih.gov) are shown.

Gene	Sequence
PLA2G4A	GACACGTGAAGTTGTCAGATGTGGGTTGAATTAGTCCATTTGAGATTGGC ATGGGCTAAATATGGTACTTTTTATGGCGCCCTGCACAAAAAAAA
LTA4H	GAAGAGTCATTTCCGGCTCGTGGGCGGAGTGGAGAACTCCAGAAT TCGATAAAGACTTTTGGGGGAGACCCAACCCCTTT
LTC4S	CGACGAACTCCGCGTTTCCTCGCCATGCTCTGGGTGGCCGGGCATC TTCTTTCACGAAGGTGCGGCAGCACATGGAACCCCC

Table 33. Sequencing results of genes using BLASTN from NCBI against nucleotide collection (nr / nt) with total score.

Gene	Best hits	Score
PLA2G4A	Bos taurus phospholipase A2, group IVA (cytosolic, calcium-dependent)	91.5
LTA4H	Bos taurus leukotriene A4 hydrolase (LTA4H), mRNA	78.8
LTC4S	Bos taurus leukotriene C4 synthase (LTC4S), mRNA	95.1

SUMMARY AND CONCLUSIONS

The overall objective of this dissertation was to evaluate neutrophil function, metabolic and inflammation indices and gene expression affected by the plane of dietary energy prepartum and an early postpartum inflammatory challenge. Our general hypothesis was that overfeeding dietary energy during the dry period, accompanied by the metabolic challenges associated with the onset of lactation would render the cow's immune function less responsive early postpartum.

In chapters 1, 2 and 3 we evaluated we evaluated different aspects related with the transition period: Preparum diet effect on early lactation (Chapter 1), early lactation inflammation/challenge (LPS challenge; Chapter 2) and Prepartum diet effect in lactation with and early lactation LPS challenge (Chapter 3). The parameters evaluated at this point included performance, immune cell function (phagocytosis and chemotaxis of neutrophils).

In chapter 1, our data indicated that the more positive energy status prepartum resulted in a surge of insulin with a temporary but significant effect on metabolism. We obtained evidence that the greater insulin concentration decreased muscle protein turnover and perhaps increased the circulating TAG uptake by adipose tissue prior to parturition. However, elevated insulin concentration also could have impaired phagocytic capacity of the PMN prior to calving. Our blood data showed some indications of negative carry over effects of overfeeding energy to the point of calving (haptoglobin, bilirubin, and ROM).

In chapter 2, the presence of an intra-mammary *E. coli* LPS challenge represented rapid changes in metabolic indices that can affect the dairy cow during the days following days (immune function) and might represent adaptations in a longer term that can negatively affect performance (Liver TAG accumulation).

Chapter 3 revealed that controlling the level of dietary energy to meet the cow's requirements during the dry period is an effective management strategy to improve neutrophil function. In the other hand overfeeding during the dry period does not represent any advantage to the dairy cow and when coupled with an early inflammatory event the immune status is fragile compared to cows that meet strictly the dietary requirements. In addition, in this chapter gene expression was evaluated showing evidence of a better immune and metabolic status in the animals fed to meet their energy requirements during the dry period.

Based on the findings on chapters 1, 2 and 3; in chapter 4 we narrowed or investigation to the period of early lactation where most of the changes were occurring. The effect of LPS challenge and the effect of prepartum diet were evaluated in mammary and liver tissues. The use of microarray technology provided specific functions, processes and pathways that can facilitate the comprehension of the underlying mechanism between metabolic status during the transition period and the risk of mastitis during early lactation. These results may lead into more specific research that can turn into improved nutritional management strategies and better prevention and treatment of the disorders that occur during the transition period. However, there are still computational challenges that have to be improved.

In chapter 5 gene expression of immune cells was evaluated. Once again our research was narrowed to the effect of prepartum diet based on performance and immune function results from previous chapters. Expression data supported results from previous chapters; overfed cows resulted in greater level expression of genes related with

inflammation associated with the onset of lactation (*IL10, SELL, SOD2* and *IL1B*) and potentially lipid utilization/ mobilization (*PPARA, PPARG, PPARD, RXRA* and *NCOR1*).

The diet effect in this experiment was transcendental during the transition period and potentially during the entire lactation. Changes in energy balance were observed and provided a good model to study the challenges associated with the onset of lactation. Overall the LPS model provided a consistent response representing an inflammation incident; however the changes in metabolic indices were sudden and hard to detect in most of the cases during the days following the challenge. In general overfeeding dietary energy during the dry period resulted in a less responsive immune function during the early postpartum. In other words, controlling the dietary energy prepartum has more benefits for the dairy cow during transition.

Figure 18 shows an integrated model of the overall responses and benefirts of controlling the dietary energy during the dry period and considering an inflammatory incident during the first wk after parturition. In terms of performance the energetic balance observed was more favorable compared to overfed cows in the days after parturition. At this point energy balance is normally negative and implies mobilization from adipose tissue reserves. However the rate of mobilization was lower compared to the overfed group. The liver plays fundamental role uptaking the intermediates of adipose tissue mobilization; in our results less TAG accumulation was observed in the liver (Indirectly suggesting a better metabolic functioning and potentially greater rated of β oxidation). The metabolic profiling assessed confirmed an improved and more favorable metabolic status that supported the response of the liver. The metabolites results were directly related to immune function and contribute to explain a more favorable response in terms of phagocytosis.

The LPS challenge affected directly the mammary gland and indirectly affected the liver and immune cells. However is important to remember that the evaluation of prepartal energy feeding was assessed at all times in cows receiving LPS implying that cows fed to meet their energy requirements performed better with the stress of an inflammatory event.

At the gene expression level, the results reconcile most of the responses of performance and metabolic indicators. The liver revealed greater expression in genes related to metabolism of lipids. The immune cells evaluated showed an opposite response to the liver in terms of utilization of intermediates resulting from metabolism of lipids. Clearly the improved metabolic status of the control fed group (in part, attributed to the well functioning of the liver uptaking NEFA) did not obligate immune cells to dramatically adapt and change energy sources permitting a better immune function performance. Finally the mammary gland did not seem to be affected by the prepartal dietary energy effect. This response was expected since the cow instinctively prioritizes the mammary gland in the parturition of energy for self preservation. However the mammary gland was extremely responsive to the effect of LPS by activation of functions related to the immune function.

Figure 18. Responses of controlling the dietary energy to dairy cows during the dry period considering a challenge of LPS during the first week after parturition as a model for inflammation.