BLOOD AND LIVER BIOMARKERS AND TRANSCRIPTOME ALTERATIONS DURING THE TRANSITION PERIOD REVEAL BENEFICIAL EFFECTS OF RUMEN-PROTECTED METHIONINE SUPPLEMENTATION ON HEALTH STATUS AND PERFORMANCE IN DAIRY COWS

BY

JOHAN SAMIR OSORIO ESTEVEZ

DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Animal Sciences
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2014

Urbana, Illinois

Doctoral Committee:

Professor James K. Drackley, Chair and Co-Director of Research
Associate Professor Juan J. Loor, Co-Director of Research
Professor Walter L. Hurley
Associate Professor Yuan-Xiang Pan
Professor Klaus L. Ingvartsen, Aarhus University, Tjele, Denmark

ABSTRACT

The early postpartal period is characterized by marked changes in hormonal, metabolic, and immune/stress-like conditions all of which may contribute to regulating dry matter intake (DMI) and the supply of nutrients to mammary gland. Peripartal cows are likely to undergo a negative methionine (Met) balance due to increased requirements of tissues and cells for methylated compounds and Met for milk protein production. Therefore, supplementation of rumen-protected Met during the peripartal period may improve yield of milk and protein, and also help coordinate immunometabolic adaptations of the cow. Supplementation of rumen-protected Met in the form of MetaSmart (MS) or Smartamine (SM) improved milk production at least in part by increasing voluntary DMI and perhaps by optimizing the use of body lipid reserves as observed in chapter 2. In fact, an overall milk protein and milk fat was positively affected by Met supplementation. Improvements in production were accompanied by a faster recovery to a positive energy balance coupled with a lower predisposition to develop ketosis in Met-supplemented cows, which indicates that overall health was at least not compromised and perhaps improved.

Results from chapter 3 indicate that supplementing Met during the peripartal period promoted favorable alterations of inflammatory and oxidative stress status of cows. The greater albumin in response to Met-supplementation may indicate enhanced liver function and improved AA status. Overall blood and liver biomarkers analyzed indicated that improved postpartal performance when feeding SM and MS was due partly to a better immunometabolic status.

Relative mRNA expression of targeted genes associated with biological processes of interest such as the Met cycle, inflammation, and oxidative stress, among others, were evaluated in chapter 4. Results indicated that feeding MS or SM to cows during the peripartal period could profoundly affect the hepatic transcriptomics of Met metabolism, *PPARA* activation, hepatokines synthesis, and gluconeogenesis.

To better understand the molecular effects of peripartal Met-supplementation on hepatic metabolism in Holstein dairy cows, a microarray platform with advanced computational and bioinformatics techniques were used in chapter 5. Hepatic transcriptome analysis revealed a high impact on metabolism especially in pathways AA metabolism.

Overall supplementing rumen-protected Met to peripartal dairy cows will improve DMI, milk yield and components, and energy balance by enhancing liver function, increasing antioxidant capacity, and ameliorating the inflammatory response, and that in turn these effects are controlled at the molecular level by cross-talk of gene expression.

TABLE OF CONTENTS

CHAPTER 1

LITERATURE REVIEW

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 suppressed; 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 hormonal changes within the days leading to calving (i.e. decreased insulin) coupled with negative energy balance has been associated with pronounced mobilization of fatty acids in adipose tissue towards the liver, 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 the postpartal rate of lipolysis/esterification in adipose tissue and, thus, the amount of NEFA released into blood and available for metabolism in liver (Drackley et al., 2005). The current recommended feeding practices (NRC, 2001) for pregnant non-lactating cows have been questioned 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).

Besides prepartal energy intake control, Methionine (Met) supplementation to the metabolizable Met pool of peripartal cows has been recently evaluated (Socha et al.,

2005; Ordway et al., 2009). Research has shown that this could increase both the sparing of essential amino acids (EAA) pool in a cow with low dry matter intake (DMI), and the export of lipids from liver. 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 (Drackley et al., 2005). Therefore, our general hypothesis was that supplementing rumen-protected Met to peripartal dairy cows will improve DMI, milk yield and components, and energy balance by enhancing liver function, increasing antioxidant capacity, and ameliorating inflammatory response, and that in turn these effects are controlled at the molecular level by cross-talk of gene expression.

Transition period

The transition period, as defined by Drackley (1999), is the most challenging stage in the lactation cycle when most metabolic disorders and infectious diseases occur. Primarily, this is due to several conditions including immunosuppression, changes in endocrine status, and decrease in dry matter intake (DMI) that collide during this relative short period of time between late gestation and early lactation (Grummer, 1995; Drackley, 1999). Most of these negative effects occur during the last 3 wk of gestation until 3 wk after parturition, which in turn has defined the length of the transition period (Grummer, 1995; Drackley, 1999).

Physiological regulation and adaptations of the periparturient cow

Late in pregnancy and in early lactation, the nutrient demand increases quite considerably. In late pregnancy nutrient demand increases as a result of fetal development, but at the onset of lactation the nutrient demand increases dramatically (Ingvartsen, 2006). This triggers a coordinated response of biological processes in different tissues resulting in metabolic changes to ensure high milk yield concurrently with maintenance of homeostasis (Ingvartsen, 2006). From a deductive standpoint, the transition period may be broken down into three main components: from 3 wk to parturition (close-up), calving time, and calving to 3 wk in lactation (fresh); in particular this may give a better insight into the physiological and metabolic adaptations but also allows more accurate interpretations from research experiments.

As the cow enters the close-up period, nutrient requirements for pregnancy and colostrogenesis increase exponentially (Bauman and Currie, 1980; Bell, 1995), while DMI often decreases over time due to several factors such as physical compression of the rumen from the growing fetus and uterus as well as endocrine and metabolic changes. These include decreasing insulin, and increasing glucocorticoids, growth hormone, and non-esterified fatty acids (NEFA). At the same time tissue sensitivity to glucocorticoids increases and sensitivity to insulin decreases (Bell, 1995; Ingvartsen and Andersen, 2000). This array of changes has been described by Bauman and Currie (1980) as homeorhesis, a coordinated control in metabolism of body tissues necessary to support a physiological state. This underlines the importance of the close-up period to “prime” the cow for her next lactation. In contrast, at calving the cow will experience the most drastic and abrupt hormonal and physiological changes during the entire lactation. Therefore,

abundant literature (Gundelach et al., 2009; Paolucci et al., 2010) has underlined this relative short period of time as a major checkpoint to prevent infectious diseases and metabolic disorders and eventually transduce an improvement of both productive and reproductive performance of the cow. Finally, the fresh period represents an additional adaptation period for the transition cow, where further increases in the nutrient demand sustains a constantly increasing exportation of nutrients through milk production. For instance, a cow with a maximum milk yield of 50 kg secretes approximately 2 kg of milk fat daily, 1.6 kg of milk protein, 2.5 kg of lactose, 65 g of Ca, 50 g of P, and 8 g of Mg daily, which, of course, increases the demand for dietary energy, protein and minerals (Ingvartsen, 2006).

Energy balance and metabolism

Energy balance has been conventionally characterized as the balance between energy needs and energy supply (Grummer, 1993; Ingvartsen and Andersen, 2000; Janovick and Drackley, 2010). Although pregnancy requirements increase exponentially during late gestation, energy intake is likely sufficient to sustain pregnancy until 1 wk prepartum (Grummer, 1995); on the contrary energy intake shortly after calving is not adequate to sustain milk production requirements. In fact, negative energy balance (NEB) is universal among dairy cows for a transient period of days around calving, and the extent of this condition may predispose cows to metabolic disorders. The latter is associated with a greater breach in energy balance between energy intake and increased energy requirements before and after calving for fetal growth and milk production, respectively. Consequently, NEB precipitated by the lactating mammary gland places a

large demand on the body and requires orchestrated changes to ensure that needs for milk production are met.

Canonical metabolism during the postpartum period

During extended episodes of NEB the rate of lipid esterification in adipose tissues is lower than the rate of lipolysis. During the postpartum period blood NEFA concentrations commonly increase, where NEFA are initially hydrolyzed from adipose tissue triglycerides (TAG) and then transported through the bloodstream to peripheral tissues for use as an energy source. However, the liver is the most important site for removal of NEFA from circulation (Bell, 1979). Extreme rates of lipid mobilization lead to increased uptake of NEFA by liver and increased TAG accumulation (Drackley, 1999). Once taken up by liver, NEFA have several fates including secretion in bile, oxidization to CO_2, partial oxidation to ketone bodies, storage in the liver as TAG, or secretion as lipoproteins (Emery et al., 1992). Biliary lipid secretion by ruminant liver is a minor source of fatty acid removal from the liver primarily as phosphatidylcholine for dietary FA absorption in the small intestine (Christie, 1978). Oxidation and partial oxidation are initiated by cytosolic NEFA being activated to fatty acyl-CoA by the enzyme acyl-CoA synthase. Translocation of the fatty acyl-CoA into the mitochondria matrix, where β-oxidation will occur, is carried out by a carnitine shuttle involving carnitine palmitoyltransferase I (CPT-I) and carnitine palmitoyltransferase II (CPT-II), where CPT-I converts fatty acyl-CoA into acylcarnitine that can be transported over the internal mitochondrial membrane and CPT-II, in the internal membrane, re-converts acylcarnitine into acyl-CoA (Ingvartsen, 2006). The product of β-oxidation of acyl-CoA is acetyl-CoA,

which can be further oxidized for energy in the citric acid (TCA) cycle. However, fatty liver can develop when the hepatic uptake of NEFA exceeds the oxidation and secretion of lipids by the liver (Bobe et al., 2004). Additionally, if the amount of acetyl-CoA generated via fatty acid β-oxidation overwhelms the capacity of the TCA cycle or if the normal activity of the TCA cycle is truncated due to lower amounts of intermediate components such as oxaloacetate, then acetyl-CoA will be used for biosynthesis of ketone bodies (Drackley, 1999).

Ketosis

Ketogenesis has been associated with the degree of NEFA infiltration (West, 1990). The ketone bodies acetoacetate (ACAC), β-hydroxybutyrate (BHBA), and acetone are then exported from the liver into the blood (Bell, 1979). If the rate of ketone body uptake by peripheral tissues is lower that the rate of hepatic ketone body production then an increase in the concentration of ketone bodies in blood will be observes, also known as hyperketonemia (Ingvartsen, 2006).

Fatty liver and lipotropic nutrients

Fate of newly synthesized TAG in the liver between secretion and storage has been discussed extensively (Grummer, 1993; Drackley, 1999; Ingvartsen, 2006) as a major determinant of the onset of fatty liver or "fat cow syndrome" (Bauchart et al., 1996; Hocquette and Bauchart, 1999; Bobe et al., 2004). Hepatic secretion of TAG in the form of lipoproteins, of which very low density lipoproteins (VLDL) constitutes the largest part (Ingvartsen, 2006), occurs in most species but it is well known that ruminants have a

small capacity to synthesize VLDL (Kleppe et al., 1988; Pullen et al., 1990). Perhaps this is related to a natural inadequacy of some the constituents of VLDL, such as TAG, cholesterol, phospholipids, and apolipoproteins. In fact, it was suggested (Marcos et al., 1990) that the latter is reduced during cases of fatty liver. Similarly, precursors of phospholipids, e.g., choline, have been identified as promoters of VLDL secretion in rats (Yao and Vance, 1990), and comparable effects have been suggested in dairy cattle (Grummer, 2008). Finally, apolipoproteins such as apolipoprotein B_{100} and apolipoprotein E play important roles in the nascent VLDL in terms of stabilization and the assembly/secretion cascade (Fazio and Yao, 1995; Bernabucci et al., 2004). In parallel to this finding, methionine (Met) supplementation has been observed to exert lipotropic effects in cows (Durand et al., 1992).

Peroxisomal β-oxidation

Besides mitochondrial oxidation of fatty acids, peroxisomal β-oxidation can account for up to 50% of initial β-oxidation in cows in contrast to 26% in rats (Grum et al., 1994). This pathway is similar to mitochondrial oxidation except the first step is catalyzed by acyl-CoA oxidase, producing hydrogen peroxide (H_2O_2) instead of NAD, and oxidation is not coupled to an electron transport chain so less ATP is produced (Drackley, 1999). Thus, peroxisomal β-oxidation may play an important role in processes such as oxidative stress, by promoting reactive oxygen metabolites (ROM) such as H_20_2 and further hepatic accumulation of ROM could lead to tissue damage and inflammation.

The immune system

The immune system of vertebrates consists of a number of organs and cell types that specialize in pathogen recognition and elimination as a defense mechanism (Perdigon et al., 1995). The immune system can be divided into two systems: 1) innate immunity and 2) adaptive immunity (Sordillo et al., 1997). Innate immunity is rapidly activated and is the primary immune defense in the early stages of an infection. Innate immunity is carried by leukocytes such as polymorphonuclear neutrophils (PMN) and macrophages among others (Paape et al., 1991). In contrast, the adaptive immune system relies on bone marrow-derived and thymus-derived lymphocytes (B cells and T cells, respectively) that generate distinct and unique receptors during development by rearrangement and rejoining of a relatively small number of genes in a 'combination gene' encoding the receptor. This ability to modify the immune response to substances encountered in multiple occasions is the basis for immunologic memory, one of the hallmarks distinguishing the adaptive from the innate immune system (Doan, 2013).

Polymorphonuclear neutrophils

Granulocytes, a subcategory of leukocytes, comprise PMN, eosinophils, and basophils based on differential cytoplasmic characteristics and cellular morphology. For example, PMN present azurophilic granulocytes and a multilobed nucleus (Goldsby et al., 2007). The PMN constitute 50% to 70% of circulating leukocytes. Before circulation, PMN are synthesized via hematopoiesis and matured in the bone marrow, a process that takes about ~14 d (Bainton et al., 1971). Once released in the peripheral blood PMN can circulate for 7 to 10 h before migrating into tissues undergoing inflammation or an acute

phase response, where PMN have a life span of only a few days (Paape et al., 2002; Goldsby et al., 2007). In response to an infection the bone marrow will release significant number of PMN (leukocytosis). Upon pathogen recognition PMN will eliminate the pathogen by phagocytosis, a process that involves engulfing and killing pathogens via production of ROM. Because ROM is non-specific to pathogens, they can also damage the host's cells tissues. Therefore, the life span of PMN is tightly regulated in order to minimize host tissue damage (Capuco et al., 1986).

Macrophages

After being synthesized in the bone marrow, promonocytes are released into the bloodstream where they differentiate in mature monocytes (MO). Once they migrate into tissues, MO differentiate into tissue-specific macrophages, for example intestinal macrophages in the gut, Kupffer cells in the liver, and osteoclasts in bone (Goldsby et al., 2007). Although macrophages can be activated by a variety of stimuli including cytokines from epithelium and white blood cells (Paape et al., 2000) the difference between activated and non-activated macrophages is substantial. For example, activated macrophages have greater phagocytic activity, increased ability to kill ingested microbes, increased secretion of inflammatory mediators, and expression of higher levels of class II MHC molecules, allowing them increased ability to activate T cells through the antigen-presenting process (Goldsby et al., 2007). Therefore, upon activation, macrophages will 1) detect and recognize non-specific foreign pathogens; 2) release cytokines that will initiate immune response as well as recruit PMN to the site of infection; 3) phagocytize and kill invading pathogens; and 4) serve as a bridge between the innate and adaptive

immune response through antigen presentation to activate T cells (Rainard and Riollet, 2006).

Lymphocytes

Lymphocytes constitute 20% to 40% of the body's white blood cells and 99% of the cells in the lymph (Goldsby et al., 2007). Lymphocytes are produced in bone marrow by white blood cells through hematopoiesis and become activated due to response to local antigenic stimulation (Sordillo and Streicher, 2002). They proliferate and recognize foreign antigens through membrane receptors. Lymphocytes consist of T and B lymphocytes (Sordillo et al., 1997). 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 central molecules of the specific immunity that have a short half-life and that produce and secrete antibodies.

Infection and the innate immune response

In general, an acute inflammatory response has a rapid onset and lasts a short period of time, which is generally followed by a systemic reaction known as the acute-phase response. When pathogens trespass natural barriers such as skin and mucous membranes, the injured tissue will initiate a cascade of events known as the inflammatory response. Within minutes the damaged tissue will increase the vascular diameter (vasodilation),

consequently increasing the blood flow to the area. This, coupled with the release of chemotactic factors, will attract leukocytes to the site of infection. It is likely that a small number of tissue macrophages in the vicinity will arrive first. Macrophages will be activated by gamma interferon (released from T-helper cells), antigens produced via phagocytosis, or by components of bacterial cell walls such as lipopolysaccharide (LPS) or lipoteichoic acid (LTA) on Gram-negative and Gram-positive bacteria, respectively (Schroder et al., 2003). Activated macrophages release a broad spectrum of mediators in which cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF) play an important role recruiting additional immune cells (Baumann and Gauldie, 1994). Consequently, these cytokines will induce the expression of glycoproteins such as E-selectin and P-selectin in the tissue endothelial cells. Then, the circulating neutrophils express mucins such as PSGL-1 that will bind to either E- and P-selectin. Following this fundamental PMN-endothelial interaction during the inflammatory response, the PMN will adhere to endothelial cells in the inflamed region and pass through the walls of capillaries into the tissue spaces, through a process call extravasation. Neutrophil extravasation can be divided into four steps: 1) rolling, 2) activation by chemoattractant stimulus, 3) arrest and adhesion, and 4) trans-endothelial migration. Rolling is initiated during PMN-endothelial low affinity interactions between glycoproteins and mucins. As the PMN rolls over the inflamed region it will encounter chemoattractants and chemokines such as IL-8 that in turn will trigger the G-protein-mediated activating signal that leads to a conformational change in the integrin adhesion molecules, resulting in neutrophil strong affinity adhesion (between β2 integrins and ICAMs from PMN and endothelium, respectively) and subsequent transendothelial migration (Goldsby et al.,

2007). Once the endothelial barrier has been traversed, the PMN submerge into a complex soup of chemoattractants and inflammatory stimulants, both host derived and of pathogenic origin. In the interstitial space, the PMN follows chemotactic gradients toward the invading microbes. During this process several chemokines (e.g IL-8), pathogen-derived chemoattractants (e.g. fMLP), and specific pathogen-recognition receptors (e.g. TLRs) will further activate the PMN oxidative burst machinery and enhance effective functional responses in PMN (Amulic et al., 2012). Upon finally reaching a point of highest chemoattractant concentration, the neutrophil halts and begins the final release of its antimicrobial arsenal; the neutrophil is now fully functional. At this stage the primary killing functions are phagocytosis, degranulation, NETosis (Neutrophils extracellular traps; (Amulic et al., 2012). The deployment of PMN is tightly regulated since PMNs not only are destructive toward invading microbes but also towards host cells. Therefore, resolution of inflammation is an active process that limits further leukocyte infiltration and removes apoptotic cells from inflamed sites. The latter is carried out mainly by macrophages that upon phagocytosis of apoptotic PMN will be re-programmed to adopt an anti-inflammatory phenotype. This process is essential for maintenance of tissue homeostasis and, if impeded, leads to unresolved inflammation, a problematic condition that contributes to many diseases (Amulic et al., 2012).

Changes in immunometabolic and innate immune response

The inflammatory response provides early protection following infection or tissue injury by restricting the tissue damage to the affected site. The inflammatory response involves both localized and systemic responses. Here, the mechanism by which the

systemic response interacts with the innate immune system will be discussed with a focus on the transition period. The most common phenomena during a systemic response, also known as acute phase response, include fever, leukocytosis, and the over- or under-expression of a large family of structurally un-related proteins, the acute phase proteins (APP) (Ceciliani et al., 2012). The liver is a central organ during an acute phase response in the organism. It is responsible for determining the level of essential metabolites during the critical stages of stress. The APP posses a pathogen pattern recognition capacity and their main role is the activation of the complement system cascade that concludes in the pathogen destruction (Goldsby et al., 2007). Most of the APP concentration 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 (SAA), α2-macroglobulin (A2M) and α1-cysteine proteinase inhibitor (CPI). Oppositely, the concentration of some APP decrease (negative APP) during inflammation; these proteins include albumin (BSA) and transferrin (Koj et al., 1988; Bertoni et al., 2008). As mentioned above tissue macrophages will release the first wave of cytokines that includes IL-1 and TNFα and a small amount of IL-6. Absorption of the first wave of cytokines into surrounding cells is followed by a second wave of cytokine release, including a large amount of IL-6 that promotes massive production of APP by hepatocytes (Parker and Picut, 2005). Additionally, Mackiewicz (1992) proposed other mediators of APP production, including IL-1, insulin-like growth factors (IGF), and glucocorticoids. In fact, these mediators can be grouped into four major categories: IL-6 type cytokines, IL-1 type cytokines, glucocorticoids, and growth factors (Baumann and

Gauldie, 1994). Inteleukin-6 type cytokines include IL-6, interleukin-11, leukemia inhibitor factor (LIF), oncostatin M (OSM) and ciliary neutrophic factor (CNTF); these groups, in particular IL-6, have been recognized to be the principal regulator of most APP including HP, FBG, API and A2M (Mackiewicz, 1992). The IL-1 type cytokines (i.e. IL-1α, IL-1β, TNFα and TNFβ) regulate the 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 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).

The periparturient cow is regarded as a immune-compromised animal, characterized by inflammatory conditions such as infectious diseases and metabolic disorders that often result in proinflammatory cytokine release, e.g. IL-1, IL-6, and TNFα (Bertoni et al., 2008). In turn, any increased amount and time of exposure to these proinflammatory cytokines will likely induce the hepatic production of positive APP (e.g. haptoglobin and ceruloplasmin) or negative APP (e.g. albumin, retinol binding protein, apolipoproteins) (Drackley et al., 2005; Bionaz et al., 2007; Bertoni et al., 2008). 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., 2007). In fact, the most sensitive acute phase proteins are HP and SAA, the concentration of which in serum can increase over 100-fold in response to acute inflammation. Also, a moderate APP in cattle is AGP, which has a lower relative increase indicating chronic conditions (Pyorala, 2003).

Haptoglobin is mostly produced in the liver and has been identified as a hemoglobin binding protein, which essentially prevents hemoglobin-driven oxidative tissue damage during intravascular hemolysis due to iron stabilization of the hemoglobin and preventing dissociation of ferric heme from globin (Melamed-Frank et al., 2001). In the same context, haptoglobin can exert an anti-inflammatory function through the haptoglobin-hemoglobin complex by binding to monocytes/macrophages, inducing the release of anti-inflammatory mediators such as in IL-10 (Ceciliani et al., 2012) . Additionally, haptoglobin can down-regulate the activity of PMN by inhibiting both lipoxygenase and cycloxygenase (Saeed et al., 2007). Haptoglobin concentration in healthy bovine plasma is commonly under 0.1 g/L; in contrast, during the onset of acute phase response, the concentration of haptoglobin increased to 1.62 ± 0.47 g/L (Ceciliani et al., 2012) . Also, within the same range Trevisi et al. (2010) observed concentrations of haptoglobin of 0.14 ± 0.1 and 0.75 ± 0.6 for pre-calving and post-calving, respectively, for cows with lower liver functionality based on inflammatory and immune parameters in blood and milk. Therefore, haptoglobin is an important APP during peripeturient period.

The SAA is so named due to its involvement in reactive amyloidosis (Ceciliani et al., 2012) , which is associated with the accumulation of insoluble proteins sharing structural traits, usually inappropriate protein folding. They have been classified as acute phase SAA (A-SAA) and the constitutive SAA (C-SAA). Three genes, i.e. *SAA1*, *SAA2*, and *SAA3* encode A-SAA, while *SAA4* encodes for C-SAA. During the induction of acute phase response by pro-inflammatory cytokines such as IL6 and TNFα the liver produces SAA1 and SAA2 (Uhlar and Whitehead, 1999). Although other biological functions of SAA are still to be uncovered, at least three main functions have been explored,

encompassing binding of cholesterol, immunomodulatory activity and opsonization. From a structural standpoint SAA is an apolipoprotein, thus it can replace ApoA1 in HDL after being synthesized (A-SAA) during an acute phase response, resulting in the uptake and removal of cholesterol from the inflammatory site (Coetzee et al., 1986). Enhancing cholesterol binding is especially important to reduce the accumulation of atheriosclerotic plaques from dying cells (Manley et al., 2006). Additionally, SAA has been associated to exert effects on immune cells such as neutrophils and monocytes by acting as a chemoattractant and mediating migration, adhesion and tissue infiltration (Badolato et al., 1994). Human SAA can act as a pathogenic recognition protein by opsonizing a wide range of gram-negative bacteria (Ceciliani et al., 2012) . Unlike human, the range of bovine SAA activity is probably wider, since it is directed toward both gram-negative and gram-positive bacteria. Under healthy conditions SAA concentration in bovine blood has been reported to be around 1.3 ± 0.4 mg/L, which can increase to 115 ± 37 mg/L under acute phase conditions (Ceciliani et al., 2012) . Comparably, Meglia et al. (2005) observed an increase from 2.4 ± 1.4 mg/L at -10 to -7 day prepartum to 77.5 ± 1.3 mg/L between 0 and 3 days postpartum. Furthermore, cows in early lactation that had been diagnosed with left or right displaced abomasum had an increase of 43 mg/L and 78 mg/L in SAA compared to healthy cows (Guzelbektes et al., 2010). Besides Hp and SAA, several other positive acute phase response proteins such as AGP, LBP, and ceruloplasmin have been studied in order to extrapolate their known function to the context of the periparturient cow.

Nutritional management of the periparturient cow

Previous reviews (Drackley, 1999; Bobe et al., 2004; Ingvartsen, 2006) have provided comprehensive and insightful knowledge not only about the condition of cows undergoing the transition from pregnant to early lactation but also suggestions on what nutritional management approaches can be taken to minimize the incidence of metabolic and reproductive related disorders or diseases postpartum. One of the main conclusions pointed out by Drackley (1999) is that prepartum intake has a major effect on postpartum DMI and periparturient lipid metabolism, that in turn have a direct influence on DMI and incidence of health disorders. Later, in the same context, Ingvartsen (2006) suggested that because of the link between body fatness at calving and subsequent degree of lipid mobilization, avoiding prepartum over-conditioning of cows will eventually decrease the risk of developing health disorders. However, other factors such as hormonal changes and incidence of infections during parturition can increase mobilization of NEFA from adipose tissue (Goff and Horst, 1997). Therefore, besides manipulation of energy intake prepartum in order to control body-fatness, additionally interest has been directed to enhancing clearance of TAG from liver via VLDL synthesis and export into bloodstream (Bobe et al., 2004). Thus, lipotropic factors such as certain amino acids (AA), propylene glycol, niacin, and carnitine have received attention by ruminant researchers and nutritionists (Bauchart et al., 1998). Among the AA, the sulfur-containing Met has been reported to have beneficial effects in dairy cow performance, especially milk fat and protein production (St-Pierre and Sylvester, 2005; Ordway et al., 2009). Most of these effects have been associated with stimulation of synthesis and secretion of VLDL particles rich in TAG by the liver (Bauchart et al., 1998). Although Met has been reported

to have an effect on dairy cow performance, the extent of the effect of Met during the periparturient period has been evaluated in relatively few experiments (Phillips et al., 2003; Socha et al., 2005; Johnson-VanWieringen et al., 2007; Ordway et al., 2009).

Amino acid balance for optimal metabolizable protein

Commonly dietary protein is divided into dietary protein metabolized in the rumen, called rumen degradable protein (RDP), and rumen undegradable protein (RUP), which is dietary protein that bypasses the rumen. After being metabolized in the rumen, RDP can become microbial protein and is later digested in the small intestine. In contrast, RUP is readily digested in the small intestine without making further changes to its AA profile (NRC, 2001).

The importance of balancing AA has been denoted by Schwab (2012) to include the following: 1) AA are the building blocks of protein synthesis; 2) the ideal profile of essential AA (EAA) may differ among physiological states (e.g. maintenance, growth, pregnancy and milk production) and even at different genetic merits (e.g. high vs low milk production cows); and 3) providing a more balanced profile of absorbable EAA allows meeting AA requirements with less dietary protein. Lysine and Met have been frequently used to balance the profile of AA as they have been identified as the two most limiting AA for lactating dairy cows in North America (NRC, 2001). Thus, by supplementing the most limiting AA such as Lys and Met, AA requirements are being met with lower concentrations of dietary protein. The NRC (2001) suggests that the required amounts of Lys and Met in metabolizable protein for optimal milk protein

production are 7.2% and 2.4%, respectively. Thus, when balancing diets it is important to maintain a 3:1 ratio of Lys and Met.

Rumen-protected methionine

Although a considerable amount of rumen microbial protein reaches the small intestine where it is digested and utilized as metabolizable protein, the AA profile within microbial protein might not allow cows to reach optimal performance. Therefore, providing an RUP source with optimal balance of AA to escape the rumen and consequently be utilized as metabolizable protein could enhance the performance in dairy cows. A considerable amount of research has been done in promoting a more efficient RUP in terms of AA profile (NRC, 2001). Diets where supplemental RUP was provided as soybean products, animal-derived proteins, or a combination of the two have been observed to be most limiting in Met (Armentano et al., 1997; Rulquin and Delaby, 1997). Current dairy systems generally supply RUP through soybean products and animal-derived proteins, thus increasing the importance of supplemental rumen-protected Met because of lower contents of that AA. Besides, physical-chemical properties of Lys are such that applications of most rumen protection technologies have been challenging (NRC, 2001).

According to the NRC (2001) the methods for protection of AA from ruminal degradation include 1) surface coating with fatty acid/pH-sensitive polymer mixture, 2) surface coating or matrices involving fat or saturated fatty acids and minerals, and 3) liquid sources of Met hydroxy analog (DL-2-hydroxy-4-methylthiobutanoic acid; HMB). Recently an isopropyl ester of HMB (HMBi) has been evaluated (Graulet et al., 2005).

For the purpose of this document we will focused on the pH-sensitive polymer mixture and HMBi. Due to the context of our research we will discuss the pH-sensitive polymer mixture and hydroxyl analog as they pertain to Smartamine and MetaSmart, respectively (Adisseo, Alpharetta, GA).

pH-sensitive polymer mixture

As a postruminal delivery system this method is independent of digestive enzyme function but is dependent on the differences in pH between the rumen and abomasum. This technology provides characteristics of a ruminally inert product, while providing high intestinal (~80% bioavailability) release of the coated AA (NRC, 2001; Schwab, 2007). Studies have shown that these forms of rumen-protected Met were effective for increasing both milk and milk component yields (Armentano et al., 1997; Rulquin and Delaby, 1997).

Methionine hydroxy analog

Esterification of HMB with isopropanol decreases the extent of its ruminal breakdown (Robert et al., 2001). In fact, Graulet et al. (2005) observed that as much as 50% of orally fed HMBi was absorbed across the rumen wall, with the remaining 50% being metabolized by rumen microbes, which are eventually utilized as microbial protein. Thus, HMBi offers the potential to both stimulate ruminal microbial activity and supply metabolizable Met (Chen et al., 2011). Therefore, HMBi has been proposed to improve three aspects of production: milk volume, milk fat percentage, and milk protein percentage, by acting to increase microbial protein and increase the supply of

metabolizable Met. Greater milk yield response in postpartal dairy cows supplemented with HMBi has been previously reported (St-Pierre and Sylvester, 2005). Similarly, secretions of milk fat and protein have been improved when supplementing cows with HMBi (Rulquin et al., 2006; Chen et al., 2011).

Transcriptomics in bovine hepatic and immune cells

Development of high-throughput sequencing and transcriptomics technologies has dramatically accelerated the rate at which biological and genetic information can be gathered (Loor, 2010). These technologies allow study of genes from a cell or tissue, not only at the mRNA (transcriptome) level but also at a DNA (genotype) or protein (proteome) level. Microarray technology, in particular, is a powerful tool for the simultaneous analysis of the expression of thousands of genes in tissues, organs or cells. The University of Illinois developed a 13,257 oligonucleotide bovine microarray, which essentially represented an expansion of the original 7,000 cDNA microarray platform (Everts et al., 2005). Details of the development of the microarray platform used can be found in the Supplementary Materials and Methods from Loor et al. (2007). Functional genomics, generally defined as the study of the transcriptome, uses the expression profiling of mRNA to provide a condition-specific and time-specific genome-scale snapshot of the transcriptome (Schoolnik, 2002).

The liver, as a central organ that orchestrates metabolism and other functions, has been most studied in terms of defining changes in mRNA expression of genes encoding proteins that participate in various aspects of lipoprotein assembly (Bernabucci et al., 2004), ketogenesis (Loor et al., 2007), growth hormone signaling (Rhoads et al., 2004),

gluconeogenesis (Rhoads et al., 2004), and ureagenesis (Hartwell et al., 2001). Different studies have explored and linked large-scale liver (Loor et al., 2005; Loor et al., 2006; Loor et al., 2007) 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 information (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). Similarly, other studies have used the periparturient period or induction of negative energy balance to study the transcriptome of bovine PMN and its adaptations during these stressed periods (Madsen et al., 2004; Wang et al., 2009; Moyes et al., 2010). In fact, Madsen et al. (2004) observed a decrease in the expression of genes associated with apoptosis, oxidative stress responses, and pro-inflammatory genes. Evaluation of NEB effects on PMN function revealed a down-regulation of genes associated with antigen presentation, respiratory burst, and cytokine secretion, but an up-regulation of TLR signaling (Moyes et al., 2010). Additionally, Burton et al. (2005) proposed that changes of hormones such a glucocorticoids during calving can trigger substantial alterations in the expression of a large number of genes that cluster into ~20 ontological categories and that primarily induce rapid translocation of glucocorticoids receptors into the nucleus of the PMN, inducing potent signals that delay apoptosis, activate immune response, and promote tissue remodeling.

Dynamic impact approach (DIA): A new approach for functional analysis of microarray datasets with time-course and multiple-treatments

Enrichment analysis, also called the overrepresented approach (ORA), has been commonly used as the gold standard for high-throughput datasets (Huang et al., 2009). Biological functions represented through Gene Ontology (GO; http://www.geneontology.org/) annotations have been used in ORA analysis to investigate whether gene sets are statistically overrepresented or enriched in the identified gene groups within a biological term. Consequently, the enrichment of genes of a particular biological term is a strong indicator of the modulation of the functions associated with the biological term within the cell in a non-random fashion (Bionaz et al., 2012). Furthermore, this particular biological term is functionally relevant under the conditions studied. Although ORA can provide quick and reliable information regarding important biological terms in a list of annotated genes/proteins, this approach has some limitations, including the inability to compare results from multiple gene lists particularly with time-course experiments or those involving multiple treatments. To overcome such limitations, a novel method termed DIA has been proposed (Bionaz et al., 2012). The DIA provides an estimate of the biological impact of the experimental conditions and the direction of the impact. Biological impact is a function of the number of differentially expressed genes (DEG) with log2 mean fold change and mean –log P-value from all the genes associated with the biological term. The direction of the impact is calculated as the difference of the impact of the up-regulated DEG and down-regulated DEG associated with the biological term. Overall, DIA represents an alternative to ORA for functional

analysis of time-course experiments and those involving multiple treatments (Bionaz et al., 2012).

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 and render cows to a state of immunosuppression that leads to increased susceptibility to metabolic disorders (Drackley, 1999; Ingvartsen, 2006). 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). Therefore, it is important to avoid overconditioning of cows during gestation, which will eventually reduce excessive lipid mobilization after calving (Ingvartsen, 2006). However, as NEB postpartum is universal for cows, in the same way lipid mobilization is ubiquitous during this time. In this context, it is possible that small alterations in management or complications at calving might trigger consecutive effects of hormonal and metabolic alterations that eventually predispose cows to infectious diseases and metabolic disorders.

Methionine supplementation of cows during lactation has a consistent effect in improving cow performance (Armentano et al., 1997; Rulquin and Delaby, 1997; Chen et al., 2011). Although similar effects have been observed when supplementing Met to peripartal cows (Phillips et al., 2003; Socha et al., 2005; Ordway et al., 2009), there is still a shortage of data in this regard. Rumen-protected Met is expected to spare EAA

once metabolized; consequently it can serve as a methyl donor or a backbone for gluconeogenesis, and also can stimulate the clearance of TAG from liver via assembly and secretion of VLDL. Taken together, it seems likely that liver functionality in peripartal cows supplemented with Met may be increased after calving. Furthermore, Met may have important implications in terms of acute phase response. In fact, cows with high liver functionality also have high levels of immune markers and low levels for inflammatory markers even before calving (Trevisi et al., 2010). Additionally, as the AA pool is spared by Met supplementation, the concentration of certain AA such as cysteine can increase, which subsequently should increase endogenous antioxidants such as glutathione.

Our general hypothesis was that supplementing rumen-protected Met to peripartal dairy cows will improve DMI, milk yield and components, and energy balance by enhancing liver function and antioxidant capacity, and decreasing the inflammatory response, and that in turn these effects are controlled at the molecular level by cross-talk of gene expression. The overall objective of this dissertation was to evaluate the performance parameters, blood and liver biomarkers, and hepatic transcriptomics alterations on diary cows supplemented with Met in the forms of Smartamine (SM) or MetaSmart (MS) during the peripartal period.

REFERENCES

Amulic, B., C. Cazalet, G. L. Hayes, K. D. Metzler, and A. Zychlinsky. 2012. Neutrophil function: from mechanisms to disease. Annu Rev Immunol. 30:459-489.

Armentano, L. E., S. J. Bertics, and G. A. Ducharme. 1997. Response of lactating cows to methionine or methionine plus lysine added to high protein diets based on alfalfa and heated soybeans. J Dairy Sci. 80:1194-1199.

Badolato, R., J. M. Wang, W. J. Murphy, A. R. Lloyd, D. F. Michiel, L. L. Bausserman, D. J. Kelvin, and J. J. Oppenheim. 1994. Serum amyloid A is a chemoattractant: induction of migration, adhesion, and tissue infiltration of monocytes and polymorphonuclear leukocytes. J Exp Med. 180:203-209.

Bainton, D. F., J. L. Ullyot, and M. G. Farquhar. 1971. The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. J Exp Med. 134:907-934.

Bauchart, D., D. Durand, D. Gruffat, and Y. Chilliard. 1998. Mechanism of liver steatosis in early lactation cows: effects of hepatoprotector agents. Pages 27-37 in Proc: Cornell Nutr. Conf. Feed Manuf.

Bauchart, D., D. Gruffat, and D. Durand. 1996. Lipid absorption and hepatic metabolism in ruminants. Proc Nutr Soc. 55:39-47.

Bauman, D. E. and W. B. Currie. 1980. Partitioning of nutrients during pregnancy and lactation: a review of mechanisms involving homeostasis and homeorhesis. J Dairy Sci. 63:1514-1529.

Baumann, H. and J. Gauldie. 1994. The acute phase response. Immunol Today. 15:74-80.

Beever, D. E. 2006. The impact of controlled nutrition during the dry period on dairy cow health, fertility and performance. Anim Reprod Sci. 96:212-226.

Bell, A. W. 1979. Lipid metabolism in liver and selected tissues and in the whole body of ruminant animals. Prog Lipid Res. 18:117-164.

Bell, A. W. 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. J Anim Sci. 73:2804-2819.

Bernabucci, U., B. Ronchi, L. Basirico, D. Pirazzi, F. Rueca, N. Lacetera, and A. Nardone. 2004. Abundance of mRNA of apolipoprotein b100, apolipoprotein e, and microsomal triglyceride transfer protein in liver from periparturient dairy cows. J Dairy Sci. 87:2881-2888.

Bertoni, G., E. Trevisi, X. Han, and M. Bionaz. 2008. Effects of inflammatory conditions on liver activity in puerperium period and consequences for performance in dairy cows. J Dairy Sci. 91:3300-3310.

Bionaz, M., K. Periasamy, S. L. Rodriguez-Zas, W. L. Hurley, and J. J. Loor. 2012. A novel dynamic impact approach (DIA) for functional analysis of time-course omics studies: validation using the bovine mammary transcriptome. PLoS One. 7:e32455.

Bionaz, M., E. Trevisi, L. Calamari, F. Librandi, A. Ferrari, and G. Bertoni. 2007. Plasma paraoxonase, health, inflammatory conditions, and liver function in transition dairy cows. J Dairy Sci. 90:1740-1750.

Bobe, G., J. W. Young, and D. C. Beitz. 2004. Invited review: pathology, etiology, prevention, and treatment of fatty liver in dairy cows. J Dairy Sci. 87:3105-3124.

Burton, J. L., S. A. Madsen, L. C. Chang, P. S. Weber, K. R. Buckham, R. van Dorp, M. C. Hickey, and B. Earley. 2005. Gene expression signatures in neutrophils exposed to glucocorticoids: a new paradigm to help explain "neutrophil dysfunction" in parturient dairy cows. Vet Immunol Immunopathol. 105:197-219.

Capuco, A. V., M. J. Paape, and S. C. Nickerson. 1986. In vitro study of polymorphonuclear leukocyte damage to mammary tissues of lactating cows. Am J Vet Res. 47:663-668.

Ceciliani, F., J. J. Ceron, P. D. Eckersall, and H. Sauerwein. 2012. Acute phase proteins in ruminants. J Proteomics. 75:4207-4231.

Chen, Z. H., G. A. Broderick, N. D. Luchini, B. K. Sloan, and E. Devillard. 2011. Effect of feeding different sources of rumen-protected methionine on milk production and N-utilization in lactating dairy cows. J Dairy Sci. 94:1978-1988.

Christie, W. W. 1978. The composition, structure and function of lipids in the tissues of ruminant animals. Prog Lipid Res. 17:111-205.

Coetzee, G. A., A. F. Strachan, D. R. van der Westhuyzen, H. C. Hoppe, M. S. Jeenah, and F. C. de Beer. 1986. Serum amyloid A-containing human high density lipoprotein 3. Density, size, and apolipoprotein composition. J Biol Chem. 261:9644-9651.

Dann, H. M., N. B. Litherland, J. P. Underwood, M. Bionaz, A. D'Angelo, J. W. McFadden, and J. K. Drackley. 2006. Diets during far-off and close-up dry periods affect periparturient metabolism and lactation in multiparous cows. J Dairy Sci. 89:3563-3577.

Doan, T. 2013. Immunology. 2nd ed. Lippincott's illustrated reviews. Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia.

Drackley, J. K. 1999. ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: the final frontier? J Dairy Sci. 82:2259-2273.

Drackley, J. K., H. M. Dann, G. N. Douglas, N. A. Guretzky, N. B. Litherland, J. P. Underwood, and J. J. Loor. 2005. Physiological and pathological adaptations in dairy cows that may increase susceptibility to periparturient diseases and disorders. Ital J Anim Sci. 4:323-344.

Drackley, J. K., S. S. Donkin, and C. K. Reynolds. 2006. Major advances in fundamental dairy cattle nutrition. J Dairy Sci. 89:1324-1336.

Drackley, J. K., T. R. Overton, and G. N. Douglas. 2001. Adaptations of Glucose and Long-Chain Fatty Acid Metabolism in Liver of Dairy Cows during the Periparturient Period. Journal of dairy science. 84:E100-E112.

Durand, D., Y. Chilliard, and D. Bauchart. 1992. Effect of lysine and methionine on in vivo hepatic secretion of VLDL in dairy cow. J Dairy Sci. 75:279.

Emery, R. S., J. S. Liesman, and T. H. Herdt. 1992. Metabolism of long chain fatty acids by ruminant liver. J Nutr. 122:832-837.

Everts, R. E., M. R. Band, Z. L. Liu, C. G. Kumar, L. Liu, J. J. Loor, R. Oliveira, and H. A. Lewin. 2005. A 7872 cDNA microarray and its use in bovine functional genomics. Vet Immunol Immunopathol. 105:235-245.

Fazio, S. and Z. Yao. 1995. The enhanced association of apolipoprotein E with apolipoprotein B-containing lipoproteins in serum-stimulated hepatocytes occurs intracellularly. Arterioscler Thromb Vasc Biol. 15:593-600.

Goff, J. P. 2006. Major advances in our understanding of nutritional influences on bovine health. J Dairy Sci. 89:1292-1301.

Goff, J. P. and R. L. Horst. 1997. Physiological changes at parturition and their relationship to metabolic disorders. J Dairy Sci. 80:1260-1268.

Goldsby, R. A., R. J. Kindt, and B. A. Osborne. 2007. Kuby Immunology. 4th Ed ed. W. H. Freeman and Co, New York, NY.

Graulet, B., C. Richard, and J. C. Robert. 2005. Methionine availability in plasma of dairy cows supplemented with methionine hydroxy analog isopropyl ester. J Dairy Sci. 88:3640-3649.

Grum, D. E., L. R. Hansen, and J. K. Drackley. 1994. Peroxisomal beta-oxidation of fatty acids in bovine and rat liver. Comp Biochem Physiol B Biochem Mol Biol. 109:281-292.

Grummer, R. R. 1993. Etiology of lipid-related metabolic disorders in periparturient dairy cows. J Dairy Sci. 76:3882-3896.

Grummer, R. R. 1995. Impact of changes in organic nutrient metabolism on feeding the transition dairy cow. J Anim Sci. 73:2820-2833.

Grummer, R. R. 2008. Nutritional and management strategies for the prevention of fatty liver in dairy cattle. Vet J. 176:10-20.

Gundelach, Y., K. Essmeyer, M. K. Teltscher, and M. Hoedemaker. 2009. Risk factors for perinatal mortality in dairy cattle: cow and foetal factors, calving process. Theriogenology. 71:901-909.

Guzelbektes, H., I. Sen, M. Ok, P. D. Constable, M. Boydak, and A. Coskun. 2010. Serum amyloid A and haptoglobin concentrations and liver fat percentage in lactating dairy cows with abomasal displacement. J Vet Intern Med. 24:213-219.

Hartwell, J. R., M. J. Cecava, and S. S. Donkin. 2001. Rumen undegradable protein, rumen-protected choline and mRNA expression for enzymes in gluconeogenesis and ureagenesis in periparturient dairy cows. J Dairy Sci. 84:490-497.

Hocquette, J. F. and D. Bauchart. 1999. Intestinal absorption, blood transport and hepatic and muscle metabolism of fatty acids in preruminant and ruminant animals. Reprod Nutr Dev. 39:27-48.

Huang, D. W., B. T. Sherman, and R. A. Lempicki. 2009. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Research. 37:1-13.

Ingvartsen, K. L. 2006. Feeding- and management-related diseases in the transition cow. Anim Feed Sci and Tech. 126:175-213.

Ingvartsen, K. L. and J. B. Andersen. 2000. Integration of metabolism and intake regulation: a review focusing on periparturient animals. J Dairy Sci. 83:1573-1597.

Janovick, N. A. and J. K. Drackley. 2010. Prepartum dietary management of energy intake affects postpartum intake and lactation performance by primiparous and multiparous Holstein cows. J Dairy Sci. 93:3086-3102.

Johnson-VanWieringen, L. M., J. H. Harrison, D. Davidson, M. L. Swift, M. A. von Keyserlingk, M. Vazquez-Anon, D. Wright, and W. Chalupa. 2007. Effects of rumen-undegradable protein sources and supplemental 2-hydroxy-4-(methylthio)-butanoic acid and lysine-HCl on lactation performance in dairy cows. J Dairy Sci. 90:5176-5188.

Kleppe, B. B., R. J. Aiello, R. R. Grummer, and L. E. Armentano. 1988. Triglyceride accumulation and very low density lipoprotein secretion by rat and goat hepatocytes in vitro. J Dairy Sci. 71:1813-1822.

Koj, A., D. Magielska-Zero, J. Bereta, A. Kurdowska, H. Rokita, and J. Gauldie. 1988. The cascade of inflammatory cytokines regulating synthesis of acute phase proteins. Tokai J Exp Clin Med. 13:255-264.

Loor, J. J. 2010. Genomics of metabolic adaptations in the peripartal cow. Animal. 4:1110-1139.

Loor, J. J., H. M. Dann, R. E. Everts, R. Oliveira, C. A. Green, N. A. Guretzky, S. L. Rodriguez-Zas, H. A. Lewin, and J. K. Drackley. 2005. Temporal gene expression profiling of liver from periparturient dairy cows reveals complex adaptive mechanisms in hepatic function. Physiol Genomics. 23:217-226.

Loor, J. J., H. M. Dann, N. A. Guretzky, R. E. Everts, R. Oliveira, C. A. Green, N. B. Litherland, S. L. Rodriguez-Zas, H. A. Lewin, and J. K. Drackley. 2006. Plane of nutrition prepartum alters hepatic gene expression and function in dairy cows as assessed by longitudinal transcript and metabolic profiling. Physiol Genomics. 27:29-41.

Loor, J. J., R. E. Everts, M. Bionaz, H. M. Dann, D. E. Morin, R. Oliveira, S. L. Rodriguez-Zas, J. K. Drackley, and H. A. Lewin. 2007. Nutrition-induced ketosis alters metabolic and signaling gene networks in liver of periparturient dairy cows. Physiol Genomics. 32:105-116.

Mackiewicz, A. 1992. Cytokine, cytokine receptors and glucocorticoid network controlling N-glycosylation of acute phase proteins in vitro. Folia Histochem Cytobiol. 30:165-166.

Mackiewicz, A., M. K. Ganapathi, D. Schultz, A. Brabenec, J. Weinstein, M. F. Kelley, and I. Kushner. 1990. Transforming growth factor beta 1 regulates production of acute-phase proteins. Proc Natl Acad Sci U S A. 87:1491-1495.

Madsen, S. A., L. C. Chang, M. C. Hickey, G. J. Rosa, P. M. Coussens, and J. L. Burton. 2004. Microarray analysis of gene expression in blood neutrophils of parturient cows. Physiol Genomics. 16:212-221.

Manley, P. N., J. B. Ancsin, and R. Kisilevsky. 2006. Rapid recycling of cholesterol: the joint biologic role of C-reactive protein and serum amyloid A. Med Hypotheses. 66:784-792.

Marcos, E., A. Mazur, P. Cardot, and Y. Rayssiguier. 1990. Serum apolipoproteins B and A-I and naturally occurring fatty liver in dairy cows. Lipids. 25:575-577.

Meglia, G. E., A. Johannisson, S. Agenas, K. Holtenius, and K. P. Waller. 2005. Effects of feeding intensity during the dry period on leukocyte and lymphocyte sub-populations, neutrophil function and health in periparturient dairy cows. Vet J. 169:376-384.

Melamed-Frank, M., O. Lache, B. I. Enav, T. Szafranek, N. S. Levy, R. M. Ricklis, and A. P. Levy. 2001. Structure-function analysis of the antioxidant properties of haptoglobin. Blood. 98:3693-3698.

Moyes, K. M., J. K. Drackley, D. E. Morin, and J. J. Loor. 2010. Greater expression of TLR2, TLR4, and IL6 due to negative energy balance is associated with lower expression of HLA-DRA and HLA-A in bovine blood neutrophils after intramammary mastitis challenge with Streptococcus uberis. Funct Integr Genomics. 10:53-61.

NRC. 2001. Nutrient Requirements of Dairy Cattle. Vol. 7th. Natl. Acad. Press, Washington, DC.

Ordway, R. S., S. E. Boucher, N. L. Whitehouse, C. G. Schwab, and B. K. Sloan. 2009. Effects of providing two forms of supplemental methionine to periparturient Holstein dairy cows on feed intake and lactational performance. J Dairy Sci. 92:5154-5166.

Paape, M., J. Mehrzad, X. Zhao, J. Detilleux, and C. Burvenich. 2002. Defense of the bovine mammary gland by polymorphonuclear neutrophil leukocytes. J Mammary Gland Biol Neoplasia. 7:109-121.

Paape, M. J., R. H. Miller, and G. Ziv. 1991. Pharmacologic enhancement or suppression of phagocytosis by bovine neutrophils. Am J Vet Res. 52:363-366.

Paape, M. J., K. Shafer-Weaver, A. V. Capuco, K. Van Oostveldt, and C. Burvenich. 2000. Immune surveillance of mammary tissue by phagocytic cells. Adv Exp Med Biol. 480:259-277.

Paolucci, M., L. Sylla, A. Di Giambattista, C. Palombi, A. Elad, G. Stradaioli, P. Pascolo, and M. Monaci. 2010. Improving calving management to further enhance reproductive performance in dairy cattle. Vet Res Commun. 34 Suppl 1:S37-40.

Parker, G. A. and C. A. Picut. 2005. Liver immunobiology. Toxicol Pathol. 33:52-62.

Perdigon, G., S. Alvarez, M. Rachid, G. Aguero, and N. Gobbato. 1995. Symposium - Probiotic Bacteria for Humans - Clinical-Systems for Evaluation of Effectiveness - Immune-System Stimulation by Probiotics. J Dairy Sci. 78:1597-1606.

Phillips, G. J., T. L. Citron, J. S. Sage, K. A. Cummins, M. J. Cecava, and J. P. McNamara. 2003. Adaptations in body muscle and fat in transition dairy cattle fed differing amounts of protein and methionine hydroxy analog. J Dairy Sci. 86:3634-3647.

Pullen, D. L., J. S. Liesman, and R. S. Emery. 1990. A species comparison of liver slice synthesis and secretion of triacylglycerol from nonesterified fatty acids in media. J Anim Sci. 68:1395-1399.

Pyorala, S. 2003. Indicators of inflammation in the diagnosis of mastitis. Vet Res. 34:565-578.

Rainard, P. and C. Riollet. 2006. Innate immunity of the bovine mammary gland. Vet Res. 37:369-400.

Rhoads, R. P., J. W. Kim, B. J. Leury, L. H. Baumgard, N. Segoale, S. J. Frank, D. E. Bauman, and Y. R. Boisclair. 2004. Insulin increases the abundance of the growth hormone receptor in liver and adipose tissue of periparturient dairy cows. J Nutr. 134:1020-1027.

Robert, J. C., C. Richard, T. d'Alfonso, N. Ballet, and E. Depres. 2001. Investigation of the site of absorption and metabolism of a novel source of metabolizable methionine: 2-Hydroxy-4-(methylthio) butanoic acid isopropyl ester (HMBi). . J Dairy Sci. 84(Suppl.1):35.

Rulquin, H. and L. Delaby. 1997. Effects of the energy balance of dairy cows on lactational responses to rumen-protected methionine. J Dairy Sci. 80:2513-2522.

Rulquin, H., B. Graulet, L. Delaby, and J. C. Robert. 2006. Effect of different forms of methionine on lactational performance of dairy cows. J Dairy Sci. 89:4387-4394.

Saeed, S. A., N. Ahmad, and S. Ahmed. 2007. Dual inhibition of cyclooxygenase and lipoxygenase by human haptoglobin: its polymorphism and relation to hemoglobin binding. Biochem Biophys Res Commun. 353:915-920.

Sayers, T. J., L. H. Mason, and T. A. Wiltrout. 1990. Trafficking and activation of murine natural killer cells: differing roles for IFN-gamma and IL-2. Cell Immunol. 127:311-326.

Schoolnik, G. K. 2002. Functional and comparative genomics of pathogenic bacteria. Curr Opin Microbiol. 5:20-26.

Schroder, N. W., S. Morath, C. Alexander, L. Hamann, T. Hartung, U. Zahringer, U. B. Gobel, J. R. Weber, and R. R. Schumann. 2003. Lipoteichoic acid (LTA) of Streptococcus pneumoniae and Staphylococcus aureus activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. J Biol Chem. 278:15587-15594.

Schwab, C. G. 2007. Protected proteins and amino acids for ruminants. Pages 115-141 in Biotechnology in Animal Feeds and Animal Feeding. Wiley-VCH Verlag GmbH.

Schwab, C. G. 2012. The principles of balancing diets for amino acids and their impact on N utilization effiency. in Proc: of the 23rd Annual Florida Ruminant Nutrition Symposium. University of Florida, Gainesville, Florida.

Socha, M. T., D. E. Putnam, B. D. Garthwaite, N. L. Whitehouse, N. A. Kierstead, C. G. Schwab, G. A. Ducharme, and J. C. Robert. 2005. Improving intestinal amino acid supply of pre- and postpartum dairy cows with rumen-protected methionine and lysine. J Dairy Sci. 88:1113-1126.

Sordillo, L. M., K. Shafer-Weaver, and D. DeRosa. 1997. Immunobiology of the mammary gland. J Dairy Sci. 80:1851-1865.

Sordillo, L. M. and K. L. Streicher. 2002. Mammary gland immunity and mastitis susceptibility. J Mammary Gland Biol Neoplasia. 7:135-146.

St-Pierre, N. R. and J. T. Sylvester. 2005. Effects of 2-hydroxy-4-(methylthio) butanoic acid (HMB) and its isopropyl ester on milk production and composition by Holstein cows. J Dairy Sci. 88:2487-2497.

Trevisi, E., A. Zecconi, G. Bertoni, and R. Piccinini. 2010. Blood and milk immune and inflammatory profiles in periparturient dairy cows showing a different liver activity index. J Dairy Res. 77:310-317.

Uhlar, C. M. and A. S. Whitehead. 1999. Serum amyloid A, the major vertebrate acute-phase reactant. Eur J Biochem. 265:501-523.

Wang, Y. Q., S. B. Puntenney, J. L. Burton, and N. E. Forsberg. 2009. Use of gene profiling to evaluate the effects of a feed additive on immune function in periparturient dairy cattle. J Anim Physiol Anim Nutr (Berl). 93:66-75.

West, H. J. 1990. Effect on liver function of acetonaemia and the fat cow syndrome in cattle. Res Vet Sci. 48:221-227.

Yao, Z. M. and D. E. Vance. 1990. Reduction in VLDL, but not HDL, in plasma of rats deficient in choline. Biochem Cell Biol. 68:552-558.

CHAPTER 2

SUPPLEMENTAL SMARTAMINE M OR METASMART DURING THE TRANSITION PERIOD BENEFITS POSTPARTAL COW PERFORMANCE AND BLOOD NEUTROPHIL FUNCTION

INTRODUCTION

Nutritional requirements of dry cows increase as gestation progresses because of a function of fetal growth, which is exponential in late gestation (NRC, 2001). Conditions such as increased blood glucocorticoids, lipid mobilization, and fetal size contribute to reducing voluntary DMI. In turn, nutrient availability for the cow and fetus decreases (Ingvartsen and Andersen, 2000). Clearly, the amount of metabolizable protein (MP) flowing to the intestine from both dietary and microbial sources can be diminished by lower DMI around calving.

Rumen undegradable protein (RUP) is ca. 50% of the total MP and limited research suggests that increasing RUP during late gestation improves subsequence lactation performance (Huyler et al., 1999; Greenfield et al., 2000). Thus, the RUP is important as a source of essential AA (EAA), e.g. methionine (Met), for body tissues and are the building blocks of enzymes and hormones of importance in a number of biological functions. Therefore, an adequate profile of EAA in RUP is crucial for a successful transition for both the cow and the unborn calf. Research has determined that Met and Lys in MP are the most-limiting AA in a wide-range of diets for dairy cows (NRC, 2001). In fact, Met is typically first limiting, supplementation of Met alone improved overall lactation performance in dairy cows (Armentano et al., 1997; Rulquin and Delaby, 1997).

Among the various biological functions besides milk protein synthesis for which Met availability is important, some of the most relevant to the peripartal period include its role in liver lipoprotein synthesis, as substrate for antioxidant reactions, and the immune function (Durand et al., 1992; Soder and Holden, 1999; Chen et al., 2007). Perhaps the most important metabolic role of Met at the level of liver is as a lipotropic agent that can stimulate the synthesis of very-low density lipoproteins (VLDL), and consequently help minimize the accumulation of triacylglycerol (TAG) (Bauchart et al., 1998; Martinov et al., 2010). Oxidative stress status also could be influenced by Met availability because it could serve as a substrate for glutathione synthesis via homocysteine produced in the Met cycle (Martinov et al., 2010). Glutathione is one of the most abundant natural antioxidants produced within liver, and a reduction in its synthesis in rodents leads to steatosis, mitochondrial damage, and marked increases in lipid peroxidation (Chen et al., 2007).

There is still a limited amount of data on the efficacy of supplementing Met during the peripartal period, i.e. the last 3 wk through the first 3 wk relative to parturition (Phillips et al., 2003; Socha et al., 2005; Johnson-VanWieringen et al., 2007), and specifically for the isopropyl ester of 2-hydroxy 4-(methylthio)-butanoic acid (HMBi, MetaSmart® (MS); Adisseo Inc., Antony, France) (Ordway et al., 2009). A common objective of previous experiments (Armentano et al., 1997; Rulquin and Delaby, 1997) has been to achieve optimal or near optimal level of Lys in MP to maintain a ratio of 3:1 of Lys to Met as estimated by NRC (2001), especially after calving. These previous studies have not reported incidence of clinical disease, thus, it is difficult to ascertain what affects MS or SM might have in that regard.

Based on previous research in lactating dairy cows (Rulquin et al., 2006; Chen et al., 2011) and peripartal cows (Socha et al., 2005; Ordway et al., 2009) we hypothesized that either Smartamine® M (SM) and MS would improve DMI, milk yield, and milk protein. Because ca. 50% of HMBi in MS is hydrolyzed into HMB and further degraded by rumen microorganisms, we also hypothesized a greater response in milk fat for cows fed MS. Additionally, L-Met supplementation has been previously associated with an improvement of hepatic lipid metabolism in calves (Auboiron et al., 1994; Auboiron et al., 1995) and dairy cows (Durand et al., 1992), thus, it is conceivable that inclusion of SM or MS during the peripartal period might alleviate overload of fatty acids in liver and consequently decrease the incidence of fatty liver and ketosis. The objective of this experiment was to evaluate the effects of supplementing during the peripartal period (-21 through 30 DIM) SM or MS in amounts that would result in a predicted 2.9:1 ratio of Lys to Met.

MATERIALS AND METHODS

Experimental design and dietary treatments

The Institutional Animal Care and Use Committee (IACUC) of the University of Illinois approved all procedures for this study (protocol #09214). The experiment was conducted as a randomized complete block design where 45 cows were blocked according to parity, previous lactation milk yield, and expected day of calving. Eleven cows were later included to substitute cows that had to be removed inadvertently from the experiment. A total of 56 multiparous Holstein cows were fed experimental treatments

consisting of a basal control diet (CO, n = 24) with no Met supplementation, CO plus MS (n =15) at a rate of 0.19% of DM, and CO plus SM (n = 18) at a rate of 0.07% of DM. After calving a total of 17 cows were removed from the experiment based on clinical disease (per IACUC guidelines) or twinning. This translated into removal of 10, 3, and 4 in CO, MS, and SM, respectively (Table 1). Therefore, the numbers of cows in Table 1 reflect the fact that more cows had to be allocated to CO diet in an attempt to balance the replicates among the treatments. The complete dataset from 14, 12, and, 13 cows in CO, MS, and SM, respectively, was used for statistical analysis. All cows received the same far-off diet (1.24 Mcal/kg DM, 14.3% CP) from -50 to -21 d before expected calving, close-up diet (1.54 Mcal/kg DM, 15% CP) from -21 d to expected calving, and lactation diet from calving (1.75 Mcal/kg DM, 17.5% CP) through 30 DIM (Table 2). Methionine supplements were top-dressed from -21 to 30 DIM.

MetaSmart® was supplied as a dry powder consisting of 57% isopropyl ester of HMBi, which in turn is 78% Met equivalent, of which 50% is absorbed through the rumen wall (Graulet et al., 2005); therefore, for each 10 g of MS, the cow received 2.22 g of Met. In contrast, SM contains 75% DL-Met, physically protected by a pH-sensitive coating, which is considered to have a Met bioavailability of 80% (Schwab, 2007) therefore, per 10 g of SM, the cows received 6 g of metabolizable Met. Using these Met estimates for MS and SM, the quantity of product to be top dressed for the respective diets was calculated to result in predicted concentrations of 6.19% and 2.12% of MP for Lys and Met for the MS and SM diets according to the NRC (2001) (Table 3). Additionally, TMR DM for close-up and lactation diets was measured weekly in order to

estimate daily TMR DM offered. In turn, the amount of Met supplements (g) for MS (0.19%) and SM (0.07%) were calculated over the amount of TMR DM-basis offered.

Animal management

All cows were enrolled in the experiment from mid-October 2009 until early-July 2010 with average temperature of 9.6 ± 10.5 °C (Illinois State Water Survey, http://www.isws.illinois.edu/atmos/statecli/cuweather/index.htm). Cows were fed individually once daily at 0630 h using an individual gate system (American Calan, Northwood, NH). Cows were housed in a ventilated enclosed barn during the dry period and had access to sand-bedded free stalls until 3 d before expected parturition, when they were moved to individual maternity pens bedded with straw until parturition. After parturition, cows were housed in a tie-stall barn and were fed a common lactation diet once daily (Table 2) and milked 3 times daily. At 30 DIM cows returned to the farm herd. Feed offered was adjusted daily to achieve 5 to 10% refusal.

Body weight was measured weekly before the mid-day milking for each cow at the same time after the morning feeding. A BCS (scale 1 = thin to 5 = obese, with quarter-point increments) was assigned to each cow weekly by two individuals and the average score was used for statistical analysis. Intake of DM was recorded daily. Milk yield was recorded daily during the first 30 DIM. Also, milk composition was analyzed while ECM and EB were calculated from calving to 30 DIM.

Feed and milk samples

Dry matter of individual feed ingredients was determined weekly and rations were adjusted accordingly to maintain DM ratios of ingredients in the TMR. Weekly samples of ingredients and TMR were frozen at -20°C and composited monthly for analysis of DM, CP, NDF, ADF, Ca, P, K, and Mg by standard wet chemistry techniques at a commercial laboratory (Dairy One, Ithaca, NY). Consecutive morning, mid-day, and evening milk samples were taken weekly until 30 DIM. Composite milk samples were prepared in proportion to milk yield at each milking, preserved (800 Broad Spectrum Microtabs II; D & F Control Systems, Inc., San Ramon, CA), and analyzed for contents of fat, protein, lactose, SNF, milk urea N, and SCC (Dairy Lab Services, Dubuque, IA). Based on milk sample analysis, the energy corrected milk (ECM) (at 3.5 % fat) was calculated daily as:

$$ECM = (12.82 * Fat\ yield\ (kg)) + (7.13 * Protein\ yield\ (kg)) + (0.323 * Milk\ yield\ (kg))$$

(Hutjens, 2010).

Energy balance (EB) was calculated for each cow using equations from the NRC (2001). Intake of NE_L was determined using daily DMI multiplied by NE_L density of the diet. Maintenance NE_L was calculated as $BW^{0.75} \times 0.080$. Requirements of NE_L for milk production were calculated as NE_{MILK} = (0.0929 × fat% + 0.0547 × protein % + 0.0395 × lactose%) × milk yield. 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. The equation used to calculate prepartal EB (Mcal/d) was $EB_{PRE} = NE_I - (NE_M + NE_P)$ and EB (% requirements) $EB_{PRE} = [NE_I / (NE_M + NE_P)] \times 100$. The equation used to calculate

postpartal EB was EB_{POST} (Mcal/d) = NE_I – (NE_M + NE_{MILK}) and EB (% requirements) (Mcal/d) = [NE_I / (NE_M + NE_{MILK})] × 100.

Blood collections and analyses

Blood was sampled from the coccygeal vein every Monday and Thursday before the morning feeding from -25 to 30 d. Samples were collected into evacuated serum tubes (BD Vacutainer, BD and Co., Franklin Lakes, NJ) containing either clot activator or lithium heparin for serum and plasma, respectively. After blood collection, tubes with lithium heparin were placed on ice and tubes with clot activator were kept at 21 °C 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 autoanalyzer at the University of Illinois Veterinary Diagnostic Laboratory (Urbana). Glucose and TAG was measured using a commercial kit (LabAssay Triglyceride; Wako Chemicals Inc., Richmond, VA). Insulin concentration was quantified using a commercial bovine insulin ELISA kit (Cat# 10-1201-01; Mercodia AB, Uppsala, Sweden). Concentration of VLDL was analyzed using an HDL and LDL/VLDL cholesterol quantification kit (Cat# K613-100; Biovision, Mountain View, CA). Apolipoprotein B-100 was measured using a commercial kit (Bovine Apolipoprotein B100 ELISA kit, ABO Switzerland Co., Ltd., China). Quantification of growth hormone (GH), insulin-like growth factor 1 (IGF1), and leptin concentration was as described by Graugnard et al. (2013).

Liver tissue composition

Liver was sampled via puncture biopsy (Dann et al., 2006) from cows under local anesthesia at approximate 0800 h on d -10, 7, and 21 relative to parturition. Liver was frozen immediately in liquid nitrogen and stored until further analysis for concentration of total lipid and TAG.

Whole blood phagocytosis

The phagocytic capacity of PMN isolated from heparinized whole blood was determined at 21 d postpartum using the Phagotest® kit (ORPEGEN Pharma, Heidelberg, Germany) (Ballou, 2012) following the manufacturer's instructions. In brief, 20 µL of bacteria *E. coli* was added to 1 of 3 whole blood samples (100 µL) in test tubes (Falcon, Becton Dickinson, Franklin Lakes, NJ) and incubated for 10 min at 37 °C. The cells were resuspended in 200 µL of DNA-staining solution, and light-protected in an ice bath until analyzed by flow cytometry (LSR II, Becton Dickinson, San Jose, CA).

Statistical analysis

Data were analyzed using the MIXED procedure of SAS with the preplanned contrasts CO vs. SM+MS and SM vs. MS according to the following model:

$$Y_{ijklm} = \mu + D_i + P_j + DP_{ij} + B_k + C_{ijkl} + T_m + DT_{im} + DPT_{ijm} + e_{ijklm},$$

where Y_{ijklm} is the dependent, continuous variable; μ is the overall mean; D_i is the fixed effect of the ith diet (i = 1, 2, 3); P_j is the fixed effect of the jth parity (j = 1, 2, 3); B_k is the random effect of the kth block (k = 1, ….15); C_{ijkl} is the random effect of lth cow within the ith treatment, within the jth parity, and within the kth block (l = 1,…., n_{ijk});

T_m is the fixed effect of the mth time (day or week) of the experiment (m= 1,…,n); DT_{im} is the fixed effect of the ith treatment by the mth time of the experiment interaction; DPT_{ijm} is the fixed effect of the ith treatment by the jth parity by the mth time of the experiment interaction; and e_{ijklm} is the residual error. Blood metabolites, insulin, and liver composition were analyzed at various time points that were not equally spaced therefore an exponential correlation covariance structure SP (POW) was used for repeated measures. The covariate of previous 305-d milk yield was maintained in the model for all variables for which it was significant ($P < 0.05$). Health and twinning data were analyzed with the FREQ procedure in SAS, and interpreted using Fisher's exact test probabilities. Statistical differences were declared significant at $P \leq 0.05$ and tendencies at $P \leq 0.15$.

RESULTS

Ingredient and nutrient composition of diets

The ingredient compositions of the diets are presented in Table 2. The nutrient composition (Table 3) was determined by analyzing each individual feed ingredient for its chemical composition and then entering the feed analysis results into the NRC (2001) model.

Prepartal DMI, BW, and BCS

An interaction ($P = 0.009$) of diet by time (D × T) was observed for prepartal BCS (Table 4; Figure 1C); however, this could be more associated to transient

differences between cows rather than a direct dietary effect ($P = 0.90$). In fact, prepartal BW, BCS, DMI (Figure 1E), DMI as % BW (Figure 1G), and prepartal EB (Figure 2A and 2C) were not affected overall by dietary treatments (Table 4). Analysis of MS+SM vs. CO indicated a tendency ($P = 0.12$) for lower prepartal BW for cows fed MS+SM; however, this effect was primarily due to a lower ($P = 0.03$) BW during the last week prepartum for Met-supplemented cows in comparison to CO (Figure 1A). Prepartal BW and EB were the only variables for which parity did not explain some of the variation.

Postpartal DMI, BW, and BCS

Main effects and interactions for postpartal BW, BCS, DMI, DMI as % BW, and postpartal EB are presented in Table 4. In Met-supplemented cows tendencies for postpartal BCS ($P = 0.11$), DMI ($P = 0.18$), and DMI as % BW ($P = 0.12$) were reflected via the contrast MS+SM vs. CO in lower BCS ($P = 0.06$), greater DMI ($P = 0.06$), and greater DMI as % BW ($P = 0.04$). A noticeable lower BCS was observed for MS and SM from calving to 3 wk postpartum (Figure 1D). An increase in DMI (kg/d and as % of BW) for MS+SM vs CO was evident from 7 d until 21 d postpartum (Figure 1F and 1H). There was an interaction ($P = 0.11$) of D × T for EB postpartum, which can be explained by the differences in DMI and milk production among them. Although a pronounced ($P = 0.06$) drop in EB of 4.8 Mcal/d (Figure 2B) was observed within 1 wk postpartum for MS+SM vs CO cows, EB as % requirements was 12.7% lower (Figure 2D) for MS+SM vs CO cows and did not differ ($P = 0.82$). Most of this effect was associated with a numerical deficit of 5.6 Mcal/d ($P \leq 0.14$) for SM vs CO+MS during 1 wk (Figure 2B).

Although EB at 4 wk vs 1 wk for MS+SM was different ($P \leq 0.001$), EB for CO cows did not differ ($P = 0.39$) throughout the 4 wk of lactation studied.

Milk production and composition

Main effects and interactions for postpartal production variables, milk:DMI, and ECM:DMI are presented in Table 5. An interaction ($P \leq 0.03$) was observed for milk fat %, fat milk fat yield (Figure 3B), Milk:DMI, and ECM:DMI. A large degree of the effect observed in milk fat % was not only due to a greater ($P = 0.006$) fat content in SM cows during 1 wk postpartum, but also to a greater fat content in MS vs SM during wk 2 ($P = 0.12$) and wk 3 ($P = 0.05$) (Figure 3A). That effect coupled with numerically greater milk production (Figure 3E) for SM cows during wk 1, was associated with the greater ($P < 0.001$) fat yield observed during the same time-frame (Figure 3B). Similarly, MS cows tended ($P < 0.13$) to have greater fat yield during 7 d to 21 d postpartum (Figure 3B). Although D × T was significant ($P = 0.006$) for ECM:DMI, neither diet nor Met contrasts were significant (Figure 3G). Therefore, this effect was mainly due to the greater ($P = 0.07$) Met effect during 1 wk postpartum.

Overall milk yield (Figure 3E), milk protein % (Figure 3C) and yield (Figure 3D), milk fat yield (Figure 3B), and ECM (Figure 3F) tended ($P \leq 0.15$) to be affected by diet. Contrast analysis, however, revealed a greater response due to feeding the Met diets than control for milk yield ($P = 0.08$), milk protein % ($P = 0.05$) and yield ($P = 0.03$), milk fat yield ($P = 0.04$), and ECM ($P = 0.03$) (Table 5).

Health

Health-related problems that occurred during the experiment are summarized in Table 1. Among the 4 main health-related problems observed, ketosis was clearly associated with diet as indicated by the tendency for fewer ($P = 0.15$) clinical cases in cows fed SM and MS than controls. It should be noted, however, that incidence of ketosis in two cows one fed MS, the other SM was confounded by retained placenta. Similarly, two other cows one fed CO and the other SM was diagnosed with ketosis and displaced abomasum after calving. The least-affected ($P = 1.00$) health-related problem was displaced abomasum.

Blood and liver metabolites

The main effects of parity, diet, time, and their interactions on blood metabolites, insulin, GH, IGF1, leptin, PMN phagocytosis, and liver tissue concentration of total lipid and TAG are presented in Table 6. The only significant D×T effect was observed for NEFA ($P < 0.001$), which increased substantially after calving and to a greater extent in cows fed MS on d 7 postpartum (Figure 4D). Although, diet did not significantly affect any of the blood metabolites and hormones or liver tissue concentration of lipid and TAG, there were strong tendencies observed for NEFA ($P = 0.12$), GH ($P = 0.07$), PMN phagocytosis ($P = 0.07$), and liver TAG ($P = 0.15$), where Met-supplemented cows had greater ($P < 0.07$) GH (7.51 vs 5.03) and PMN phagocytosis (50.4 % vs 38.5%). Unlike GH and phagocytosis, NEFA and liver TAG concentration was greater with MS and lower with SM in comparison with CO (Table 6). Unlike ApoB-100, all other blood and liver parameters had a time effect with concentrations changing over time, i.e. insulin,

glucose, TAG, and VLDL decreased between at least -17 d through 14 d postpartum (Figure 4 and 5). However, concentration of VLDL increased to values observed prepartum on d 21 (Figure 5).

The parity effect was significant for GH ($P = 0.03$), IGF1 ($P = 0.04$), total liver lipid ($P = 0.023$), and TAG ($P < 0.001$). As observed with blood data, the concentration of total lipid and TAG in liver changed over time due to marked increases between d -10 and 7 relative to the postpartum (Figure 6). Despite the numerically greater concentration of total lipid on d 21 in cows fed CO, there was no statistical difference in concentrations of lipid and TAG between d 7 and 21.

DISCUSSION

Ingredient and nutrient composition of diets

Mean chemical composition of feed ingredients throughout the experiment were used to evaluate prepartal and postpartal diets through the NRC (2001) model. Unlike prepartum, postpartal MP balance was negative across dietary treatments. Met-supplemented diets (MS+SM) provide ca. 28 g/d more MP during the close-up than CO. However, during the postpartal period MS+SM vs CO had a relative more negative MP/d balance of 42.5g due to a greater (321.5 g/d) MP requirement in MS+SM vs CO in order to sustain a greater yield of milk (Table 5) during the same period.

The importance of adequate RUP supply during the transition period was further confirmed by a15 times increase in required RUP regardless of treatment from the close-up to postpartal period. Therefore, Met-supplemented diets provided ca. 0.34 percentage

units more Met in terms of % MP, which was enough to maintain a more desirable Lys:Met ratio of 2.93 vs 3.4 for MS+SM and CO. Concentrations of NE_L, CP, NDF, and ADF throughout the experiment did not differ greatly between dietary treatments. Overall, postpartal MP balance in Met-supplemented cows was lower (-153 g/d vs -617 g/d) in comparison with levels reported previously by Ordway et al. (2009). Those could have been associated with greater Lys and Met (as % MP) of 0.85 and 0.16 percentage units more than those reported in our experiment. That effect could be attributed to supplying 0.35% and 0.06% of DM of MS and SM from -21 to calving and increase this rate to 0.54% and 0.10% of MS and SM from calving to 140 d (Ordway et al., 2009). It is likely that these differences in MP balance between studies were due to greater inclusion rates of Met supplements and length of experimental period. During the close-up period, cows on the MS and SM diets received similar amounts of MP-lysine and 7 g more MP-Met than the CO cows. After calving Met-supplemented cows received an extra ca. 15 g of MP-Lys and ca. 11 g of MP-Met (Table 3). Similar to our results, (Chen et al., 2011) achieved an increase of 0.37 percentage units of Met over controls in terms of % MP with Met supplementation of 0.17% of DM in the form of MS and 0.06% of DM in the form of SM.

Effects on DMI, BW, and BCS

Although a significant interaction of D × T was observed for BCS prepartum, this result was not associated with main effects of diet ($P = 0.90$) or Met supplements ($P = 0.72$); rather, the marked changes in time plus the effect of between-cow variation appeared to explain this interaction. Our results are in agreement with previous research

(Socha et al., 2005; Ordway et al., 2009) where providing Met supplements prepartum did not affect DMI, BW, or BCS.

Methionine supplementation after calving was associated with an overall increase in DMI (Table 4), along with an even more pronounced effect on DMI and DMI as % BW after 7 d postpartum and throughout d 30 (Figure 1F and 1H). In this regard, previous work with MS and SM supplementation has yielded mixed results. For instance, Ordway et al. (2009) observed an increase in postpartum DMI when supplementing MS while SM did not differ from controls. Another study (Chen et al., 2011) found no effect of MS supplementation during a 12-wk period with cows at >88 DIM (0.17% of DM) on DMI. In contrast, Socha et al. (2005) reported a tendency ($P \leq 0.15$) for a decrease of DMI postpartum when supplementing 15 g/d of SM to a basal diet producing an imbalance in the Lys:Met, particularly before calving (2.7:1) but also after calving (3.22:1), i.e. too much Met relative to Lys or vice versa.

The inconsistent results reported to date might have been related with differences in level of Met supplementation, length of feeding, and stage of lactation, i.e. greatest responses to optimal Lys and Met nutrition occur during the early stages of lactation when the need for absorbed AA, relative to absorbed energy, is the highest (Socha et al., 2005). Our results underscore the benefit of achieving an optimal Lys:Met via MS or SM during the peripartal period on enhancing voluntary feed intake during this critical physiological stage of the lactation cycle. Furthermore, it is unlikely that the intake response was solely due to supplemental Met postpartum, i.e. there might have been carryover effects of supplemental Met during the close-up period.

Milk production and composition

The strong tendency ($P = 0.08$) for an effect of supplemental Met on milk yield underscores the importance of optimal Met to fine-tune the profile of essential AA in MP available to the early lactation cow. In fact, greater responses in milk yield to postpartum supplementation of SM or MS have been reported previously (St-Pierre and Sylvester, 2005). Although D × T interactions were observed for milk fat % and milk fat yield, this was primarily associated with Met supplementation ($P \leq 0.08$) effects because at least one Met treatment behaved differently than the other. The latter is further supported in Figure 3B where SM improved milk fat yield during wk 1, whereas MS had the effect during wk 2 and 3. Similar to our findings others (Chen et al., 2011) have observed an increase in milk fat % or milk fat yield when supplementing cows with Met and especially MS. Milk protein % and yield was greatly affected by Met supplementation increasing by ca. 0.18 percentage units and 0.12 kg/d, respectively, over CO. This effect has been consistently reported throughout the literature, underscoring that milk protein is affected in direct proportion with adequacy of Met in MP (NRC, 2001).

Feed intake and apparent efficiency

The interaction observed for ECM:DMI could be explained mainly by an increase for MS (3.75) and SM (3.52) vs CO cows (3.0, P=0.07) during wk 1 postpartum (Figure 3G). These results are in agreement with those of Socha et al. (2005) and Chen et al. (2011) where an increase in ECM:DMI was observed when supplementing basal diets with rumen-protected Met or rumen-protected Met and Lys. Although EB had a D × T interaction ($P = 0.11$), this result was not associated with a significant main effect of Met;

however, evaluation of Figure 2B and 2D suggested that cows receiving MS or SM experienced a greater demand for nutrients, especially during 1 wk, which was noticeable across milk yield, milk fat yield, and ECM (Figures 3E, 3B, and 3F, respectively).

Although EB in cows fed MS+SM vs CO was lower during wk 1, EB in CO cows did not improve at 4 wk vs wk 1, which was opposite to the response observed in cows fed MS or SM. When evaluating data from Figure 2B the EB as % change with respect to wk 1 (Figure 2E) we observed that EB in MS and SM increased by >40% at 4 wk vs 1 wk; whereas, CO cows remained below 20% of EB at wk 1. This response was consistent with both the observed 4 kg/d increase in ECM for cows supplemented with MS+SM (Table 5) and the increase in DMI (Figure 1H).

Socha et al. (2005) and Ordway et al. (2009) reported mean values of EB between -4.15 and -2.15 Mcal/d between the period of calving to 105 d and 140 postpartum. However, it is likely that differences between those studies and ours are mostly due to experimental design, i.e. starting supplementation during the close-up period (-14 vs -21 d). Interestingly, Ordway et al. (2009) reported an improvement in EB when supplementing MS, while SM did not alter EB over CO. That response could have been associated with a greater (0.54% vs 0.17% of DM) rate of inclusion of MS in the diet in comparison with our experiment.

Blood and liver tissue metabolites as it relates to health

Ketosis is a common disease during starvation or negative EB episodes, when the large uptake of adipose tissue-derived long chain fatty acids by liver results in incomplete oxidation to ketone bodies (Bauchart et al., 1998). Other studies reported an increase in

the occurrence of ketosis when feeding moderate-energy diets similar to ours during the dry period (Van den Top et al., 1996; Dann et al., 2006; Janovick et al., 2011). Thus, the tendency for lower incidence of clinical ketosis due to inclusion of SM and MS during the peripartal period suggests that supplemental Met might have influenced lipid metabolism in liver.

Any lipotropic agent such as Met or choline could help to clear lipid accumulation from the liver (Durand et al., 1992) at least in part by stimulating hepatic VLDL formation and export (Bauchart et al., 1998). Such a response consequently might lead to a reduction in liver TAG accumulation and of ketone body production (Waterman and Schultz, 1972; Bauchart et al., 1998). The lack of change in liver TAG when SM and MS were fed was not entirely surprising as similar responses were observed previously in peripartal cows fed a different Met analog (2-hydroxy-4-methylthiobutanoic acid, Alimet, Novus Intl.) (Piepenbrink et al., 2004). In addition, a recent study with peripartal cows fed rumen-protected choline did not observe differences in NEFA and BHBA despite lower liver TAG postpartum with choline (Zom et al., 2011).

Although speculative, the pattern of NEFA and blood TAG observed with MS between the prepartum and d 7 and 14 could be taken as indication that despite the numerically-greater liver TAG the ability of liver to secrete TAG was not compromised. The actual mechanism behind such response is not readily apparent from our data; however, it might be related with ApoB-100 synthesis as we observed a numerically-greater concentration postpartum in cows fed MS relative to controls. Overall, the concentrations of liver tissue TAG we observed on d 7 and 21 in response to MS and SM are within the range (4-5% wet wt basis) reported recently in cows supplemented with

rumen-protected choline (Zom et al., 2011). More importantly, however, our data underscored that liver TAG in cows fed MS or SM did not impact DMI and the ability of the cows fed SM or MS to produce more milk.

Blood neutrophil killing capacity

Human lymphocytes seem to have an absolute requirement for Met to proliferate (Hall et al., 1986), which is partly responsible for the positive effect of supplemental Met on immune function of monogastrics (Nauss et al., 1982; Tsiagbe et al., 1987) and likely ruminants (Soder and Holden, 1999). For instance, mid-lactation cows supplemented with 30 g/d of rumen-protected Met (Mepron® 85) compared with 0 or 15 g/d had greater T lymphocyte proliferation in vitro in response to various mitogens (Soder and Holden, 1999). The greater blood killing capacity that we observed postpartum with SM and SM provides additional evidence of an important role for Met in the immune response during the transition period. Whether the greater phagocytosis was a result of more cells or a more pronounced oxidative burst response (or both) remains to be established.

Endocrine responses

The temporal changes observed for the concentration of insulin, GH, IGF1, and leptin agree with the expected patterns for these hormones around parturition (Radcliff et al., 2003; Rabelo et al., 2005; Janovick et al., 2011). To our knowledge, endocrine effects to supplemental rumen-protected Met in peripartal cows have not been reported. However, studies performed with post-peak lactating dairy cows have reported (Blum et al., 1999; Misciatteilli et al., 2003) greater insulin in response to SM at doses greater than

the ones used in the present study which would be expected because amino acids are insulinotropic.

The tendency for greater GH with Met supplementation, namely MS, was unexpected because in a previous study with lactating dairy cows supplemental SM did not alter GH or IGF1 concentration (Misciatteilli et al., 2003). It is well-established that in non-lactating ruminants low-protein diets reduce circulating IGF1 and the responsiveness of liver to GH (Breier et al., 1988; Wynn et al., 1991). Work with sheep hepatocytes in vitro demonstrated that Met availability is essential for IGF1 mRNA and protein synthesis, and that limitations in Met dampen the ability of GH to stimulate IGF1 synthesis (Stubbs et al., 2002). The reduction of GH receptor expression in liver of peripartal cows is one factor that accounts for the low mRNA expression and blood circulation of IGF1 particularly after calving (Radcliff et al., 2003). Whether supplemental Met is functionally related with the GH/IGF1 axis during the peripartal period remains to be determined.

CONCLUSIONS

The findings of this study reveal that supplementation with MS or SM, when Lys is adequate to achieve a ~2.9:1 Lys:Met ratio can improve milk production at least in part by increasing voluntary DMI and perhaps by optimizing the use of body lipid reserves. Such responses were more evident during the first week postpartum when SM or MS-supplemented cows increased ECM:DMI while in more negative EB with respect to CO cows, and regaining similar EB as CO by 2 wk. As originally hypothesized, the overall milk protein and milk fat was positively affected by Met supplementation. To the

authors' knowledge this is the first study where a simultaneous improvement in postpartal DMI, milk production-related traits, and better leukocyte killing capacity have been observed while supplementing basal diets with MS or SM, further supporting the benefit of using those products for fine-tuning of EAA in MP.

REFERENCES

Armentano, L. E., S. J. Bertics, and G. A. Ducharme. 1997. Response of lactating cows to methionine or methionine plus lysine added to high protein diets based on alfalfa and heated soybeans. J Dairy Sci. 80:1194-1199.

Auboiron, S., D. Durand, D. Bauchart, J. C. Robert, and M. J. Chapman. 1994. Lipoprotein metabolism in the preruminant calf: effect of a high fat diet supplemented with L-methionine. J Dairy Sci. 77:1870-1881.

Auboiron, S., D. Durand, J. C. Robert, M. J. Chapman, and D. Bauchart. 1995. Effects of dietary fat and L-methionine on the hepatic metabolism of very low density lipoproteins in the preruminant calf, Bos spp. Reprod Nutr Dev. 35:167-178.

Ballou, M. A. 2012. Immune responses of Holstein and Jersey calves during the preweaning and immediate postweaned periods when fed varying planes of milk replacer. J Dairy Sci. 95:7319-7330.

Bauchart, D., D. Durand, D. Gruffat, and Y. Chilliard. 1998. Mechanism of liver steatosis in early lactation cows: effects of hepatoprotector agents. Pages 27-37 in Proc: Cornell Nutr. Conf. Feed Manuf.

Blum, J. W., R. M. Bruckmaier, and F. Jans. 1999. Rumen-protected methionine fed to dairy cows: bioavailability and effects on plasma amino acid pattern and plasma metabolite and insulin concentrations. J Dairy Sci. 82:1991-1998.

Breier, B. H., P. D. Gluckman, and J. J. Bass. 1988. Influence of nutritional status and oestradiol-17 beta on plasma growth hormone, insulin-like growth factors-I and -II and the response to exogenous growth hormone in young steers. J Endocrinol. 118:243-250.

Chen, Y., Y. Yang, M. L. Miller, D. Shen, H. G. Shertzer, K. F. Stringer, B. Wang, S. N. Schneider, D. W. Nebert, and T. P. Dalton. 2007. Hepatocyte-specific Gclc deletion leads to rapid onset of steatosis with mitochondrial injury and liver failure. Hepatology. 45:1118-1128.

Chen, Z. H., G. A. Broderick, N. D. Luchini, B. K. Sloan, and E. Devillard. 2011. Effect of feeding different sources of rumen-protected methionine on milk production and N-utilization in lactating dairy cows. J Dairy Sci. 94:1978-1988.

Dann, H. M., N. B. Litherland, J. P. Underwood, M. Bionaz, A. D'Angelo, J. W. McFadden, and J. K. Drackley. 2006. Diets during far-off and close-up dry periods affect periparturient metabolism and lactation in multiparous cows. J Dairy Sci. 89:3563-3577.

Durand, D., Y. Chilliard, and D. Bauchart. 1992. Effect of lysine and methionine on in vivo hepatic secretion of VLDL in dairy cow. J Dairy Sci. 75:279.

Graugnard, D. E., K. M. Moyes, E. Trevisi, M. J. Khan, D. Keisler, J. K. Drackley, G. Bertoni, and J. J. Loor. 2013. Liver lipid content and inflammometabolic indices in peripartal dairy cows are altered in response to prepartal energy intake and postpartal intramammary inflammatory challenge. J Dairy Sci. 96:918-935.

Graulet, B., C. Richard, and J. C. Robert. 2005. Methionine availability in plasma of dairy cows supplemented with methionine hydroxy analog isopropyl ester. J Dairy Sci. 88:3640-3649.

Greenfield, R. B., M. J. Cecava, T. R. Johnson, and S. S. Donkin. 2000. Impact of dietary protein amount and rumen undegradability on intake, peripartum liver triglyceride, plasma metabolites, and milk production in transition dairy cattle. J Dairy Sci. 83:703-710.

Hall, C. A., J. A. Begley, and R. C. Chu. 1986. Methionine dependency of cultured human lymphocytes. Proc Soc Exp Biol Med. 182:215-220.

Hutjens, M. F. 2010. Benchmarking your feed efficiency, feed costs, and income over feed cost. Pages 3-10 in Proc: Western Canadian Dairy Seminar, Red Deer, Alberta.

Huyler, M. T., R. L. Kincaid, and D. F. Dostal. 1999. Metabolic and yield responses of multiparous Holstein cows to prepartum rumen-undegradable protein. J Dairy Sci. 82:527-536.

Ingvartsen, K. L. and J. B. Andersen. 2000. Integration of metabolism and intake regulation: a review focusing on periparturient animals. J Dairy Sci. 83:1573-1597.

Janovick, N. A., Y. R. Boisclair, and J. K. Drackley. 2011. Prepartum dietary energy intake affects metabolism and health during the periparturient period in primiparous and multiparous Holstein cows. J Dairy Sci. 94:1385-1400.

Johnson-VanWieringen, L. M., J. H. Harrison, D. Davidson, M. L. Swift, M. A. von Keyserlingk, M. Vazquez-Anon, D. Wright, and W. Chalupa. 2007. Effects of rumen-undegradable protein sources and supplemental 2-hydroxy-4-(methylthio)-butanoic acid and lysine-HCl on lactation performance in dairy cows. J Dairy Sci. 90:5176-5188.

Martinov, M. V., V. M. Vitvitsky, R. Banerjee, and F. I. Ataullakhanov. 2010. The logic of the hepatic methionine metabolic cycle. Biochim Biophys Acta. 1804:89-96.

Misciatteilli, L., V. F. Kristensen, M. Vestergaard, M. R. Welsbjerg, K. Sejrsen, and T. Hvelplund. 2003. Milk production, nutrient utilization, and endocrine responses to increased postruminal lysine and methionine supply in dairy cows. J Dairy Sci. 86:275-286.

Nauss, K. M., A. M. Connor, A. Kavanaugh, and P. M. Newberne. 1982. Alterations in immune function in rats caused by dietary lipotrope deficiency: effect of age. J Nutr. 112:2333-2341.

NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th ed. Natl. Acad. Press, Washington, DC.

Ordway, R. S., S. E. Boucher, N. L. Whitehouse, C. G. Schwab, and B. K. Sloan. 2009. Effects of providing two forms of supplemental methionine to periparturient Holstein dairy cows on feed intake and lactational performance. J Dairy Sci. 92:5154-5166.

Phillips, G. J., T. L. Citron, J. S. Sage, K. A. Cummins, M. J. Cecava, and J. P. McNamara. 2003. Adaptations in body muscle and fat in transition dairy cattle fed differing amounts of protein and methionine hydroxy analog. J Dairy Sci. 86:3634-3647.

Piepenbrink, M. S., A. L. Marr, M. R. Waldron, W. R. Butler, T. R. Overton, M. Vazquez-Anon, and M. D. Holt. 2004. Feeding 2-hydroxy-4-(methylthio)-butanoic acid to periparturient dairy cows improves milk production but not hepatic metabolism. J Dairy Sci. 87:1071-1084.

Rabelo, E., R. L. Rezende, S. J. Bertics, and R. R. Grummer. 2005. Effects of pre- and postfresh transition diets varying in dietary energy density on metabolic status of periparturient dairy cows. J Dairy Sci. 88:4375-4383.

Radcliff, R. P., B. L. McCormack, B. A. Crooker, and M. C. Lucy. 2003. Plasma hormones and expression of growth hormone receptor and insulin-like growth factor-I mRNA in hepatic tissue of periparturient dairy cows. J Dairy Sci. 86:3920-3926.

Rulquin, H. and L. Delaby. 1997. Effects of the energy balance of dairy cows on lactational responses to rumen-protected methionine. J Dairy Sci. 80:2513-2522.

Rulquin, H., B. Graulet, L. Delaby, and J. C. Robert. 2006. Effect of different forms of methionine on lactational performance of dairy cows. J Dairy Sci. 89:4387-4394.

Schwab, C. G. 2007. Protected proteins and amino acids for ruminants. Pages 115-141 in Biotechnology in Animal Feeds and Animal Feeding. Wiley-VCH Verlag GmbH.

Socha, M. T., D. E. Putnam, B. D. Garthwaite, N. L. Whitehouse, N. A. Kierstead, C. G. Schwab, G. A. Ducharme, and J. C. Robert. 2005. Improving intestinal amino acid supply of pre- and postpartum dairy cows with rumen-protected methionine and lysine. J Dairy Sci. 88:1113-1126.

Soder, K. J. and L. A. Holden. 1999. Lymphocyte proliferation response of lactating dairy cows fed varying concentrations of rumen-protected methionine. J Dairy Sci. 82:1935-1942.

St-Pierre, N. R. and J. T. Sylvester. 2005. Effects of 2-hydroxy-4-(methylthio) butanoic acid (HMB) and its isopropyl ester on milk production and composition by Holstein cows. J Dairy Sci. 88:2487-2497.

Stubbs, A. K., N. M. Wheelhouse, M. A. Lomax, and D. G. Hazlerigg. 2002. Nutrient-hormone interaction in the ovine liver: methionine supply selectively modulates growth hormone-induced IGF-I gene expression. J Endocrinol. 174:335-341.

Tsiagbe, V. K., M. E. Cook, A. E. Harper, and M. L. Sunde. 1987. Enhanced immune responses in broiler chicks fed methionine-supplemented diets. Poult Sci. 66:1147-1154.

Van den Top, A. M., M. J. Geelen, T. Wensing, G. H. Wentink, A. T. Van 't Klooster, and A. C. Beynen. 1996. Higher postpartum hepatic triacylglycerol concentrations in dairy cows with free rather than restricted access to feed during the dry period are associated with lower activities of hepatic glycerolphosphate acyltransferase. J Nutr. 126:76-85.

Waterman, R. and L. H. Schultz. 1972. Methionine hydroxy analog treatment of bovine ketosis: effects on circulating metabolites and interrelationships. J Dairy Sci. 55:1513-1516.

Wynn, P. C., M. C. Stuart, A. L. Wallace, A. C. Kirby, and E. F. Annison. 1991. Influence of nutritional status on growth hormone-dependent circulating somatomedin-C activity in mature sheep. J Endocrinol. 130:313-320.

Zom, R. L., J. van Baal, R. M. Goselink, J. A. Bakker, M. J. de Veth, and A. M. van Vuuren. 2011. Effect of rumen-protected choline on performance, blood metabolites, and hepatic triacylglycerols of periparturient dairy cattle. J Dairy Sci. 94:4016-4027.

TABLES AND FIGURES

Table 1. Frequency of occurrence of health problems and twinning in cows supplemented with MetaSmart® or Smartamine® M during the peripartal period.

	Diet[1]			P-value	
Variable	CO	MS	SM	Diet	Met
n	24	15	17	---	---
Twins	2	0	1	0.78	0.57
Ketosis[2]	6	1	2	0.34	0.15
Displaced abomasum	3	2	2	1.00	1.00
Retained placenta[3]	0	1	1	0.32	0.50
Excluded cows[4]	10	3	4	---	---

[1]CO = Control, MS = MetaSmart® (Control + MS 0.19% of DMI), and SM = Smartamine® M (Control + SM 0.07% of DMI).
[2]Defined as cows having moderate (~40 mg/dL) or large ketone concentrations (>80 mg/dL) in urine as detected using a reagent strip and treated by veterinarians with oral propylene glycol or intravenous dextrose.
[3]Defined as fetal membranes retained >24 h postpartum.
[4]Actual number of cows excluded from the experiment, where 4 cows were diagnosed with two clinical diseases after calving.

Table 2. Ingredient composition of diets fed during far-off (-50 d to -21 d relative to expected calving), close-up (-21 d to calving), and early lactation[1].

Component[1]	Far-off	Close-up	Lactation
Ingredient, % of DM			
Alfalfa silage	12.00	8.20	5.00
Alfalfa hay	---	3.50	4.00
Corn silage	33.00	35.90	33.00
Wheat straw	36.00	15.40	4.00
Cottonseed	---	---	3.50
Wet brewers grains	---	6.00	10.00
Ground shelled corn	4.00	13.00	22.20
Soy hulls	2.00	4.00	4.00
Soybean meal, 48% CP	7.92	3.10	3.30
Expeller soybean meal[2]	---	2.00	6.20
SoyChlor	0.15	3.80	---
Blood meal 85% CP	1.00	1.00	0.30
Urea	0.45	0.30	0.14
Rumen-inert fat[3]	---	---	1.00
Limestone	1.30	1.30	1.18
Salt (plain)	0.32	0.30	0.27
Dicalcium phosphate	0.12	0.18	0.27
Magnesium oxide	0.21	0.08	0.14
Magnesium sulfate	0.91	0.97	---
Sodium bicarbonate	---	---	0.75
Potassium carbonate	---	---	0.10
Calcium sulfate	---	---	0.10
Mineral-vitamin mix[4]	0.20	0.20	0.20
Vitamin A[5]	0.015	0.015	---
Vitamin D[6]	0.025	0.025	---
Vitamin E[7]	0.38	0.38	---
Biotin	---	0.35	0.35

[1]Basal close up and lactation diets were considered as CO = Control, MS = MetaSmart® (Control + MS 0.19% of DMI), and SM = Smartamine® M (Control + SM 0.07% of DMI).
[2]SoyPLUS (West Central Soy, Ralston, IA)
[3]Energy Booster 100® (MSC, Carpentersville, IL)
[4]Contained a minimum of 5% Mg, 10% S, 7.5% K, 2.0% Fe, 3.0% Zn, 3.0% Mn, 5,000 mg/kg of Cu, 250 mg/kg of I, 40 mg/kg of Co, 150 mg/kg of Se, 2,200 kIU/kg of vitamin A, 660 kIU/kg of vitamin D3, and 7,700 IU/kg of vitamin E.
[5]Contained 30,000 kIU/kg.
[6]Contained 5,009 kIU/kg.
[7]Contained 44,000 IU/kg.

Table 3. Nutrient composition and evaluation (NRC, 2001) of prepartal and postpartal diets fed to cows supplemented with MetaSmart® or Smartamine® M during the peripartal period[1].

	Prepartum				Postpartum		
		Close-up					
Chemical component[2]	Far-off	CO	MS	SM	CO	MS	SM
Chemical analysis							
NE_L, Mcal/ kg DM	1.24	1.54	1.53	1.54	1.76	1.74	1.73
CP, % DM	14.30	15.0	15.1	15.1	17.4	17.5	17.4
NDF, % DM	51.2	42.3	42.2	42.3	35.2	35.3	35.2
ADF, % DM	35.5	28.4	28.3	28.4	22.6	22.6	22.6
RDP supplied, g/d	1,277	1,217	1,265	1,235	1,453	1,631	1,655
RDP balance, g/d	153	32	35	40	35	30	16
RUP supplied, g/d	499	618	654	631	859	1,022	1,062
RUP required, g/d	138	119	103	114	1,535	1,745	1,790
RUP balance, g/d	361	499	551	518	-676	-723	-728
MP supplied, g/d	1,059	1,191	1,248	1,209	1,563	1,812	1,869
MP balance, g/d	282	395	437	410	-574	-613	-620
Lys:Met	3.84:1	3.59:1	2.82:1	2.76:1	3.43:1	2.82:1	2.82:1
Lys ,% of MP	7.30	6.66	6.60	6.62	6.17	6.09	6.06
MP-Lys, g	77	79	82	80	96	110	113
Met, % of MP	1.87	1.86	2.35	2.38	1.81	2.15	2.15
MP-Met, g	20	22	29	29	28	39	40

[1]NRC evaluation of diets was based on final DMI and production data and feed analysis.

[2]Composition MetaSmart® (MS) and Smartamine® M (SM) supplied by Adisseo Inc. (Antony, France, and Alpharetta, GA).

Table 4. Effects of supplementing cows with MetaSmart® or Smartamine® M during the peripartal period on DMI, BW, and BCS.

	Diets[1]				*P*				
Parameter	CO	MS	SM	SEM[2]	Diet	Met[3]	Par[4]	Time	DxT[5]
Prepartum									
BW, kg	773.7	762.8	766.2	4.8	0.26	0.12	--	<0.001	0.14
BCS	3.18	3.17	3.16	0.04	0.90	0.72	0.04	<0.001	0.009
DMI, kg/d	12.2	12.7	12.3	0.48	0.67	0.49	0.05	<0.001	0.42
DMI, % BW	1.54	1.68	1.60	0.07	0.35	0.23	0.003	<0.001	0.70
Energy balance, Mcal/d	8.6	9.0	9.1	0.81	0.85	0.58	--	<0.001	0.70
Postpartum									
BW, kg	670.8	660.3	645.3	20.5	0.56	0.37	--	<0.001	0.23
BCS	2.79	2.52	2.66	0.09	0.11	0.06	--	<0.001	0.75
DMI, kg/d	13.3	15.2	15.6	1.01	0.18	0.06	--	<0.001	0.78
DMI, % BW	1.88	2.24	2.31	0.18	0.12	0.04	--	<0.001	0.50
Energy balance, Mcal/d	-7.6	-8.9	-9.5	1.71	0.69	0.43	--	<0.001	0.11

[1]CO = Control, MS = MetaSmart® (Control + MS 0.19% of DMI), and SM = Smartamine® M (Control + SM 0.07% of DMI).
[2]Greatest SEM.
[3]Contrast statement of CO vs MS+SM.
[4]Parity effect was used in the model depending on significance.
[5]Interaction of diet × time.

Table 5. Effects of supplementing cows with MetaSmart® or Smartamine® M during the peripartal period on production variables.

	Diet[1]				*P*				
Parameter	CO	MS	SM	SEM[2]	Diet	Met[3]	Par[4]	Time	DxT[5]
Milk yield, kg/d	35.7[b]	38.1[ab]	40.0[a]	1.6	0.15	0.08	--	<0.001	0.86
Milk fat, %	4.27	4.68	4.09	0.22	0.59	0.36	0.05	<0.001	0.004
Milk protein, %	3.04[b]	3.26[a]	3.19[ab]	0.08	0.13	0.05	--	<0.001	0.23
Milk fat yield, kg/d	1.64	1.84	1.81	0.08	0.11	0.04	--	0.04	0.009
Milk protein yield, kg/d	1.11[a]	1.23[a]	1.24[a]	0.05	0.08	0.03	--	0.02	0.14
ECM, kg/d	41.0[b]	44.8[a]	45.0[a]	1.55	0.09	0.03	--	<0.001	0.07
Milk:DMI	2.89	2.81	2.69	0.18	0.71	0.50	--	0.089	0.03
ECM:DMI	3.00	3.39	3.05	0.22	0.42	0.40	--	<0.001	0.006

[1]CO = Control, MS = MetaSmart® (Control + MS 0.19% of DMI), and SM = Smartamine® M (Control + SM 0.07% of DMI).
[2]Greatest SEM.
[3]Contrast statement of CO vs MS+SM.
[4]Parity effect was used in the model depending on significance.
[5]Interaction of diet × time.

Table 6. Effects of supplementing cows with MetaSmart® or Smartamine® M during the peripartal period on blood metabolites, liver composition, and phagocytosis.

	Treatment				*P*-value				
Parameter	CO	MS	SM	SEM[1]	Diet	Met[2]	Par[3]	Time	D×T[4]
Blood									
NEFA, mEq/L	0.432	0.494	0.420	0.029	0.12	0.43	--	<0.001	<0.001
BHBA, mmol/L	0.687	0.697	0.645	0.057	0.80	0.82	--	<0.001	0.33
TAG, mg/dL	300.9	327.2	300.1	23.8	0.65	0.66	--	<0.001	0.65
Insulin, μg/L	0.43	0.50	0.45	0.07	0.79	0.65	--	<0.001	0.68
Glucose, mg/dL	55.6	55.6	55.4	1.3	0.74	0.74	--	<0.001	0.82
GH, ng/mL	5.03	7.62	7.40	0.89	0.07	0.02	0.03	<0.001	0.73
IGF1, ng/mL	58.9	52.0	57.3	4.9	0.59	0.47	0.04	<0.001	0.97
Leptin, ng/mL	5.42	4.36	4.40	1.24	0.78	0.49	--	<0.001	0.12
VLDL, μg/μL	0.43	0.45	0.47	0.05	0.86	0.65	--	<0.001	0.57
ApoB-100, ng/mL	17.4	23.2	20.6	2.1	0.16	0.14	--	0.97	0.75
Phagocytosis, %[5]	38.5	55.1	45.8	5.6	0.07	0.07	--	--	--
Liver, % wet wt									
Total lipid	10.55	9.53	8.66	1.09	0.39	0.24	0.023	<0.001	0.17
TAG	4.27	4.55	3.14	0.54	0.15	0.50	<0.001	<0.001	0.46

[1]Greatest Standard error mean of all treatments
[2]Contrast statement of CO vs MS+SM
[3]Parity effect was used in the model depending on significance
[4]Interaction of treatment by time
[5]Whole blood leukocyte phagocytosis at 21 DIM.

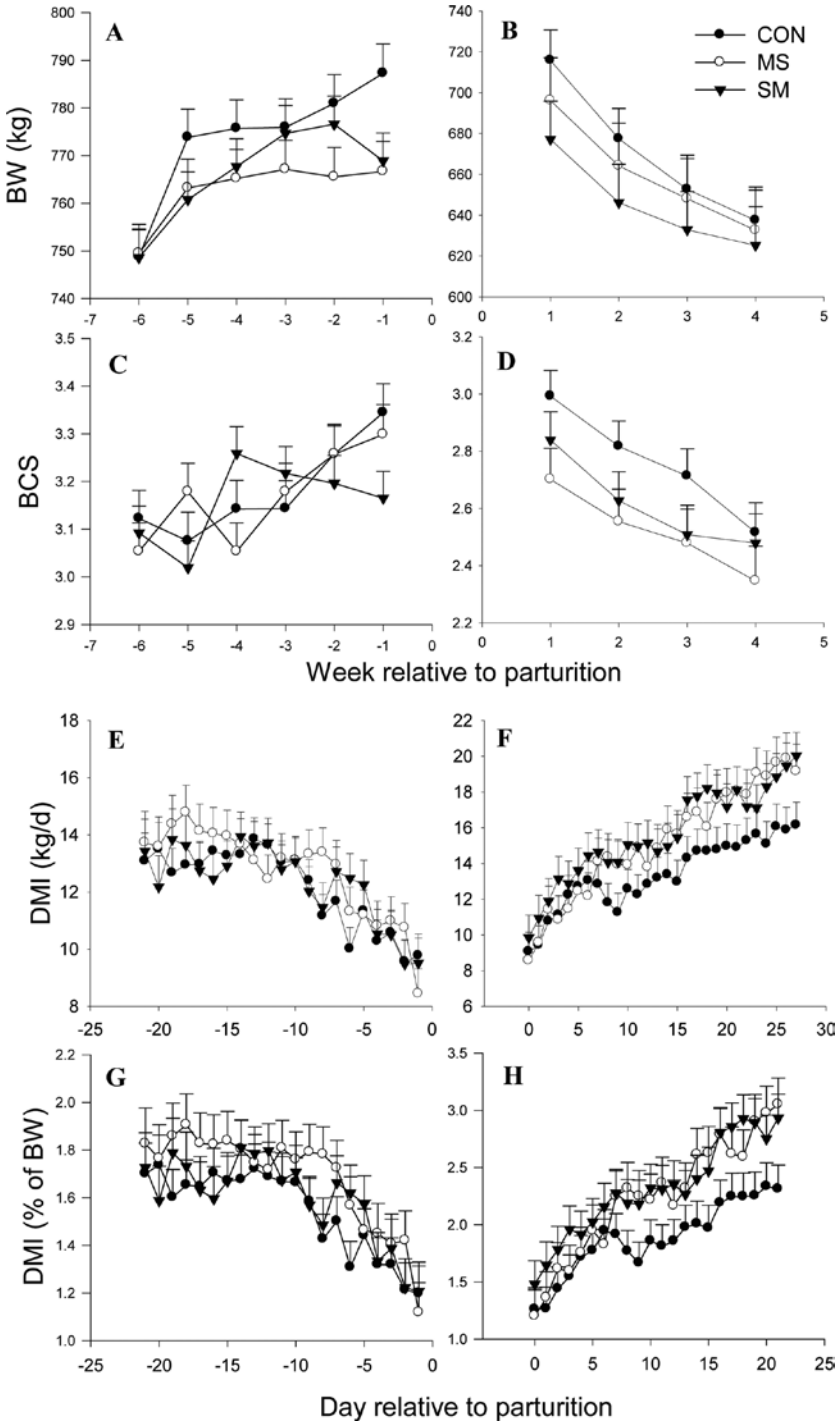

Figure 1. Prepartal (A) and postpartal (B) BW, BCS (C, D), DMI (E, F), and DMI as percentage of BW (G, H) in cows supplemented with MetaSmart (MS; Adisseo Inc., Antony, France) or Smartamine M (SM; Adisseo Inc.) during the peripartal period. CON = control. Values are means, with standard errors represented by vertical bars.

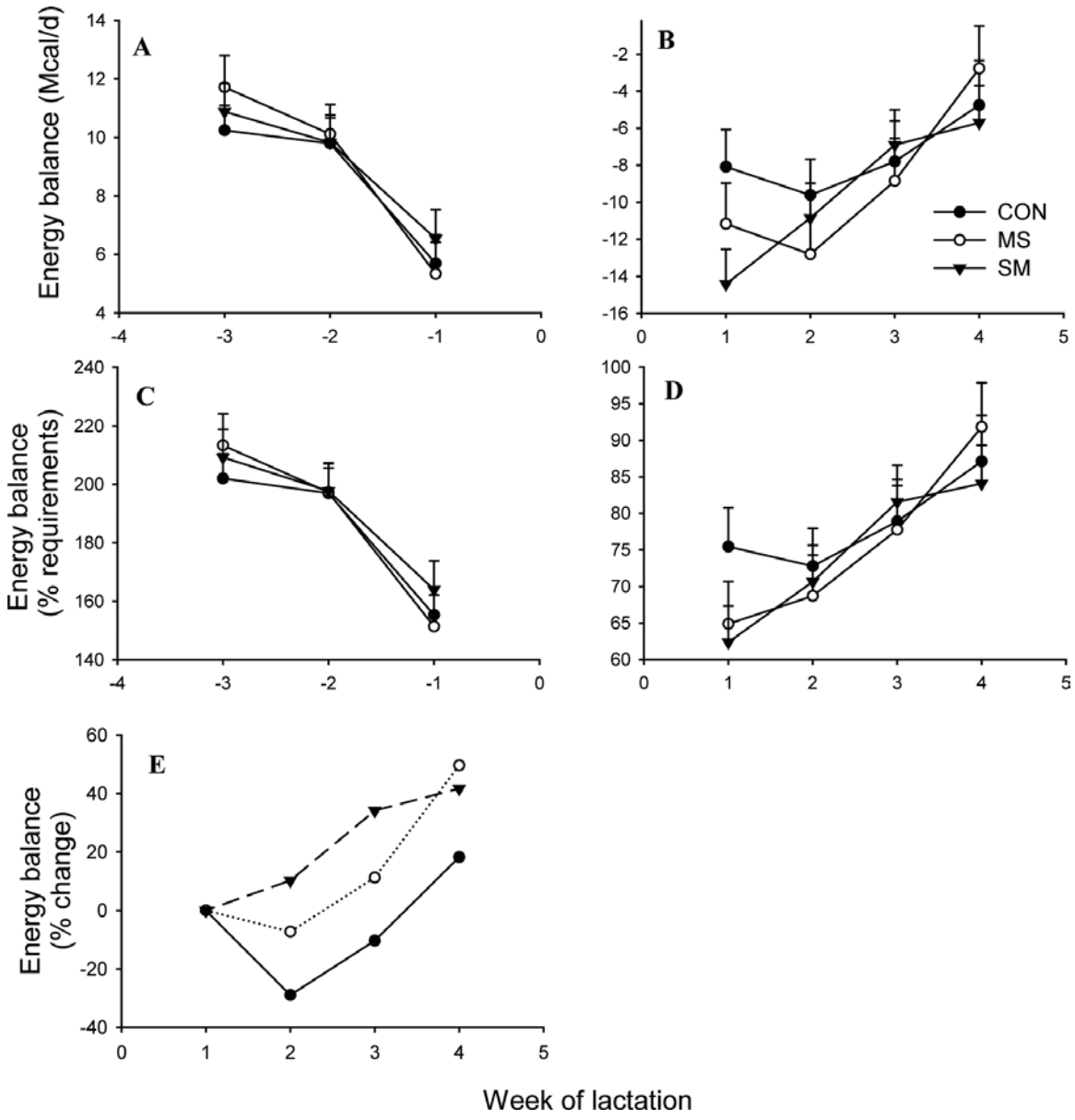

Figure 2. Prepartal energy balance (EB; Mcal/d; A), postpartal EB (Mcal/d; B), prepartal EB (% of requirements; C), postpartal EB (% of requirements; D), and EB (% change with respect to 1 wk; E) in cows supplemented with MetaSmart (MS; Adisseo Inc., Antony, France) or Smartamine M (SM; Adisseo Inc.) during the peripartal period. CON = control. Values are means, with standard errors represented by vertical bars.

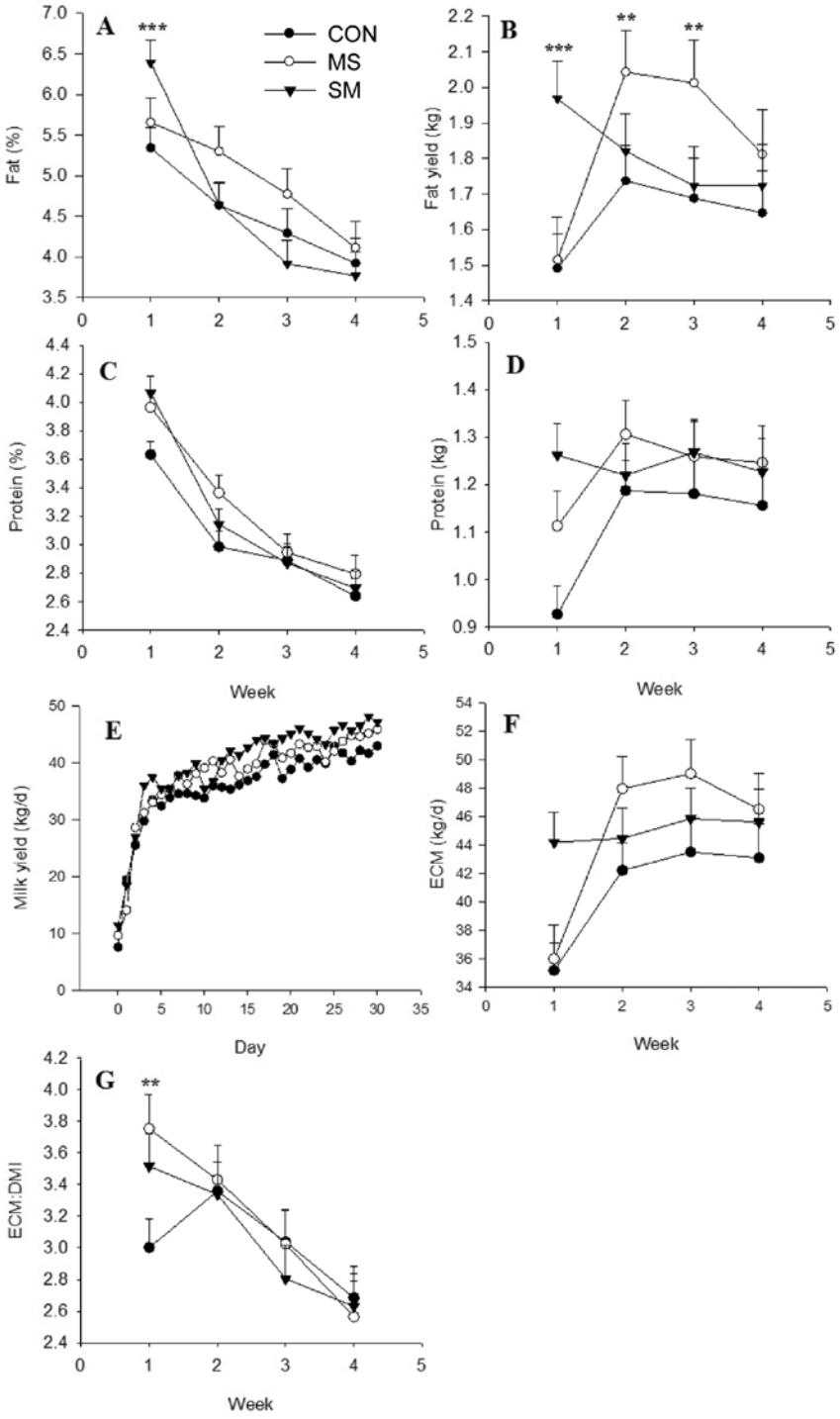

Figure 3. Milk fat percentage (A), milk fat yield (B), milk protein percentage (C), milk protein yield (D), milk yield until 30 DIM (E), ECM (F), and ECM:DMI ratio (G) in cows supplemented with MetaSmart (MS; Adisseo Inc., Antony, France) or Smartamine M (SM; Adisseo Inc.) during the peripartal period. Mean separation between diets ($P < 0.05$) were evaluated via contrasts: control (CON) versus MS + SM (*), CON versus MS (**), and CON versus SM (***). Values are means, with standard errors represented by vertical bars.

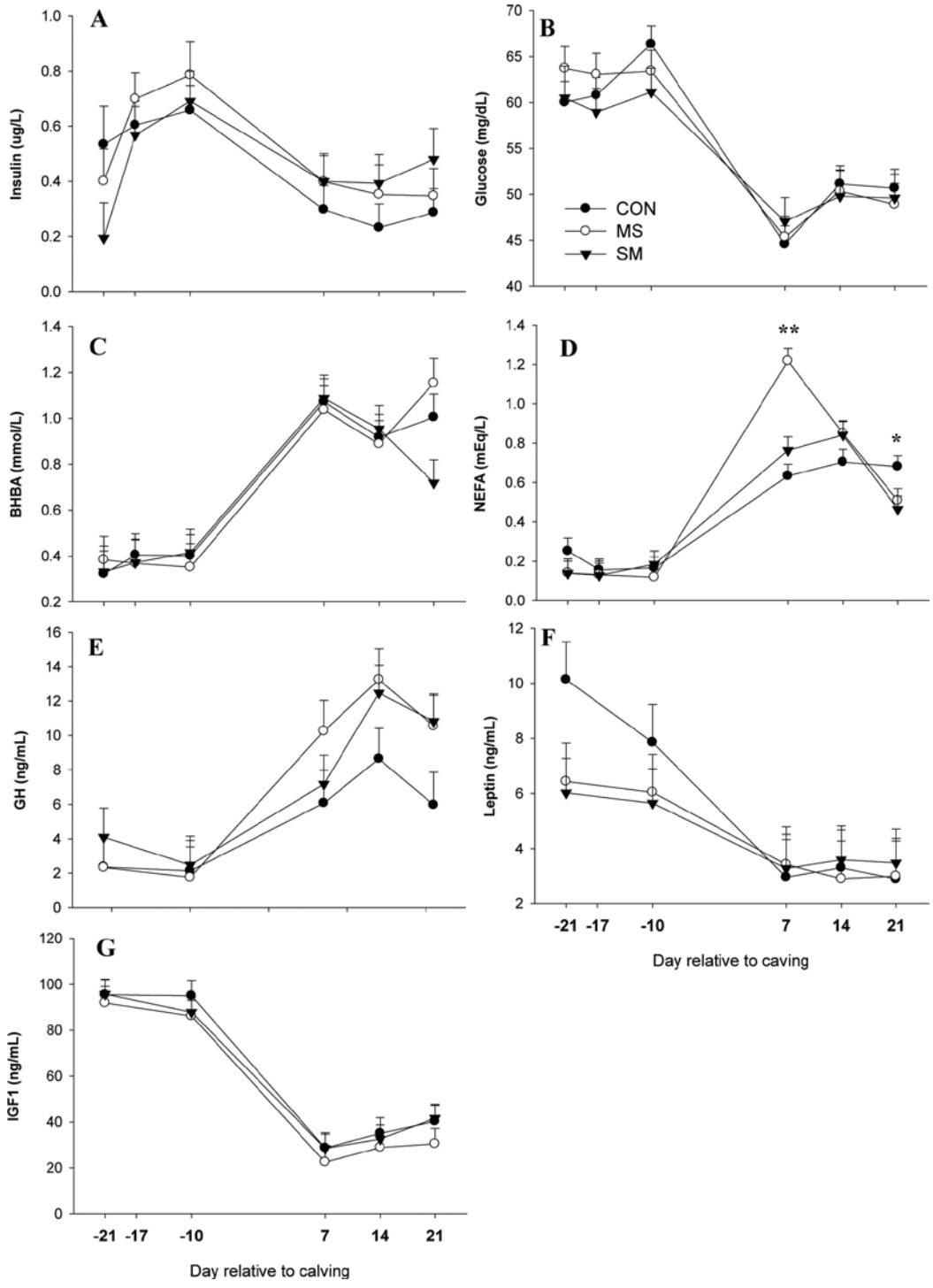

Figure 4. Effects of supplemental MetaSmart (MS; Adisseo Inc., Antony, France) or Smartamine M (SM; Adisseo Inc.) on insulin (A), glucose (B), BHBA (C), NEFA (D), growth hormone (GH; E), leptin (F), and IGF1 (G) in dairy cows during the transition period. Mean separation between diets ($P < 0.05$) were evaluated via contrasts: control (CON) versus MS + SM (*), CON versus MS (**), and CON versus SM (***). Values are means, with standard errors represented by vertical bars.

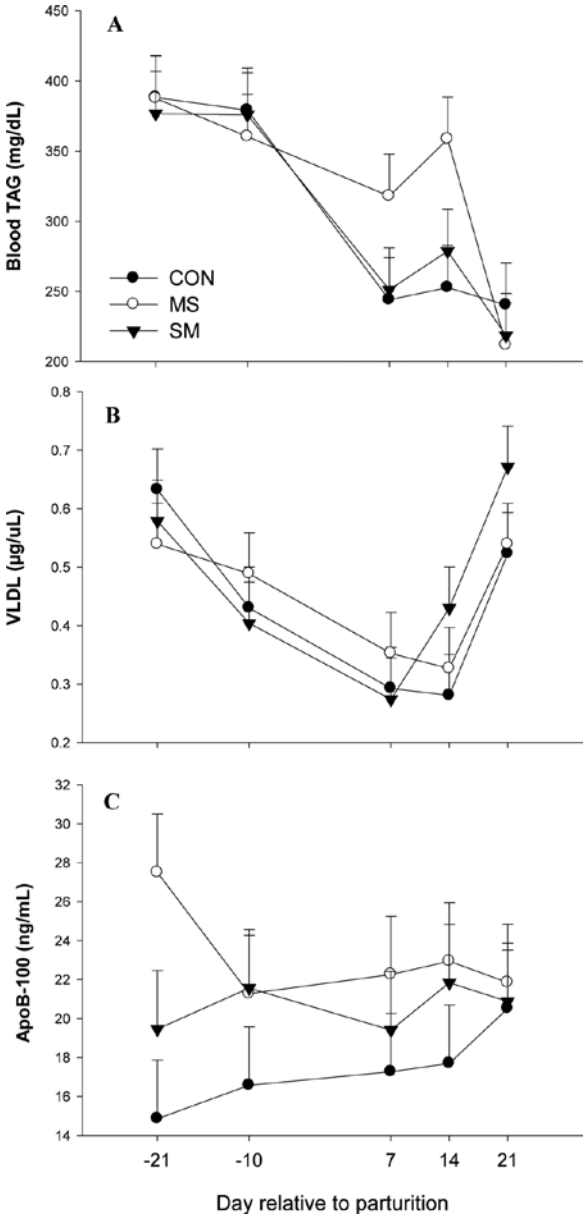

Figure 5. Effects of supplemental MetaSmart (MS; Adisseo Inc., Antony, France) or Smartamine M (SM; Adisseo Inc.) on blood triacylglycerol (TAG; A), very low density lipoproteins (VLDL; B), and apolipoprotein B-100 (ApoB-100; C) in dairy cows during the transition period. CON = control. Values are means, with standard errors represented by vertical bars.

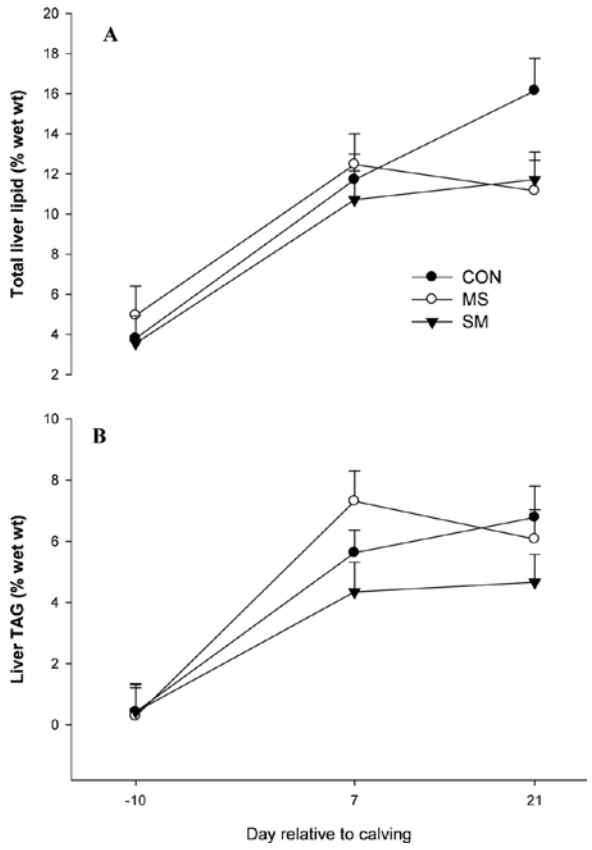

Figure 6. Effects of supplemental MetaSmart (MS; Adisseo Inc., Antony, France) or Smartamine M (SM; Adisseo Inc.) on liver composition in terms of total lipids (A) and triacylglycerol (TAG; B) in dairy cows during the transition period. Values are means, with standard errors represented by vertical bars.

CHAPTER 3

BIOMARKERS OF INFLAMMATION, METABOLISM, AND OXIDATIVE STRESS IN BLOOD AND LIVER REVEAL A BETTER IMMUNOMETABOLIC STATUS IN PERIPARTAL COWS SUPPLEMENTED WITH SMARTAMINE M OR METASMART

INTRODUCTION

There is research with ruminant animals supporting the hypothesis that increasing the supply of methionine (Met) could enhance the capacity of liver to export triacylglycerol (TAG) in the form of VLDL (Durand et al., 1992; Auboiron et al., 1994; Auboiron et al., 1995) and help ameliorate the negative effects of fatty acid accumulation in the liver soon after parturition (Drackley, 1999). Within liver, Met can also be transformed into S-adenosylmethionine (SAM) the most important methyl-donor (Martinov et al., 2010), and in turn SAM can be used to methylate phosphatidylethanolamine (PE) to produce phophatidylcholine, which is a main constituent of VLDL. Besides the methylation of PE, SAM is also involved in methylation of DNA, which can have profound regulatory effects on gene expression (Kass et al., 1997).

It is well-established that around parturition the dairy cow experiences a state of depressed liver function coupled with increased inflammation and oxidative stress (Bionaz et al., 2007; Trevisi et al., 2012). Bilirubin, glutamic-oxaloacetic transaminase (GOT), γ-glutamyltransferase (GGT) along with albumin and paraoxonase (PON) are commonly-used biomarkers of liver status around calving (Bertoni et al., 2008). While the liver is responsible for clearance of bilirubin (Bertoni et al., 2008), higher GOT and

GGT are related with liver cell damage (i.e. lysis and necrosis). The peripartal inflammatory response is characterized by an increase in the production of positive acute-phase proteins (APP) such as haptoglobin and serum amyloid A (SAA), and a concomitant decrease in the production of negative APP such as albumin. At the level of liver, the well-established triggers of these responses are the pro-inflammatory cytokines IL-6, IL-1, and TNF-α (Kindt et al., 2007). The concentrations of these cytokines in turn are driven by several factors including tissue damage, pathogen invasion, and potentially excessive fat deposition (O'Boyle et al., 2006; Loor et al., 2013).

Excessive fat deposition leading to high BCS has been proposed to increase the release of TNF-α (O'Boyle et al., 2006), while the uterine bacterial contamination that commonly occurs after calving also contributes to the release TNF-α and IL-6 (LeBlanc, 2012). In fact, the endometrial epithelium can secrete prostaglandin F during calving, and after calving prostaglandin E in response to bacteria. Both molecules can further stimulate the pro-inflammatory response, but also are related with the metabolism of arachidonic acid, which is an important contributor of reactive oxygen metabolites (ROM**)** in the circulation (Haeggstrom and Funk, 2011; LeBlanc, 2012).

Oxidative stress is driven by the imbalance between the production of ROM and the neutralizing capacity of antioxidant mechanisms in tissues and in blood. Some of the well-established antioxidants include glutathione, superoxide dismutase (SOD), and vitamins A and E (Bernabucci et al., 2005). Additionally, the concentration of PON is inversely related with oxidative stress partly because it helps protect both LDL and HDL against lipid peroxidation (Aviram and Rosenblat, 2004).

Our general hypothesis was that rumen-protected Met supplementation in the form of Smartamine (SM) and MetaSmart (MS) during the peripartal period ameliorates the negative effects of peripartal adipose tissue lipolysis that often leads to excessive liver TAG accumulation and consequently places cows at greater risk of developing ketosis (Osorio et al., 2013). As such, supplemental Met would have a positive effect on liver function, inflammation, and oxidative stress status. Blood serum and plasma from cows fed SM and MS (Osorio et al., 2013) were used to address this hypothesis. Concentrations of total bilirubin (TB), GGT, GOT, cholesterol, and PON were used to assess liver function and integrity; creatinine and urea to evaluate skeletal muscle mass catabolism; ceruloplasmin, SAA, haptoglobin, and IL-6 to assess inflammation status; and concentrations of nitric oxide (NOx) and constituents [nitrite (NO_2^-) and nitrate (NO_3^-)], oxygen radical absorbance capacity (ORAC), ROM, retinol, tocopherol, β-carotene as well as liver glutathione to assess oxidative stress status.

MATERIALS AND METHODS

Animals, experimental design and dietary treatments

The Institutional Animal Care and Use Committee (IACUC) of the University of Illinois approved all protocols for this study (protocol no. 09214). Details of the experimental design have been published previously (Osorio et al., 2013). Briefly, 45 multiparous Holstein cows were fed experimental treatments consisting of a basal control diet (CON, n = 13) with no Met supplementation, CON plus MS (n = 11) at a rate of 0.19% of DM, and CON plus SM (n = 13) at a rate of 0.07% of DM. All cows received

the same Far-off diet (1.24 Mcal/kg DM, 14.3% CP, 10.3 RDP % of DM, 4 RUP % of DM, Lys 7.3 % of MP, Met 1.87 % MP) from -50 to -21 d before expected calving, close-up diet (1.54 Mcal/kg DM, 15% CP, 10 RDP % of DM, 5.1 RUP % of DM, Lys 6.66 % MP, Met 1.86% MP) from -21d to expected calving, and from calving (1.75 Mcal/kg DM, 17.5 % CP, 10.9 RDP % of DM, 6.5 RUP % of DM, Lys 6.17 % MP, Met 1.81 % MP) through 30 DIM (Table 8). From -21 to parturition, Met supplements were top-dressed to increase the Met (% MP) from 1.86 (CO) to 2.35 (MS) and 2.38 (SM). From 1 to -21 to 30 DIM, Met supplements were top-dressed to increase the Met (% MP) from 1.81 (CO) to 2.15 (MS and SM).

As described in Osorio et al. (2013) before calving all cows were individually fed once daily at 0630 h using an individual gate system (American Calan, Northwood, NH). Cows were housed in a ventilated enclosed barn during the dry period and had access to sand-bedded free stalls until 3 d before expected parturition, when they were moved to individual maternity pens bedded with straw until parturition. After parturition, cows were housed in a tie-stall barn and were fed a common lactation diet once daily and milked 3 × daily.

Blood samples and biomarker analyses

Blood was sampled from the coccygeal vein and was collected using a 20-gauge BD Vacutainer® needles (Becton Dickinson, Franklin Lakes, NJ) at -26, -21, -10, 7, 14, and 21 d relative to calving. Blood was collected into vacutainers (5 mL, BD Vacutainer®, Becton Dickinson, Franklin Lakes, NJ) containing either serum clot activator or lithium heparin. After blood collection, tubes with lithium heparin were

placed on ice and tubes with clot activator were kept at 21°C until centrifugation (~30 min). Serum and plasma were obtained by centrifugation of clot activator and lithium heparin tubes, respectively, at 1,900 × *g* for 15 min.

Blood samples were analyzed for albumin, cholesterol, TB, creatinine, urea, GOT, haptoglobin, ceruloplasmin, IL-6, PON, NOx, NO_2^-, NO_3^-, ROM and GGT using kits purchased from Instrumentation Laboratory (IL Test) following the procedures described previously (Bionaz et al., 2007; Trevisi et al., 2012) using a clinical auto-analyzer (ILAB 600, Instrumentation Laboratory, Lexington, MA, USA). Total anti-oxidants were assessed through the ORAC assay. This method measures a fluorescent signal from a probe (fluorescein) that decreases in the presence of radical damage (Cao and Prior, 1999).

Statistical analysis

The data of blood and liver metabolites were analyzed with the PROC MIXED of SAS 9.3 (SAS Institute, Inc., Cary, NC, USA). The fixed effects in the model included diet, time, and their interactions. The random effect was cow nested within treatment. Unequally-spaced data were analyzed using the exponential correlation covariance structure SP for repeated measures. Blood results obtained at -26 d for various biomarkers were used as covariates. Least-squares means separation between time points was performed using the PDIFF statement. Statistical significance was declared at $P \leq 0.10$ and tendencies at $P \leq 0.15$.

RESULTS

Biomarkers of muscle mass catabolism and N metabolism

Main effects of diet, time, and interactions are presented in Table 7. Unlike urea, concentration of creatinine had an interaction ($P = 0.10$) of D×T that resulted from a greater ($P < 0.05$) concentration at 7 d in CON cows compared with MS + SM (Figure 7A). Although overall concentration of urea was affected ($P = 0.005$) only by time (Figure 7B), the contrast comparing prepartum versus postpartum concentrations was ($P = 0.08$) significant in CON cows but not in MS ($P = 0.33$) or SM cows ($P = 0.62$) (Table 8).

Biomarkers of liver function

Main effects of diet, time, and interactions are presented in Table 7. The interaction D×T was not significant ($P > 0.05$) for any of the liver function biomarkers.. Among the biomarkers related with liver function only albumin showed a tendency for greater albumin in Met-supplemented cows than CON throughout the experiment (Table 7). In fact, albumin decreased significantly in CON ($P = 0.01$) cows while it remained unchanged in MS ($P = 0.88$) and SM ($P = 0.21$). The nadir of albumin at 7 and 14 d postpartum in CON cows confirmed the latter (Figure 8A). The concentration of TB (Figure 8B; $P < 0.001$), GOT (Figure 8C; $P < 0.001$), and cholesterol (Figure 8D; $P = 0.02$) increased significantly from prepartum to postpartum across all treatments (Table 8). Concentration of GGT (Figure 8E) did not change from prepartum to postpartum in MS ($P = 0.20$) cows while it increased significantly in CON ($P = 0.002$) and SM ($P <$

0.001) cows (Table 8). In contrast, PON (Figure 8F) remained unchanged from prepartum to postpartum in SM ($P = 0.19$) cows while it decreased in CON ($P = 0.002$) and MS ($P = 0.02$).

Biomarkers of inflammation and APP

Main effects of diet, time, and interactions for inflammation and APP biomarkers are presented in Table 7. The interaction D×T was not significant ($P > 0.05$) for any of the inflammation and APP biomarkers. Unlike haptoglobin and IL-6, ceruloplasmin (Figure 9A) had a diet effect ($P = 0.03$) and specifically because of lower ($P = 0.009$) concentration in Met-supplemented diets (CON vs MS + SM). Similarly, SAA (Figure 9B) concentration was lower ($P = 0.06$) in Met-supplemented cows than CON. Although, diet had no effect IL-6 concentration, when comparing prepartum vs postpartum there was a significant decrease in cows fed MS ($P < 0.001$) and SM ($P = 0.05$) while it remained unchanged in CON ($P = 0.14$) cows (Table 8). Consistent with a non-significant ($P = 0.24$) overall time effect for SAA (Table 7), the concentrations did not differ ($P = 0.43$) between prepartum and postpartum regardless of treatments (Table 8). There was a tendency ($P = 0.11$) for a decrease in haptoglobin from prepartum to postpartum (Figure 9D) in MS cows while no change ($P = 0.45$) was observed in CON and SM cows (Table 8).

Biomarkers of oxidative stress

Main effects of diet, time, and interactions for biomarkers related with oxidative stress are presented in Table 7. There was a significant interaction of D×T for NOx ($P =$

0.06) and NO_3^- ($P = 0.04$). This effect was associated with a greater ($P < 0.05$) concentration of NOx in CON vs MS + SM cows at -21 d (Figure 10A), and a greater concentration of NOx ($P = 0.10$) and NO_3^- (Figure 10B; $P = 0.02$) in SM vs CON at 7 d postpartum. In fact, NO_3^- did not change from prepartum to postpartum in SM ($P = 0.12$) cows, but decreased in CON ($P < 0.001$) and MS ($P = 0.04$; Table 8). Unlike NOx and NO_3^-, ORAC (Figure 10C; $P = 0.04$) and liver glutathione concentration (Figure 10F; $P = 0.04$) were greater in MS + SM cows than CON. Although, ROM (Figure 10E) and NO_2^- (Figure 10D) were only affected by time (Table 7), there was a substantial increase in ROM from prepartum to postpartum in CON ($P = 0.06$) and SM ($P = 0.007$) but not in MS ($P = 0.11$), while NO_2^- decreased in CON ($P = 0.02$) and MS ($P = 0.04$) but not in SM ($P = 0.18$) cows (Table 8).

Vitamins

Main effects of diet, time, and interactions for vitamins are presented in Table 7. Only retinol was affected ($P = 0.06$) by the interaction D×T, namely due to an increase ($P < 0.003$) in SM at 21 d postpartum compared with other treatments (Figure 11A). In fact, retinol in SM cows did not change ($P = 0.24$) between prepartum to postpartum, while it decreased in CON ($P = 0.006$) and MS ($P = 0.06$) cows (Table 8). Tocopherol and beta-carotene were affected by time (Table 7) and they decreased ($P < 0.06$) across treatments between prepartum to postpartum (Table 8).

DISCUSSION

Supplementation with MS or SM improved milk production at least in part by increasing postpartal DMI and by reducing the loss of BCS (Osorio et al., 2013). Postpartal negative energy balance (**NEB**) in dairy cows is commonly associated with lower DMI with respect to the greater energy requirements for milk production. In the companion study we observed that Met supplementation (SM or MS) led to a faster recovery from NEB toward a positive energy balance during the first 4 wk postpartum (Osorio et al., 2013). Supplemental Met also resulted in a tendency for lower incidence of ketosis postpartum.

Biomarkers of muscle mass catabolism and N metabolism

Creatinine is an important indicator of body muscle mass and its concentration typically decrease around parturition (Kokkonen et al., 2005; Pires et al., 2013). Although Pires et al. (2013) observed a decrease in creatinine from -4 wk to 7 wk relative to calving, the differences reported between -4 wk to 4 wk were marginal. The time effect observed for creatinine in the present study did not result in an overall decrease during the peripartal period (Table 8). However, our results encompass the period from -3 wk to 3 wk, which in turn are similar to those reported by Pires et al. (2013). This suggests that although cows normally experience a decrease in body weight couple with decrease in BCS, the decrease in body muscle mass is more likely to occur during extended periods (>7 wk) of NEB as tissue mobilization of lipids precedes that of AA. Pires et al. (2013) also observed that cows with a low BCS (≤2.5) had lower creatinine concentration in comparison with medium and high BCS (≥2.75) cows. We observed a similar effect in

CON cows which had a greater postpartum BCS than MS and SM (Osorio et al., 2013), and also greater creatinine concentration (Figure 7A) at 7 d postpartum.

Decrease in ruminal ammonia concentration has been associated to a decrease in blood urea N, as the latter is transfer into the rumen in order to serve as a buffer against excessively low ruminal ammonia and to supply amino N for microbial function (Firkins et al., 2007). It's likely that decreased postpartal DMI in CON cows (Osorio et al., 2013) might had also decreased the total N intake available for microbial protein synthesis, which in turn might had lead to an increase in the recycle of blood urea into the rumen (Bach et al., 2005), and thus decreasing blood urea in CON cows (Table 8). The latter could also be associated to lower milk protein % and milk protein yield observed in CON cows (Osorio et al., 2013), where lower total N intake might had impair microbial protein reaching the small intestine.

Biomarkers of liver function

The fact that albumin is classified as a negative APP implies that hepatic production (the main site in the body) is commonly reduced during the onset of inflammation (Bertoni et al., 2008). Therefore, the substantial decrease of albumin in CON cows during the transition period (while MS and SM maintained same levels, Table 8) is consistent with the animals experiencing more pronounced inflammatory conditions. Liver function has been previously classified as upper, upper intermediate, lower intermediate, and lower in terms of blood PON concentration (Bionaz et al., 2007) or plasma negative acute phase proteins such as albumin, retinol-biding protein, and total cholesterol (Bertoni et al., 2008). Then, cows classified as upper postpartal liver function

have albumin concentrations ranging between 34 to 35 g/L (Bionaz et al., 2007; Bertoni et al., 2008), whereas in Met-supplemented cows was above 35 g/L throughout the peripartal period (Figure 8A). Although albumin in CON cows were within physiological levels, its concentration decreased ~2 g/L from prepartum to 7 d postpartum, similar decreased was observed in Bionaz et al. (2007) within cows in the lower liver function group. Furthermore, the fact that albumin in CON cows remained lower than prepartal levels for ~ 2 wk could suggest additional liver damage during this time.

The increase in GOT (58.9 versus 116.9 U/L; Figure 8C) across treatments between pre and postpartum is to some extent consistent with the increase of GGT (26.2%; Figure 8E). However, in the case of GGT it did not increase in MS cows (P = 0.2; Table 8). The mechanism by which MS supplementation did not increase concentration of GGT after parturition remains unknown. However, this lack of effect could be partly explained by transient effects due to animal variation rather than an actual treatment effect. Although this might suggest that liver damage in MS cows was less severe, it is well-known that concentration of both enzymes (GOT and GGT) in blood increases after calving (Bertoni et al., 2008; Graugnard et al., 2012).

Overall, the decrease in PON around parturition and specifically at 7 d postpartum is in agreement with previous work (Bionaz et al., 2007; Grossi et al., 2013). Regardless of this overall reduction, the concentration of PON remained unchanged in SM cows but decreased in CON and MS cows (Table 8). The decrease in serum PON at the end of pregnancy and after calving has been functionally linked with liver dysfunction due to the reduction of cholesterol HDL which is the main carrier of paraoxonase molecules in blood (Turk et al., 2005), and which is justified by inflammation and oxidative stress

(Bionaz et al., 2007). Some of these mechanisms could partly explain the results in the present study. For instance, SM cows had numerically-lower ($P = 0.15$) NEFA concentrations than CON and MS (Osorio et al., 2013), and ROM (Figure 10E) was numerically-greater but less affected ($P = 0.56$).

Biomarkers of inflammation and APP

Concentrations of ceruloplasmin and SAA are likely to increase during inflammatory episodes such as those occurring in the peripartal period (Ceciliani et al., 2012). Although, ceruloplasmin was not affected by the interaction D × T, it was 10.7% lower (Table 7) in Met supplemented diets, which was more noticeable at -10 d prepartum (Figure 9A). Despite the lack of time effect for the concentration of SAA most of the 30.9% increase in CON cows over Met-supplemented could be attributed to numerically-greater concentrations of SAA at -10 and 14 d relative to calving (Figure 9B). Although IL-6 was not directly affected by Met supplementation (Table 7), the tendency for a decrease ($P = 0.14$) in IL-6 concentration observed between prepartum and postpartum with MS and SM could be taken as indication that supplemental Met decreased the synthesis of this proinflammatory cytokine. The latter could have elicited an effect on the liver, effectively reducing the production of positive APP such as ceruloplasmin and SAA.

It could be envisioned that any factor reducing the synthesis of positive APP by the liver would spare some AA for the liver synthesis of negative APP and perhaps for utilization by the mammary gland. Under such scenario, supplemental SM and MS would have increased Met availability to the mammary gland and partly explain the greater

overall milk production and ECM (Osorio et al., 2013). Similar effects have been observed in chickens when they are raised under stressful environments that induce both an immune response and partitioning of nutrients, e.g. Lys and Met, towards immune cells (e.g. increasing IL-1) (Klasing and Barnes, 1988). The end result being a reduction in the AA requirements for growth and tissue accretion.

Biomarkers of oxidative stress

The total NO_2^- and NO_3^- concentration is a valid estimation of NOx generation in biological samples; NOx from NO_2^- and NO_3^- is referred to as NOx (Komine et al., 2004). The similar profiles in the concentration of NOx (Figure 10A), NO_{2-} (Figure 10E), and NO_{3-} (Figure 10B) agree with the proposed definition of NOx. We are unaware of previous studies reporting the pattern of NOx concentration blood around calving. However, milk secretions sampled at 4 wk intervals during the dry period and throughout lactation had greater concentration of NOx prepartum than postpartum (Bastan et al., 2013), which is similar to our results in blood.

Previous work with dairy cows demonstrated that NOx is a potent vasodilatory compound that can be produced locally by the mammary gland (Prosser et al., 1996). In turn, an increase in local NOx and NO_{3-} could increase the flow and mammary uptake of fatty acids and AA for milk synthesis (Bastan et al., 2013). Therefore, it could be possible that the greater concentration of NOx in SM at 7 d had a mechanistic role in the substantial increase in milk production (+2.7 kg/d greater than CON), fat yield (+0.48 kg/d greater than CON) and protein yield (+0.33 kg/d greater than CON) observed in SM cows within the first wk of lactation (Osorio et al., 2013).

The ORAC capacity was 5.9% greater in Met-supplemented cows, which could have been at least in part by the greater (22.6%) liver glutathione concentration and the greater concentration of vitamins such as retinol (Figure 11A). The total liver glutathione effect can be directly associated with Met supplementation because Met can be incorporated up-stream in the pathway for *de novo* glutathione synthesis (Halsted, 2013). Although, no significant D ×T interaction was observed in liver glutathione, there was an evident greater prepartal concentration in Met-supplemented cows (Figure 10D) that eventually decreased as lactation progressed. In mice, liver glutathione has been described as a reservoir for supplying AA such as Cys to the γ-glutamyl cycle (Lu, 2009). Glutathione in dairy cows can supply AA such as Cys to the mammary gland for milk synthesis (Pocius et al., 1981). Taken together, it is plausible that the accumulation of glutathione in liver during the close-up period in cows fed MS and SM served after calving as a "buffer" to spare Cys to promote milk synthesis, which was reflected by the greater milk production, milk protein %, and milk yield (Osorio et al., 2013).

The overall increase in ROM from pre to postpartum deviates slightly from findings observed previously by Bionaz et al. (2007), and Trevisi et al. (2009), where ROM concentration peaked at 7 d postpartum and substantially decreased by 28 d. Despite the lack of a D × T interaction, compared with CON and SM there was an evident reduction (15.6%) of ROM in MS from 7 d to 21 d postpartum (Figure 10E). These results coupled with the greater postpartal neutrophil activity in Met-supplemented cows (Osorio et al., 2013) indicate that ROM concentration was not entirely driven by concentrations of O_2- and H_2O_2 (e.g. produced during phagocytosis). There might be other underlying factors such as changes in NADH/NADPH oxidase, xanthine oxidase,

and arachidonic acid metabolism controlling the overall oxidative status (Cepinskas et al., 2002).

It also could be hypothesized that cows fed MS might have relied on other antioxidant sources such as vitamin E in the form of tocopherol or SOD to neutralize and lessen ROM levels arising from oxidative stress around parturition (Bionaz et al., 2007). The increases in concentration of tocopherol of 91.3% and 106% between 7 d to 21 d postpartum in cows fed MS and SM support its use as an anti-oxidant.

It has been previously observed that postpartal activity of SOD is lower particularly in cows with greater BCS before calving (Bernabucci et al., 2005). Taking that and the lower postpartal BCS in Met-supplemented cows (Osorio et al., 2013) into account, it could be possible that SOD activity partly influenced the reduction of ROM in MS cows after 7 d postpartum. Although this idea should also hold for SM cows, it is possible that rates of BCS loss postpartum (Bernabucci et al., 2005) and/or reduction in zinc and copper availability (Muehlenbein et al., 2001) after calving might have influenced SOD activity in SM cows.

Vitamins

Overall, the decrease in concentration of vitamins (Figure 11) from prepartum to 7 d postpartum is similar to results observed by Graugnard et al. (2012). The sharp increase from 7 to 21 d in concentration of retinol and tocopherol are in agreement with Bertoni et al. (2008). Moreover, those increases in retinol and tocopherol were particularly evident in the Met-supplemented cows (Figure 11A and 11B). Thus, results agree with previous data reporting that feeding methyl-deficient (choline and folate) diets

leads to a reduction of vitamin concentrations (ascorbic acid, α- and γ-tocopherol, and retinol) in tissues (i.e. lung, liver, and heart) and plasma (Henning et al., 1997).

In the case of α-tocopherol, Henning et al. (1997) attributed those effects to disturbances in VLDL synthesis (as a result of feeding choline-deficient diets) coupled with fatty acid and TAG accumulation in liver. The latter can affect the hepatic transport of α-tocopherol in the VLDL (Jenkins et al., 1993; Henning et al., 1997) where Jenkins et al. (1993) attributed a decrease in plasma retinol to an impairment of the release and/or transport of retinol in the blood via retinol-binding proteins.

From the above discussion it can be suggested that cows fed SM might have had greater ability to export/transport retinol into the blood stream at 21 d postpartum (Figure 11A).

CONCLUSION

Overall, the responses observed at the level of IL-6 and acute-phase proteins in cows supplemented with Smartamine M and MetaSmart revealed favorable alterations of the inflammatory and oxidative stress status of cows. The greater albumin in response to Met-supplementation suggested that rumen-protected Met promoted an enhanced liver function. Biomarker analyses in blood and tissue suggests that the beneficial effect of feeding SM and MS on postpartal cow performance is due partly to a better immunometabolic status.

REFERENCES

Auboiron, S., D. Durand, D. Bauchart, J. C. Robert, and M. J. Chapman. 1994. Lipoprotein metabolism in the preruminant calf: effect of a high fat diet supplemented with L-methionine. J Dairy Sci. 77:1870-1881.

Auboiron, S., D. Durand, J. C. Robert, M. J. Chapman, and D. Bauchart. 1995. Effects of dietary fat and L-methionine on the hepatic metabolism of very low density lipoproteins in the preruminant calf, Bos spp. Reprod Nutr Dev. 35:167-178.

Aviram, M. and M. Rosenblat. 2004. Paraoxonases 1, 2, and 3, oxidative stress, and macrophage foam cell formation during atherosclerosis development. Free Radic Biol Med. 37:1304-1316.

Bach, A., S. Calsamiglia, and M. D. Stern. 2005. Nitrogen metabolism in the rumen. J Dairy Sci. 88 Suppl 1:E9-21.

Bastan, A., M. Cengiz, S. Cengiz, T. Sel, B. Polat, A. Colak, M. Akan, and I. Darbaz. 2013. Nitric oxide concentrations in mammary quarters during heifer mastitis. Animal. 7:499-504.

Bernabucci, U., B. Ronchi, N. Lacetera, and A. Nardone. 2005. Influence of body condition score on relationships between metabolic status and oxidative stress in periparturient dairy cows. J Dairy Sci. 88:2017-2026.

Bertoni, G., E. Trevisi, X. Han, and M. Bionaz. 2008. Effects of inflammatory conditions on liver activity in puerperium period and consequences for performance in dairy cows. J Dairy Sci. 91:3300-3310.

Bionaz, M., E. Trevisi, L. Calamari, F. Librandi, A. Ferrari, and G. Bertoni. 2007. Plasma paraoxonase, health, inflammatory conditions, and liver function in transition dairy cows. J Dairy Sci. 90:1740-1750.

Cao, G. and R. L. Prior. 1999. Measurement of oxygen radical absorbance capacity in biological samples. Methods Enzymol. 299:50-62.

Ceciliani, F., J. J. Ceron, P. D. Eckersall, and H. Sauerwein. 2012. Acute phase proteins in ruminants. J Proteomics. 75:4207-4231.

Cepinskas, G., T. Rui, and P. R. Kvietys. 2002. Interaction between reactive oxygen metabolites and nitric oxide in oxidant tolerance. Free Radic Biol Med. 33:433-440.

Drackley, J. K. 1999. ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: the final frontier? J Dairy Sci. 82:2259-2273.

Durand, D., Y. Chilliard, and D. Bauchart. 1992. Effect of lysine and methionine on in vivo hepatic secretion of VLDL in dairy cow. J Dairy Sci. 75:279.

Firkins, J. L., Z. Yu, and M. Morrison. 2007. Ruminal nitrogen metabolism: perspectives for integration of microbiology and nutrition for dairy. J Dairy Sci. 90 Suppl 1:E1-16.

Graugnard, D. E., M. Bionaz, E. Trevisi, K. M. Moyes, J. L. Salak-Johnson, R. L. Wallace, J. K. Drackley, G. Bertoni, and J. J. Loor. 2012. Blood immunometabolic indices and polymorphonuclear neutrophil function in peripartum dairy cows are altered by level of dietary energy prepartum. J Dairy Sci. 95:1749-1758.

Grossi, P., G. Bertoni, F. P. Cappelli, and E. Trevisi. 2013. Effects of the precalving administration of omega-3 fatty acids alone or in combination with acetylsalicylic acid in periparturient dairy cows. J Anim Sci. 91:2657-2666.

Haeggstrom, J. Z. and C. D. Funk. 2011. Lipoxygenase and leukotriene pathways: biochemistry, biology, and roles in disease. Chem Rev. 111:5866-5898.

Halsted, C. H. 2013. B-Vitamin dependent methionine metabolism and alcoholic liver disease. Clin Chem Lab Med. 51:457-465.

Henning, S. M., M. E. Swendseid, B. T. Ivandic, and F. Liao. 1997. Vitamins C, E and A and heme oxygenase in rats fed methyl/folate-deficient diets. Free Radic Biol Med. 23:936-942.

Jenkins, M. Y., N. M. Sheikh, G. V. Mitchell, E. Grundel, S. R. Blakely, and C. J. Carter. 1993. Dietary carotenoids influenced biochemical but not morphological changes in adult male rats fed a choline-deficient diet. Nutr Cancer. 19:55-65.

Kass, S. U., D. Pruss, and A. P. Wolffe. 1997. How does DNA methylation repress transcription? Trends Genet. 13:444-449.

Kindt, T. J., R. A. Goldsby, B. A. Osborne, and J. Kuby. 2007. Kuby immunology. 6th ed. W.H. Freeman, New York.

Klasing, K. C. and D. M. Barnes. 1988. Decreased amino acid requirements of growing chicks due to immunologic stress. J Nutr. 118:1158-1164.

Kokkonen, T., J. Taponen, T. Anttila, L. Syrjala-Qvist, C. Delavaud, Y. Chilliard, M. Tuori, and A. T. Tesfa. 2005. Effect of body fatness and glucogenic supplement on lipid and protein mobilization and plasma leptin in dairy cows. J Dairy Sci. 88:1127-1141.

Komine, K., T. Kuroishi, Y. Komine, K. Watanabe, J. Kobayashi, T. Yamaguchi, S. Kamata, and K. Kumagai. 2004. Induction of nitric oxide production mediated by

tumor necrosis factor alpha on staphylococcal enterotoxin C-stimulated bovine mammary gland cells. Clin Diagn Lab Immunol. 11:203-210.

LeBlanc, S. J. 2012. Interactions of metabolism, inflammation, and reproductive tract health in the postpartum period in dairy cattle. Reprod Domest Anim. 47 Suppl 5:18-30.

Loor, J. J., G. Bertoni, A. Hosseini, J. R. Roche, and E. Trevisi. 2013. Functional welfare - using biochemical and molecular technologies to understand better the welfare state of peripartal dairy cattle. Anim Prod Sci. 53:931-953.

Lu, S. C. 2009. Regulation of glutathione synthesis. Mol Aspects Med. 30:42-59.

Martinov, M. V., V. M. Vitvitsky, R. Banerjee, and F. I. Ataullakhanov. 2010. The logic of the hepatic methionine metabolic cycle. Biochimica et biophysica acta. 1804:89-96.

Muehlenbein, E. L., D. R. Brink, G. H. Deutscher, M. P. Carlson, and A. B. Johnson. 2001. Effects of inorganic and organic copper supplemented to first-calf cows on cow reproduction and calf health and performance. J Anim Sci. 79:1650-1659.

O'Boyle, N., C. M. Corl, J. C. Gandy, and L. M. Sordillo. 2006. Relationship of body condition score and oxidant stress to tumor necrosis factor expression in dairy cattle. Vet Immunol Immunopathol. 113:297-304.

Osorio, J. S., P. Ji, J. K. Drackley, D. Luchini, and J. J. Loor. 2013. Supplemental Smartamine M or MetaSmart during the transition period benefits postpartal cow performance and blood neutrophil function. J Dairy Sci. 96:6248-6263.

Pires, J. A., C. Delavaud, Y. Faulconnier, D. Pomies, and Y. Chilliard. 2013. Effects of body condition score at calving on indicators of fat and protein mobilization of periparturient Holstein-Friesian cows. J Dairy Sci. 96:6423-6439.

Pocius, P. A., J. H. Clark, and C. R. Baumrucker. 1981. Glutathione in bovine blood: possible source of amino acids for milk protein synthesis. J Dairy Sci. 64:1551-1554.

Prosser, C. G., S. R. Davis, V. C. Farr, and P. Lacasse. 1996. Regulation of blood flow in the mammary microvasculature. J Dairy Sci. 79:1184-1197.

Trevisi, E., M. Amadori, A. M. Bakudila, and G. Bertoni. 2009. Metabolic changes in dairy cows induced by oral, low-dose interferon-alpha treatment. J Anim Sci. 87:3020-3029.

Trevisi, E., M. Amadori, S. Cogrossi, E. Razzuoli, and G. Bertoni. 2012. Metabolic stress and inflammatory response in high-yielding, periparturient dairy cows. Res Vet Sci. 93:695-704.

Turk, R., D. Juretic, D. Geres, N. Turk, B. Rekic, V. Simeon-Rudolf, M. Robic, and A. Svetina. 2005. Serum paraoxonase activity in dairy cows during pregnancy. Res Vet Sci. 79:15-18.

TABLES AND FIGURES

Table 7. Effects of supplementing cows with MetaSmart® or Smartamine® M during the peripartal period on liver glutathione and blood biomarkers for muscle body mass catabolism, liver function, inflammation and APP, oxidative stress, and vitamins.

	Treatment				*P*-value			
Parameter	CON	MS	SM	SEM[1]	Diet	Met[2]	Time	D×T[4]
Muscle mass catabolism and N metabolism								
Creatinine, µmol/L	97.5	96.6	95.7	1.56	0.38	0.43	< 0.001	0.10
Urea, mmol/L	4.87	4.87	4.87	0.19	0.70	0.97	0.005	0.90
Liver function								
Albumin, g/L	35.1	36.1	35.7	0.53	0.28	0.15	0.04	0.20
Total bilirubin,[3] µmol/L	0.88(1.84)	0.58(1.49)	0.57(1.48)	0.49	0.66	0.70	< 0.001	0.97
GGT, U/L	24.9	24.1	27.2	2.26	0.61	0.78	< 0.001	0.48
AST/GOT, U/L	98.7	95.8	86.6	7.04	0.39	0.34	< 0.001	0.63
Cholesterol, mmol/L	3.22	3.29	3.36	0.15	0.77	0.52	< 0.001	0.96
PON, U/mL	64.9	68.3	63.6	3.74	0.64	0.80	< 0.001	0.81
Inflammation and APP								
Ceruloplasmin, µmol/L	3.02	2.68	2.71	0.10	0.03	0.009	< 0.001	0.67
SAA, µg/mL	61	40.7	43.5	9.3	0.17	0.06	0.24	0.47
Haptoglobin,[3] g/L	-1.48(0.36)	-1.70(0.31)	-1.67(0.31)	0.18	0.6	0.32	0.01	0.60
IL-6, pg/mL	612.1	546.3	447.4	93	0.40	0.29	< 0.001	0.73
Oxidative stress								
NOx, µmol/L	23.5	22.8	23.5	0.67	0.69	0.69	< 0.001	0.06
NO_3^-, µmol/L	17	16.5	16.9	0.51	0.7	0.57	< 0.001	0.04
ORAC, mol/L	11.9	12.9	12.4	0.29	0.05	0.04	< 0.001	0.66
Liver glutathione, mM	1.27	1.55	1.73	0.14	0.09	0.04	< 0.001	0.45
ROM, mg H_2O_2/100 mL	13.7	12.8	14.2	0.61	0.24	0.73	< 0.001	0.62
NO_2^-, µmol/L	6.42	6.7	6.6	0.54	0.92	0.70	< 0.001	0.27
Vitamins								
Retinol, µg/100 mL	40	37.7	42.3	2.3	0.40	0.98	< 0.001	0.06
Tocopherol, µg/mL	3.44	3.79	3.06	0.51	0.58	0.98	< 0.001	0.21
β-carotene, mg/100 mL	0.19	0.21	0.24	0.02	0.34	0.22	< 0.001	0.22

[1]Greatest standard error of the mean.
[2]Contrast statement of CON vs MS+SM.
[3]Data were log-transformed before statistics. Back-transformed least square means are shown in parentheses. The standard error of the means associated with log-transformed data are in log scale.
[4]Interaction of treatment by time.

Table 8. Contrast estimates of blood biomarkers concentrations for muscle body mass, liver function, inflammation and APP, oxidative stress, and vitamins between prepartum (-21 and -10 d relative to calving) versus postpartum (7, 14, and 21 d relative to calving) in cows supplemented with MetaSmart or Smartamine M during the peripartal period.

	Contrast prepartum vs postpartum					
	CON		MS		SM	
Parameter	Estimate[1]	*P*-value	Estimate	*P*-value	Estimate	*P*-value
Muscle mass and N metabolism						
Urea, mmol/L	0.49	0.08	0.29	0.33	0.16	0.62
Creatinine, µmol/L	0.06	0.98	-3.4	0.14	0.44	0.85
Liver function						
Total bilirubin, µmol/L	-3.2(0.11)	< 0.001	-3.7(0.10)	< 0.001	-3.2(0.11)	< 0.001
AST/GOT, U/L	-67.0	< 0.001	-53.1	< 0.001	-54.0	< 0.001
Cholesterol, mmol/L	-0.43	0.02	-0.47	0.02	-0.65	0.02
GGT, U/L	-5.68	0.002	-2.41	0.2	-9.5	< 0.001
PON, U/mL	13.5	0.002	11.1	0.02	6.4	0.19
Inflammation and APP						
Haptoglobin, g/L	-0.23(0.85)	0.45	0.53(1.44)	0.11	-0.03(0.98)	0.94
Ceruloplasmin, µmol/L	-0.53	0.002	-0.55	0.003	-0.75	< 0.001
IL-6, pg/mL	70.4	0.14	192.9	< 0.001	95.5	0.05
Albumin, g/L	1.43	0.01	-0.09	0.88	-0.81	0.21
SAA, µg/mL	4.8	0.75	-13.9	0.43	-12.9	0.46
Oxidative stress						
ROM, mg H_2O_2/100 mL	-1.6	0.06	-1.5	0.11	-2.6	0.007
ORAC, mol/L	-653	0.06	-820	0.02	-911	0.02
NOx, µmol/L	4.3	< 0.001	3.2	0.001	2.1	0.04
NO_2^-, µmol/L	1.65	0.02	1.63	0.04	1.12	0.18
NO_3^-, µmol/L	2.67	< 0.001	1.30	0.04	1.02	0.12
Vitamins						
Retinol, µg/100 mL	9.29	0.006	7.43	0.06	4.78	0.24
Tocopherol, µg/mL	1.54	< 0.001	0.92	0.06	1.76	0.002
β-carotene, mg/100 mL	0.08	0.008	0.06	0.06	0.10	0.007

[1]Estimate of the contrast of blood biomarkers concentrations prepartum [-21 plus -10 d relative to calving] versus postpartum [7, 14, and 21 d relative to calving], where positive (+) estimates represent a decrease and negative estimates (-) an increase in concentrations.

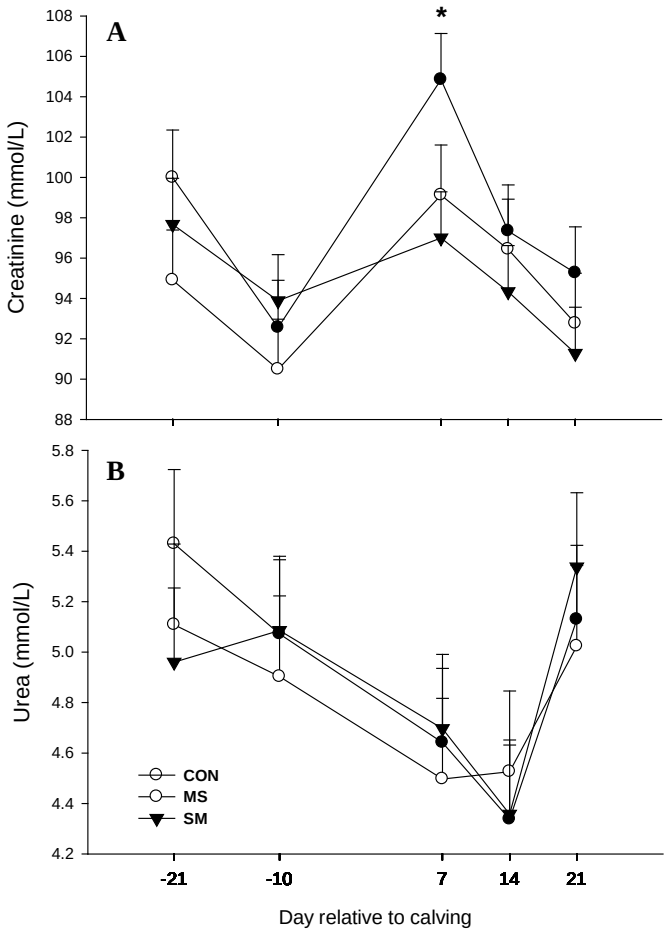

Figure 7. Effects of supplemental MetaSmart (MS; Adisseo Inc., Antony, France) or Smartamine M (SM; Adisseo Inc.) on biomarkers of muscle body mass catabolism (urea and creatinine) in dairy cows during the transition period. Mean separation between diets ($P < 0.05$) were evaluated via contrast: control (CON) versus SM (*).

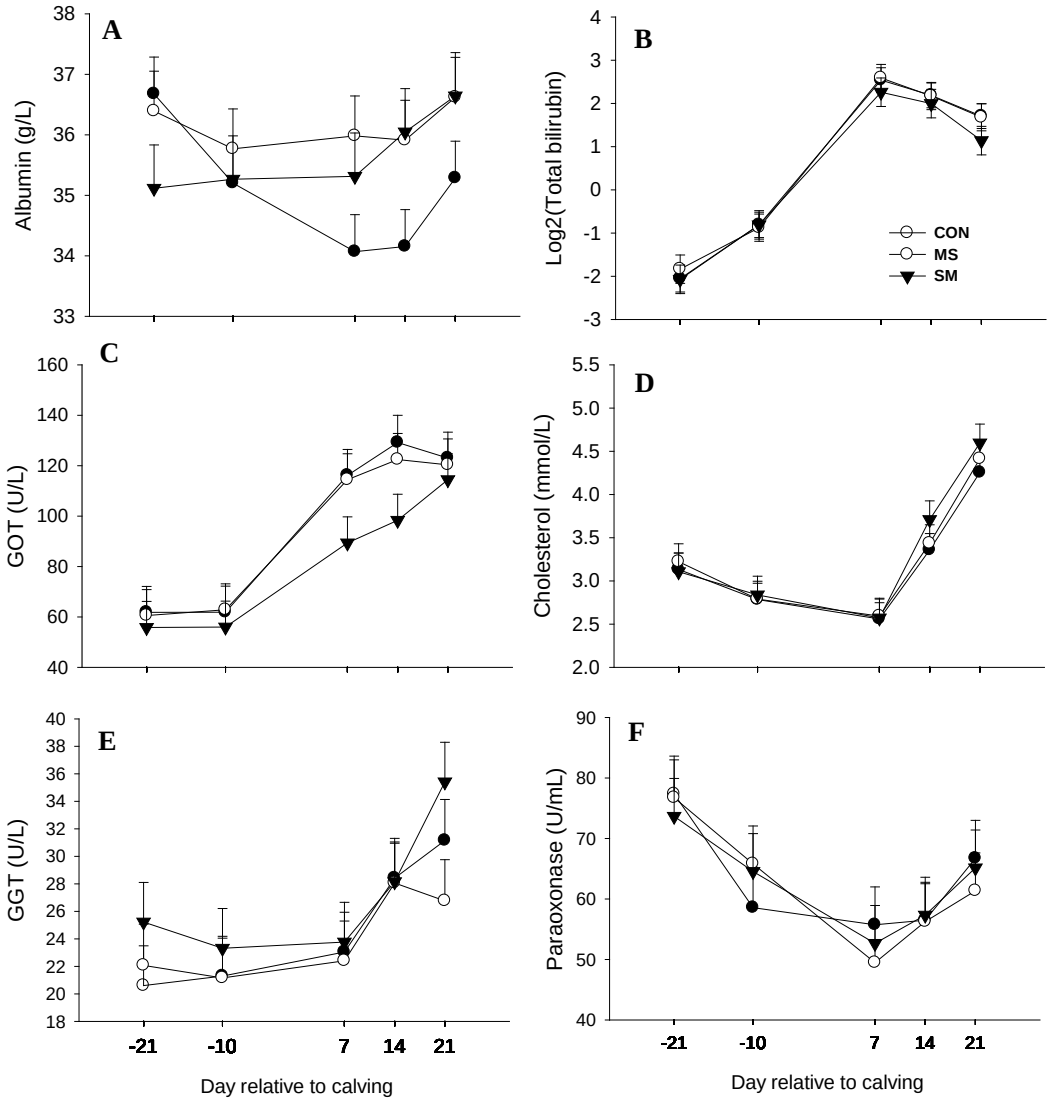

Figure 8. Effects of supplemental MetaSmart (MS; Adisseo Inc., Antony, France) or Smartamine M (SM; Adisseo Inc.) on liver function-related biomarkers in dairy cows during the transition period. Mean separation between diets ($P < 0.05$) were evaluated via contrast: control (CON) versus MS + SM (*). GOT = glutamic-oxalacetic transaminase, GGT = gamma-glutamyl transferase.

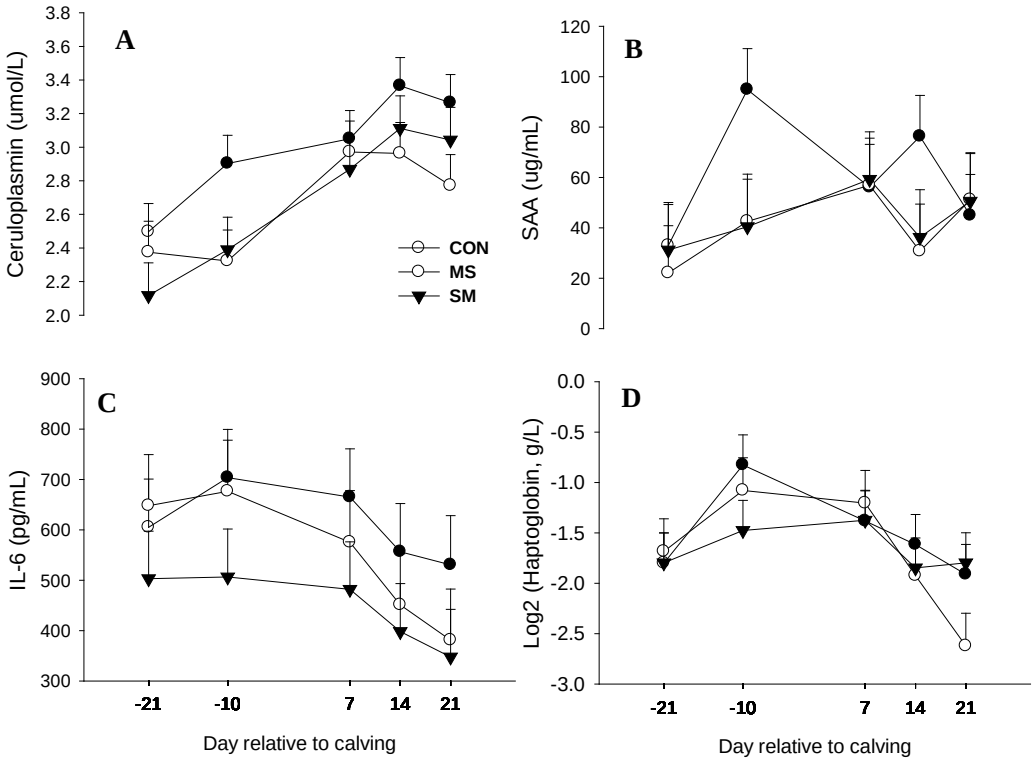

Figure 9. Effects of supplemental MetaSmart (MS; Adisseo Inc., Antony, France) or Smartamine M (SM; Adisseo Inc.) on inflammatory biomarkers in dairy cows during the transition period. Mean separation between diets ($P < 0.05$) were evaluated via contrast: control (CON) versus MS + SM (*). IL-6 = Interleukin 6, SAA = serum amyloid A.

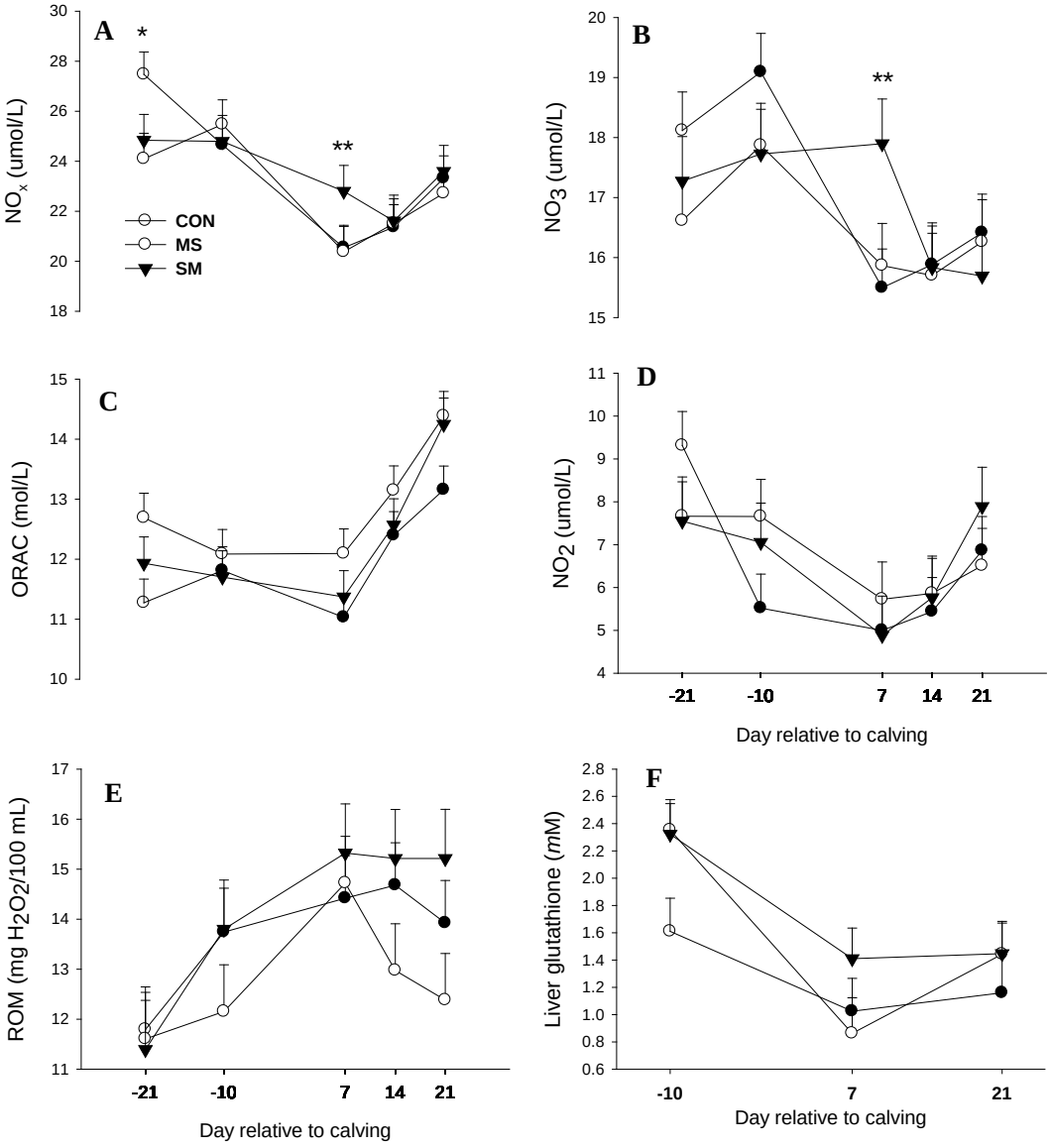

Figure 10. Effects of supplemental MetaSmart (MS; Adisseo Inc., Antony, France) or Smartamine M (SM; Adisseo Inc.) on oxidative stress biomarkers in dairy cows during the transition period. Mean separation between diets ($P < 0.05$) were evaluated via contrast: control (CON) versus MS + SM (*) and CON versus SM (**). NO = nitric oxide, NO_3 = nitrate, NO_2 = nitrite, ORAC = oxygen radical absorbance capacity, ROM = reactive oxygen metabolites.

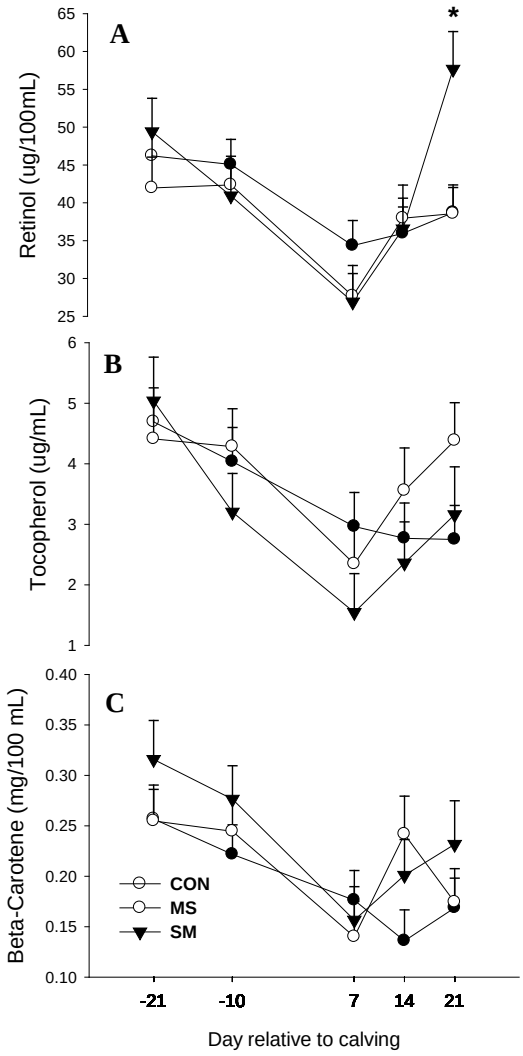

Figure 11. Effects of supplemental MetaSmart (MS; Adisseo Inc., Antony, France) or Smartamine M (SM; Adisseo Inc.) on concentration of vitamins in dairy cows during the transition period. Mean separation between diets ($P < 0.05$) were evaluated via contrast: control (CON) versus SM (*).

CHAPTER 4

HEPATIC TRANSCRIPTOMICS OF PERIPARTAL DAIRY COWS SUPPLEMENTED WITH SMARTAMINE M OR METASMART

INTRODUCTION

The importance of methionine (Met) as one of the most limiting AA for milk protein synthesis in dairy cows has been well established by previous research (Schwab et al., 1992; Pisulewski et al., 1996). Similar effects have been observed soon after calving when supplementing Met during the peripartal period leading to increased milk production and milk fat yield (Osorio et al., 2013). The latter observation could be partially associated with an increase in postpartal DMI when cows are supplemented with Met (Ordway et al., 2009; Osorio et al., 2013). Improved postpartal performance also could be linked to a reduced peripartal inflammatory response when supplementing Met (Osorio et al., 2012).

Increased Met bioavailability in Met-supplemented cows (Graulet et al., 2005) is likely to enter the Met cycle (Figure 12) that is closely related to the methylation and transsulfuration cycles. Therefore, Met is intrinsically related to several processes that can use this AA as a precursor. For instance, Met can be converted to s-adenosylmethionine (SAM) and used for methylation, or SAM can be further metabolized through the Met cycle to form cystathionine that can enter glutathione metabolism pathway or the Krebs cycle at the succinyl-CoA level.

The importance of the Met and methylation cycles has been previously studied during the peripartal period by supplementing methyl groups (e. g., rumen-protected Met, folic acid, and vitamin B_{12}), but with limited effect on dairy cow performance (Preynat et

al., 2010). In contrast, Goselink et al. (2013) observed that rumen-protected choline reduced liver triacylglycerol (TAG) by enhancing FA processing and very-low-density lipoprotein (VLDL), synthesis presumably by increasing gene expression of *APOB* and *MTTP*.

DNA methylation is one of the epigenetic modifications involved in the regulation of gene expression and hence it is an important biological function. It is well established that cells can undergo global DNA hypomethylation while having hypermethylation of a specific gene promoter; consequently the expression of the gene is silenced or attenuated when its promoter is heavily methylated (Wang et al., 2013). In fact, methyl-deficient diets have been associated with hypermethylation of specific gene promoters of genes involved in lipid metabolism (Tryndyak et al., 2011). Recently, a study testing betaine as a methyl donor in order to reduce hypermethylation of specific promoter genes in male rats uncovered an up-regulation of *PPARA* as a result of hypomethylation of its promoter (Wang et al., 2013). This finding provides a novel insight into the important lipotropic effects of methyl-donors such as betaine or Met in the form of SAM. Peripartal dairy cows may benefit from activation of *PPARA* via Met supplementation, due to several adaptions at the metabolic and inflammatory level driven by activated *PPARA* (Bionaz et al., 2013). Among these adaptations by *PPARA* is the targeting of other genes such as fibroblast growth factor-21 (*FGF21*) and angiopoietin-like 4 (*ANGPTL4*). FGF21 in turn can stimulate hepatic gluconeogenesis, glucose uptake in adipocytes, and fatty oxidation and ketogenesis (Schoenberg et al., 2011). Upon activation, *ANGPTL4*, a positive acute phase protein, inhibits LPL activity and enhances lipolysis as a response driven by undernutrition (Loor et al., 2007). Gluconeogenesis

activation via *FGF21* encompasses the activation of genes such as pyruvate dehydrogenase kinase isozyme 4 (*PDK4*) in male rats (Palou et al., 2008), and activation of phosphoenolpyruvate carboxykinase 1 (*PCK1*) and pyruvate carboxylase (*PC*) in peripartal dairy cows (Akbar et al., 2013).

Our hypothesis was that dietary Met supplementation during the peripartal period will increase the pool of Met flowing through the Met cycle, and eventually might influence the RNA expression of enzymes involved in this cycle that produce intermediate components such SAM or cystathionine that might ameliorate negative effects observed during the peripartal period. While SAM can increase global methylation and reduce hypermethylation of specific promoters, cystathionine can be drawn to glutathione metabolism since previously we observed greater glutathione in Met-supplemented cows (Osorio et al., 2013).

The objective of this experiment was to evaluate at the transcriptomics level the effects of supplementing Met in the form of Smartamine (SM) and MetaSmart (MS) specifically focusing on Met, Glutathione, lipid, and carbohydrate metabolism as well as the growth hormone (GH)-IGF1 axis and methylation during the peripartal period, with the aim to draw further understanding of previous results observed (Osorio et al., 2012; Osorio et al., 2013).

MATERIALS AND METHODS

Animals and experimental design

All procedures were conducted under protocols approved by the University of Illinois Institutional Animal Care and Use Committee. Details of the experimental design

have been published previously (Osorio et al., 2013). Briefly, 25 Holstein cows entering their second or greater lactation were enrolled in the study and were fed experimental treatments consisting of a basal control diet (CO, n = 14) with no Met supplementation, CO plus MS (n =12) at a rate of 0.19% of DM, and CO plus SM (n = 13) at a rate of 0.07% of DM (Osorio et al., 2013). All cows received the same far-off diet (1.24 Mcal/kg DM, 14.3% CP) from -50 to -21 d before expected calving, a close-up diet (1.54 Mcal/kg DM, 15% CP) from -21d to expected calving, and a fresh-cow diet from calving (1.75 Mcal/kg DM, 17.5% CP) through 30 DIM. Methionine supplements were top-dressed from -21 to 30 DIM. A subset of cows (CO, n = 8; MS, n = 8; SM, n = 9) was used to evaluate hepatic transcriptomic profile.

Animal husbandry for feeding system, sampling of ingredients and TMR, BW, BCS, milk weights, sampling for milk composition, and housing of cows pre- and postpartum were as reported previously (Osorio et al., 2013).

Liver biopsy, RNA extraction, quantitative PCR, and design and evaluation of primers

Liver was sampled via puncture biopsy (Dann et al., 2006) from cows under local anesthesia at approximately 0730 h on −10, 7, and 21 d relative to parturition. Tissue specimens were stored in liquid N_2 until RNA extraction.

Total RNA was extracted from liver samples using establish protocols in our laboratory (see supplementary materials or details: http://www.journalofdiarysciences.org/). Details of quantitative PCR, design, and primer evaluation are presented in the supplementary materials. Percentage relative abundance

of mRNA was calculated to provide additional mechanistic information on the target genes (Bionaz and Loor, 2008).

Statistical analysis

The data of gene expression were analyzed with the Proc MIXED procedure of SAS 9.3 (SAS Institute, Inc., Cary, NC, USA). The fixed effects in the model included diet, parity, time, and their interactions. Cow was designated as a random effect. Parity and its interactions were removed from the model when these effects were non-significant (P > 0.10). Unequally spaced data were analyzed using the exponential correlation covariance structure SP for repeated measures. Least-squares means separation between time points was performed using the PDIFF statement. Statistical significance was declared at $P \leq 0.10$, and tendencies at $P \leq 0.15$.

RESULTS

Methionine metabolism

Main effects of diet and time and interactions on genes associated with Met metabolism are presented in Figure 13. There was an interaction of D×T for *MAT1A* and *CBS*, where expression of *MAT1A* in MS cows was lower ($P = 0.10$) than CON at -10 d while greater ($P = 0.09$) at 21 d. Similarly, expression of *CBS* was greater ($P = 0.06$) in MS than CON at 21 d. Expression of *SAHH* ($P < 0.01$), *MTR* ($P = 0.03$), and *PEMT* ($P = 0.09$) was greater in Met-supplemented cows; in contrast *CSAD* was lower ($P = 0.03$).

Expression of *CTH*, *BHMT*, and *BHMT2* was affected by time only, where *CTH* decreased while in contrast *BHMT* and *BHMT2* increased over time.

Glutathione metabolism

Main effects of diet and time and interactions on genes associated with glutathione metabolism are presented in Figure 14. The interaction of D×T was not significant for any of the genes related to glutathione metabolism. However, the expression of *GSS* was affected ($P < 0.01$) by diet as reflected in a lower ($P = 0.05$) expression in Met-supplemented cows than CON. Similarly, the expression of *GSR* tended ($P = 0.12$) to be affected by diet, where the expression was greater ($P = 0.05$) in Met-supplemented cows than CON. Although *GPX1* and *GCLC* were not affected by diet, their expression decreased ($P < 0.01$) during the transition period.

Inflammation

Main effects of diet and time and interactions on genes associated with inflammation are presented in Figure 15. The interaction of D×T was not significant for any of the genes related to inflammation response. However, the expression of *NFKB1* was significantly ($P < 0.01$) affected by diet. Although, expression of *NFKB1* greater ($P = 0.01$) expression in Met-supplemented cows than CON, this effect was a result of greater *NFKB1* mRNA in SM cows than MS and CON. Expression of *CP* and *SAA2* decreased ($P = 0.01$) over time regardless of diet. Although similar ($P = 0.65$) expression of *HP* was observed at 21 d and -10 d there was an evident up-regulation at 7 d

postpartum primarily in Met-supplemented cows (Figure 15); however, this effect did not reach statistical significance. Expression of *STAT3* was not affected (Figure 15).

Oxidative stress

Main effects of diet and time and interactions on genes associated with oxidative stress are presented in Figure 16. There was a trend ($P = 0.13$) for the interaction D×T for *SOD1* and *BBOX1*, where at 21 d postpartum the expression of *SOD1* in MS cows was greater ($P = 0.10$) than CON, while SM cows had greater (P = 0.07) expression of *BBOX1* than CON. Additionally, Met-supplemented cows tended ($P = 0.12$) to have lower expression of *SOD1* than CON. In contrast to *SOD1*, expression of *SLC22A5* tend ($P = 0.13$) to be greater in Met-supplemented cows. Expression of *TMLHE* was affected by time (P < 0.01), while neither diet nor time affected the expression of *SOD2* (Figure 16).

Fatty acid metabolism

Main effects of diet and time and interactions on genes associated with fatty acid metabolism are presented in Figure 17. The interaction D×T was not significant for any of the genes related to fatty acid metabolism. However, the expression of *RXRA* was affected ($P = 0.02$) by diet such that expression was greater ($P = 0.06$) in Met-supplemented cows than CON. Similarly, there was a trend ($P = 0.12$) for a diet effect in the expression of *PPARA*, where the expression was greater ($P = 0.04$) in Met-supplemented cows than CON. Like *SLC22A5* and *TMLHE* (Figure 17), the expression of *CPT1A* increased from -10 d to 7 d postpartum, followed by a decrease at 21 d, which

resulted in a significant time effect (P < 0.01). Diet and time did not affect the expression of *ACOX1* and *HMGCS2* (Figure 17).

Hepatokines, gluconeogenesis, and VLDL synthesis and export

Main effects of diet and time and interactions on genes associated with hepatokines, gluconeogenesis, and VLDL synthesis and export are presented in Figure 18. The expression of *FGF21* (*P* < 0.01) and *PCK1* (*P* = 0.08) were significantly affected by the interaction D×T. Where *FGF21* expression was greater (*P* < 0.006) in SM cows than CON at -10 d, expression tended (*P* = 0.14) to be greater in MS cows than CON . Similar to *FGF21*, Met-supplemented cows had a greater (*P* < 0.04) expression of *PCK1* at -10 d and 7 d, which resulted in an overall up-regulation (*P* < 0.01) of *PCK1* in Met-supplemented cows. Expression of *ANGPTL4* resulted in a trend for the interaction D×T, which revealed a greater expression in both SM (*P* = 0.04) cows -10 d and MS (*P* = 0.07) cows at 7 d than CON cows. Additionally, SM cows tended (*P* = 0.13) to have greater expression of *ANGPTL4* than CON at 7 d postpartum. While Met-supplemented cows had lower (*P* = 0.02) expression of *MTTP*, there was a trend (*P* = 0.13) for a greater PDK4 in comparison to CON cows. The expression of *PC* (*P* = 0.03) and *APOB* (*P* = 0.10) was affected by diet, where PC was lower (*P* < 0.08) in SM cows than MS and CON, but *APOB* was greater (*P* < 0.10) in SM cows.

Growth hormone signaling

Main effects of diet and time and interactions on genes associated with growth hormone signaling are presented in Figure 19. The expression of *SOCS2* (*P* = 0.11) and

IGF1 ($P = 0.13$) were affected by the interaction D×T. The latter resulted in MS cows having greater ($P = 0.11$) *SOCS2* than CON cows at 7 d postpartum. Expression of *IGF1* was greater in SM ($P = 0.06$) and MS ($P = 0.15$) than CON cows at -10 d, followed by greater ($P = 0.15$) *IGF1* in SM than CON at 21 d. Expression of *STAT5B* decreased ($P < 0.01$) during the transition period regardless of treatment effect.

DNA Methylation

Main effects of diet and time and interactions on genes associated with DNA methylation are presented in Figure 20. The interaction D×T was not significant for any of the genes related to DNA methylation. The expression of *DNMT1* and *DNMT3A* was not affected by diet, but decreased by 21 d postpartum. Unlike *DNMT3A*, *DNMT1* expression remained unchanged from -10 d to 7 d postpartum, at which point *DNMT3A* expression decreased and remained unchanged until 21 d. In contrast, *DNMT1* expression was downregulated from 7 d to 21 d postpartum.

DISCUSSION

In previous published data from the same experiment (Osorio et al., 2013), it was reported that supplementation with MS or SM improved milk production. This effect occurred at least in part, by increasing postpartal DMI and perhaps by reduction of postpartal BCS. The latter observation was also associated with an earlier recovery towards a positive energy balance. Additionally, there was evidence for a lesser inflammatory response in Met-supplemented cows during the peripartal period (Osorio et al., 2012).

Methionine metabolism

Activity of S-adenosylhomocysteine hydrolase (SAHH) has not been extensively studied in ruminants and especially in dairy cattle. However, its importance in the methylation cycle in connection to hypermethioninemia, by hydrolyzing SAH to Hcy, has been established in humans and mice (Baric, 2009). In the current experiment *SAHH* expression was the most abundant of any gene studied (Table1), which presumably underlines the importance of this gene under the conditions and experimental design of the study. Increased Met bioavailability in Met-supplemented cows in the forms of either MS or SM (Graulet et al., 2005) was likely to increase activity of the Met cycle and therefore modify the expression of genes related to the Met cycle such as *MAT1A* and *SAHH*. Because SAHH, a substrate-dependent enzyme, can convert SAH to Hcy or vice versa, this enzyme might have played an important role in both the availability of SAM and Hcy. Conversion of SAH to Hcy might render Hcy as a competitor against a number of other methyl-receptors for use of SAM (Martinov et al., 2010). On the other hand, Hcy could serve as an important substrate in the synthesis of compounds such as glutathione. The *MAT1A* gene encodes both MATI and MATIII isoenzymes present in the liver of mammals, where MATI is tetrameric and MATIII is dimeric (Martinov et al., 2010). Besides having different configurations MATI is inhibited by SAM, whereas MATIII is activated. The latter differences between MATI and MATII coupled with a prepartal down-regulation of *MAT1A* at -10 d in MS vs CON, followed by postpartal up-regulation at 21 d, might be an indicator of the SAM status. However, the redundancy of SAM synthesis via MATI/MATIII is suggestive of a more complex regulatory system for

SAM. Besides *MAT1A*, greater expression of CBS in MS vs CON could be indicative of greater concentration of SAM, since SAM can exert allosteric activation of CBS (Martinov et al., 2010).

Greater expression of *PEMT* in Met-supplemented cows was more evident at 21 d (Figure 13), which may indicate a greater availability of phosphatidylcholine (PC; Figure 12), an important constituent of lipoproteins such as VLDL. Synthesis of VLDL is essential in lactating ruminants not only to respond to the increased demand for milk fat synthesis, but also to mitigate hepatic steatosis commonly observed after calving. In fact, the latter is in agreement with previous published data (Osorio et al., 2013) from the same experiment where Met-supplemented cows had lower liver lipid concentration at 21 d postpartum. In contrast to our results, *PEMT* has been previously reported to be unresponsive to rumen-protected Met during the transition period (Preynat et al., 2010). In fact, expression of *PEMT* decreased throughout the experiment. The previous differences among *PEMT* response to Met supplementation could be associated not only with a greater rate of Met supplementation in our experiment (2.25% vs 1.83%) but also because the ratio Lys:Met was maintained at 2.8:1 through the experiment.

Homocysteine is a sulfur-containing AA that upon remethylation by BHMT or MTR can regenerate Met from Hcy by transferring a methyl group from 5-methyltetrahydrofolate (5-MTHF) and betaine, respectively (Preynat et al., 2010). Supplementation of Met affected *MTR* expression but not *BHMT* or *BHMT2*, which indicates that regeneration of Met from Hcy relied on 5-MTHF with vitamin B_{12} as a coenzyme. However, the expression of *MTR* decreased over time while *BHMT* and *BHMT2* increased over time. This indicates that Met regeneration through methylation of

Hcy was pivotal having prepartal dependence on *MTR* and postpartal dependence on *BHMT*/*BHMT2*.

The fate of cysteine metabolism at the branch-point of cysteine sulfinic acid (CSA) between sulfate and taurine biosynthetic pathways has been observed to be regulated by CSAD activity, where decreased activity of this enzyme lead to the biosynthesis of taurine (Jerkins et al., 1998) and transamination of CSA leads to formation of pyruvate and sulfate. Feeding high-protein diets to rats resulted in a decreased *CSAD* mRNA and consequently lower CSAD enzyme activity (Jerkins et al., 1998). Similar results were observed in Met-supplemented cows with lower expression of *CSAD*, which indicates that cysteine that did not enter the glutathione metabolism and was likely to enter the sulfate pathway producing sulfate and pyruvate. The latter is an important substrate in gluconeogenesis, which is an essential process during the transition period due the greater demand of glucose for milk production.

Glutathione metabolism

The importance of anti-oxidant activity such as glutathione peroxidase during the peripartal period has been well established (Spears and Weiss, 2008), and its activity decreases after calving (Weiss et al., 1990). Increased Met bioavailability through Met supplementation can indirectly increase the production of total hepatic glutathione through the transsulfuration pathway (Osorio et al., 2013). Increased concentration of glutathione has been observed to produce a feedback inhibition effect on the enzyme GCL (Franklin et al., 2009), although this was not associated with a lower *GCLC* mRNA in Met-supplemented cows (Figure 14). In fact, lower mRNA of *GSS* and *GSR* was

observed in Met-supplemented cows, which might be related to lower oxidative stress that triggers greater demand for these enzymes (Lu, 2009). This conjecture is supported by overall lower oxidative stress status in Met-supplemented cows as indicated by oxygen radicals absorbance capacity (ORAC) previously reported (Osorio et al., 2012). Expression of *GPX1* and *GCLC* decreased over time, which may be related to several factors such as lower cysteine availability due to greater AA requirements for milk protein and hepatic glutathione depletion due to increased oxidative stress (Lu, 2009). The decrease in *GPX1* throughout the experiment (Figure 14) suggests a further decrease in the anti-oxidant capabilities in transition cows to use reduced glutathione through GPX enzyme.

Inflammation

The expression of *STAT3* was not affected by diet; therefore this transcription factor was not likely to be responsible for greater response of *NFKB1* in SM cows (Figure 15). However, the overall expression of *NFKB1* decreased over time, which is consistent with previous work in our lab (Graugnard et al., 2013). Although mRNA expression of *CP* and *SAA2* was not affected by Met supplementation, lower concentrations of these proteins were observed in Met-supplemented cows (Osorio et al., 2012), suggesting that the genes were subjected to post-transcriptional regulations such as miRNA or RNA methylation.

Oxidative stress

Between the two isozymes of SOD, the SOD1 form was primarily affected by Met supplementation, while SOD2 was not (Figure 16). This difference suggests that Met-supplementation might partially have compartmentalize oxidative stress mainly towards the cytosolic compartment (*SOD1*) rather than mitochondrial (*SOD2*). Similar to *GSS* and *GSR*, lower *SOD1* in Met-supplemented cows in comparison to CON can be associated with either a lower oxidative stress or the reliance on other antioxidants such as ascorbic acid and tocopherol in Met-supplemented cows. In fact, such of pivotal effects can be observed in greater *SOD1* in MS cows at 21 d postpartum, at which point SM cows were previously reported (Osorio et al., 2012) to have greater retinol concentration.

Genes involved in carnitine biosynthesis such as *TMLHE* and *BBOX1* and mitochondrial uptake of carnitine such as *SLC22A5* are known target genes for *PPARA*. Among these, *SLC22A5* was the only gene up-regulated via Met-supplementation, which is in agreement with *PPARA* activation (Figure 17) in Met-supplemented cows. These changes suggest a greater mitochondrial uptake of carnitine in Met-supplemented cows. Overall, *SLC22A5* and *TMLHE* expression increased at 7 d postpartum, followed by a decrease at 21 d postpartum, similar to those observed in Schlegel et al. (2012), where the expression of these genes increased at 7 d postpartum followed by a decreased at 35 d postpartum. Postpartal up-regulation of these genes at 7 d indicates a greater demand for an activation of carnitine that regulates the mitochondrial uptake of fatty acids that commonly accumulate in the liver soon after calving.

PPARA and Fatty acid metabolism

The evident up-regulation of *PPARA* via Met supplementation throughout the experiment was coupled with the greater expression of *RXRA* that further confirmed the functionality of *PPARA* as a potent metabolic regulator (Bionaz et al., 2013). Particularly interesting is the decreased expression of *PPARA* from -10 to 7 d postpartum at which point expression remained until 21 d. This contrasts with previous studies (Loor et al., 2005; Schlegel et al., 2012) where *PPARA* up-regulation occurred as soon as 1 wk after calving and even when rumen-protected choline has been supplemented (Goselink et al., 2013); however this response is not consistent in all studies (Carriquiry et al., 2009; Weber et al., 2013).

Considering our results from the DNA methylation standpoint, it is plausible that Met as promoter of DNA methylation through SAM could have induced the greater *PPARA* activation observed in Met-supplemented cows. It is well established that cells can undergo global DNA hypomethylation while having hypermethylation of a specific gene promoter, and consequently the expression of a gene is silenced or attenuated when its promoter if heavily methylated (Wang et al., 2013). In fact, methyl-deficient diets have been associated with hypermethylation of specific gene promoters of genes involved in lipid metabolism (Tryndyak et al., 2011). Recently, a study testing betaine as a methyl donor in order to reduce hypermethylation of specific promoter genes in male rats revealed an up-regulation of *PPARA* as a result of hypomethylation of its promoter (Wang et al., 2013). This finding may provide a novel insight into the important lipotropic effects of methyl-donors such as betaine or Met in the form of SAM. To the authors knowledge this is the first report of such an effect in dairy cows.

Previous research has shown that *HMGCS2* is likely to be a target gene for *PPARA* (Loor, 2010), however *HMGCS2* was not affected by Met-supplementation. This suggests that another mechanism might have overriden the effects of PPARA activation observed in Met-supplemented cows. Although *CPT1A* expression followed a similar pattern to those observed in *SLC22A5* and *TMLHE, CPT1A* was not affected by Met supplementation.

Hepatokines, gluconeogenesis, and VLDL synthesis and export

Fibroblast growth factor-21 is potent regulator of metabolism that upon activation can stimulate hepatic gluconeogenesis, glucose uptake in adipocytes, fatty acid oxidation and ketogenesis (Schoenberg et al., 2011). Consistent with previous research (Carriquiry et al., 2009), overall expression of *FGF21* increased after calving. Additionally, *FGF21* is well known to be a *PPARA* target gene (Loor, 2010); as such there tended to be a greater ($P < 0.14$) expression of *FGF21* in Met-supplemented cows. In fact expression of *FGF21* at -10 d followed that of *PPARA* with greater expression in Met-supplemented cows. Up-regulation of *FGF21* in Met-supplemented cows via *PPARA* up-regulation ,but not of *CPT1A* as another target of *PPARA*, is in agreement with Akbar et al. (2013).

Parallel to *FGF21*, *ANGPTL4* is considered to be a *PPARA* target and as such its expression overall was greater in Met-supplemented at -10 d and 7 d postpartum. Upon activation *ANGPTL4* inhibits LPL activity and enhances lipolysis, and its expression is upregulated during undernutrition episodes and NEB soon after calving (Loor et al., 2007).

Expression of *MTTP* peaked at 7 d postpartum, with lower expression in Met-supplemented cows throughout the experiment. This finding, together with greater *APOB* in SM cows, did not reveal a clear effect at a molecular level of Met supplementation on VLDL synthesis and secretion from liver. However, VLDL was not affected by Met supplementation from previous results obtained in the same experiment; in fact, APOB-100 tended ($P = 0.14$) to be greater in Met-supplemented cows (Osorio et al., 2013). Taken together these results might suggest that post-transcriptional regulation might have an effect on final translation of *MTTP* and *APOB* into proteins.

The up-regulation of *PDK4* and *PCK1* in Met-supplemented cows suggestions greater gluconeogenesis activity in these cows. This seems to confirm the role of the *PPARA-FGF21* axis as a gluconeogenesis promoter (Fisher et al., 2011). Targeting of *PDK4* via *PPARA-FGF21* axis was observed in fasted male rats (Palou et al., 2008), and *PCK1* increased along with *FGF21* expression in dairy cows (Akbar et al., 2013). However, these effects did not result in greater plasma glucose concentration in Met-supplemented cows, as glucose concentration is tightly regulated in cows. Greater gluconeogenesis would be consistent with the increased milk production observed (Osorio et al., 2013). Therefore, other factors might have regulated glucose concentration in Met-supplemented cows such as mammary gland glucose uptake required for lactose synthesis. In fact insulin, even though not significant throughout the experiment, was ~50% greater postpartum in Met-supplemented cows, which would be consistent with both transient increase in glucose concentration and earlier recovery towards positive energy balance (Osorio et al., 2013).

Growth hormone signaling

High GH not only stimulates milk production but also enhances and sustains gluconeogenesis in liver and lipolysis in adipocytes (Etherton and Bauman, 1998). We previously reported that greater plasma GH was coupled with greater milk production in Met-supplemented cows of this study (Osorio et al., 2013). Although Met-supplemented cows had similar GH concentrations, the greater *IGF1* in SM cows, but not in MS, suggests that the GH/IGF1 axis was differently regulated between Met sources. For instance, MS cows had greater GH (numerically) and NEFA at 7 d postpartum (Osorio et al., 2013), a pattern that has been associated with lower GH-receptor A and IGF-1 (Lucy, 2008). Although *STAT5B* has been proven to play a central role in the *GH-IGF1* signaling cascade, it can be activated by multiple cytokines involved in immunity (Feigerlova et al., 2013). These effects might have undermined the Met supplementation effect in expression of *STAT5B.*

DNA Methylation

DNA methylation has an essential regulatory function in tissue- and stage-specific modulation of gene expression. This mechanism is carried out through the covalent addition of a methyl group to cytosine within the context of the CpG dinucleotide, and has profound effects on the mammalian genome (Robertson and Jones, 2000). DNA methylation via non-sequence-specific binding site relies on methyl-CpG recognition and in this way the methylation is independent of DNA sequence (Kass et al., 1997). DNA methyltransferases (DNMT) are known to methylate DNA cytosine residues, and consequently creates methylated CpG patterns in the mammalian genome. Eventually

components such as MeCP2 will bind to these methylated CpG to mediate transcription (Sansom et al., 2007). There are many DNMT's known to date, i.e. DNMT1b, DNMT2, and DNMT3b, among others, that could have affected DNA methylation (Zhang and Liu, 2010). Interestingly, expression of *DNMT3A* and *DNMT1* decreased from -10 d to 21 d postpartum regardless of treatment. That might indicate that stress periods such as the peripartal period promote DNA methylation. There is a lack of mechanistic information regarding DNA methylation during stress periods such as in the peripartal period in dairy cows, and on the effect of methyl donors such as Met on DNA methylation.

CONCLUSIONS

Our findings indicate that dietary supplementation of MS or SM to cows during the peripartal period can affect the hepatic transcriptomics of Met metabolism, *PPARA* activation, hepatokines, and gluconeogenesis. Methionine metabolism was primarily affected by *MTR* by regenerating Met through methylation of Hcy. Geater expression *SAHH* might have promoted formation of Hcy that could have served as a substrate for Met or glutathione synthesis. Furthermore, the greater *SAHH* mRNA abundance among all genes evaluated reflects the importance of this gene to Met supplementation. As originally hypothesized, Met as a methyl donor in the form of SAM, presumably activate *PPARA*, which is a well-established transcription factor able to mediate important hepatokines such as *ANGPTL4* and *FGF21*. In turn, these compounds seemed to be linked to activation of genes related to gluconeogenesis. To the authors' knowledge this is the first study where a methyl donor such as Met might have increased the bioavailability of SAM and consequently activate *PPARA*, a master regulatory gene.

However, further research is needed to confirm the mechanistic connections between hypermethylation of *PPARA* promoter and decreased *PPARA* expression in ruminants.

REFERENCES

Akbar, H., E. Schmitt, M. A. Ballou, M. N. Correa, E. J. Depeters, and J. J. Loor. 2013. Dietary Lipid During Late-Pregnancy and Early-Lactation to Manipulate Metabolic and Inflammatory Gene Network Expression in Dairy Cattle Liver with a Focus on PPARs. Gene Regul Syst Bio. 7:103-123.

Baric, I. 2009. Inherited disorders in the conversion of methionine to homocysteine. J Inherit Metab Dis. 32:459-471.

Bionaz, M., S. Chen, M. J. Khan, and J. J. Loor. 2013. Functional role of PPARs in ruminants: Potential targets for fine-tuning metabolism during growth and lactation. PPAR Res. 2013:684159.

Bionaz, M. and J. J. Loor. 2008. Gene networks driving bovine milk fat synthesis during the lactation cycle. BMC genomics. 9:366.

Carriquiry, M., C. R. Dahlen, W. J. Weber, G. C. Lamb, and B. A. Crooker. 2009. Postpartum ovarian activity in multiparous Holstein cows treated with bovine somatotropin and fed n-3 fatty acids in early lactation. J Dairy Sci. 92:4876-4888.

Dann, H. M., N. B. Litherland, J. P. Underwood, M. Bionaz, A. D'Angelo, J. W. McFadden, and J. K. Drackley. 2006. Diets during far-off and close-up dry periods affect periparturient metabolism and lactation in multiparous cows. J Dairy Sci. 89:3563-3577.

Etherton, T. D. and D. E. Bauman. 1998. Biology of somatotropin in growth and lactation of domestic animals. Physiol Rev. 78:745-761.

Feigerlova, E., V. Hwa, M. A. Derr, and R. G. Rosenfeld. 2013. Current issues on molecular diagnosis of GH signaling defects. Endocr Dev. 24:118-127.

Fisher, F. M., J. L. Estall, A. C. Adams, P. J. Antonellis, H. A. Bina, J. S. Flier, A. Kharitonenkov, B. M. Spiegelman, and E. Maratos-Flier. 2011. Integrated regulation of hepatic metabolism by fibroblast growth factor 21 (FGF21) in vivo. Endocrinology. 152:2996-3004.

Franklin, C. C., D. S. Backos, I. Mohar, C. C. White, H. J. Forman, and T. J. Kavanagh. 2009. Structure, function, and post-translational regulation of the catalytic and modifier subunits of glutamate cysteine ligase. Mol Aspects Med. 30:86-98.

Goselink, R. M., J. van Baal, H. C. Widjaja, R. A. Dekker, R. L. Zom, M. J. de Veth, and A. M. van Vuuren. 2013. Effect of rumen-protected choline supplementation on liver and adipose gene expression during the transition period in dairy cattle. J Dairy Sci. 96:1102-1116.

Graugnard, D. E., K. M. Moyes, E. Trevisi, M. J. Khan, D. Keisler, J. K. Drackley, G. Bertoni, and J. J. Loor. 2013. Liver lipid content and inflammometabolic indices

in peripartal dairy cows are altered in response to prepartal energy intake and postpartal intramammary inflammatory challenge. J Dairy Sci. 96:918-935.

Graulet, B., C. Richard, and J. C. Robert. 2005. Methionine availability in plasma of dairy cows supplemented with methionine hydroxy analog isopropyl ester. J Dairy Sci. 88:3640-3649.

Jerkins, A. A., D. D. Jones, and E. A. Kohlhepp. 1998. Cysteine sulfinic acid decarboxylase mRNA abundance decreases in rats fed a high-protein diet. J Nutr. 128:1890-1895.

Kass, S. U., D. Pruss, and A. P. Wolffe. 1997. How does DNA methylation repress transcription? Trends Genet. 13:444-449.

Loor, J. J. 2010. Genomics of metabolic adaptations in the peripartal cow. Animal. 4:1110-1139.

Loor, J. J., H. M. Dann, R. E. Everts, R. Oliveira, C. A. Green, N. A. Guretzky, S. L. Rodriguez-Zas, H. A. Lewin, and J. K. Drackley. 2005. Temporal gene expression profiling of liver from periparturient dairy cows reveals complex adaptive mechanisms in hepatic function. Physiol Genomics. 23:217-226.

Loor, J. J., R. E. Everts, M. Bionaz, H. M. Dann, D. E. Morin, R. Oliveira, S. L. Rodriguez-Zas, J. K. Drackley, and H. A. Lewin. 2007. Nutrition-induced ketosis alters metabolic and signaling gene networks in liver of periparturient dairy cows. Physiol Genomics. 32:105-116.

Lu, S. C. 2009. Regulation of glutathione synthesis. Mol Aspects Med. 30:42-59.

Lucy, M. C. 2008. Functional differences in the growth hormone and insulin-like growth factor axis in cattle and pigs: implications for post-partum nutrition and reproduction. Reprod Domest Anim. 43 Suppl 2:31-39.

Martinov, M. V., V. M. Vitvitsky, R. Banerjee, and F. I. Ataullakhanov. 2010. The logic of the hepatic methionine metabolic cycle. Biochimica et biophysica acta. 1804:89-96.

Ordway, R. S., S. E. Boucher, N. L. Whitehouse, C. G. Schwab, and B. K. Sloan. 2009. Effects of providing two forms of supplemental methionine to periparturient Holstein dairy cows on feed intake and lactational performance. J Dairy Sci. 92:5154-5166.

Osorio, J. S., P. Ji, J. K. Drackley, D. Luchini, and J. J. Loor. 2013. Supplemental Smartamine M or MetaSmart during the transition period benefits postpartal cow performance and blood neutrophil function. J Dairy Sci. 96:6248-6263.

Osorio, J. S., E. Trevisi, P. Ji, D. Luchini, J. K. Drackley, G. Bertoni, and J. J. Loor. 2012. Immunometabolic indices in dairy cows supplemented with Smartamine M or MetaSmar during the peripartal period. . J Dairy Sci. 95. (Suppl. 2), T298

Palou, M., T. Priego, J. Sanchez, E. Villegas, A. M. Rodriguez, A. Palou, and C. Pico. 2008. Sequential changes in the expression of genes involved in lipid metabolism in adipose tissue and liver in response to fasting. Pflugers Arch. 456:825-836.

Pisulewski, P. M., H. Rulquin, J. L. Peyraud, and R. Verite. 1996. Lactational and systemic responses of dairy cows to postruminal infusions of increasing amounts of methionine. J Dairy Sci. 79:1781-1791.

Preynat, A., H. Lapierre, M. C. Thivierge, M. F. Palin, N. Cardinault, J. J. Matte, A. Desrochers, and C. L. Girard. 2010. Effects of supplementary folic acid and vitamin B(12) on hepatic metabolism of dairy cows according to methionine supply. J Dairy Sci. 93:2130-2142.

Robertson, K. D. and P. A. Jones. 2000. DNA methylation: past, present and future directions. Carcinogenesis. 21:461-467.

Sansom, O. J., K. Maddison, and A. R. Clarke. 2007. Mechanisms of disease: methyl-binding domain proteins as potential therapeutic targets in cancer. Nat Clin Pract Oncol. 4:305-315.

Schlegel, G., J. Keller, F. Hirche, S. Geissler, F. J. Schwarz, R. Ringseis, G. I. Stangl, and K. Eder. 2012. Expression of genes involved in hepatic carnitine synthesis and uptake in dairy cows in the transition period and at different stages of lactation. BMC Vet Res. 8:28.

Schoenberg, K. M., S. L. Giesy, K. J. Harvatine, M. R. Waldron, C. Cheng, A. Kharitonenkov, and Y. R. Boisclair. 2011. Plasma FGF21 is elevated by the intense lipid mobilization of lactation. Endocrinology. 152:4652-4661.

Schwab, C. G., C. K. Bozak, N. L. Whitehouse, and M. M. A. Mesbah. 1992. Amino Acid Limitation and Flow to Duodenum at Four Stages of Lactation. 1. Sequence of Lysine and Methionine Limitation. J Dairy Sci. 75:3486-3502.

Spears, J. W. and W. P. Weiss. 2008. Role of antioxidants and trace elements in health and immunity of transition dairy cows. Vet J. 176:70-76.

Tryndyak, V. P., T. Han, L. Muskhelishvili, J. C. Fuscoe, S. A. Ross, F. A. Beland, and I. P. Pogribny. 2011. Coupling global methylation and gene expression profiles reveal key pathophysiological events in liver injury induced by a methyl-deficient diet. Mol Nutr Food Res. 55:411-418.

Wang, L., L. Chen, Y. Tan, J. Wei, Y. Chang, T. Jin, and H. Zhu. 2013. Betaine supplement alleviates hepatic triglyceride accumulation of apolipoprotein E

deficient mice via reducing methylation of peroxisomal proliferator-activated receptor alpha promoter. Lipids Health Dis. 12:34.

Weber, C., C. Hametner, A. Tuchscherer, B. Losand, E. Kanitz, W. Otten, H. Sauerwein, R. M. Bruckmaier, F. Becker, W. Kanitz, and H. M. Hammon. 2013. Hepatic gene expression involved in glucose and lipid metabolism in transition cows: Effects of fat mobilization during early lactation in relation to milk performance and metabolic changes. J Dairy Sci. 96:5670-5681.

Weiss, W. P., D. A. Todhunter, J. S. Hogan, and K. L. Smith. 1990. Effect of duration of supplementation of selenium and vitamin E on periparturient dairy cows. J Dairy Sci. 73:3187-3194.

Zhang, T. N. and N. Liu. 2010. Effect of bovine lactoferricin on DNA methyltransferase 1 levels in Jurkat T-leukemia cells. J Dairy Sci. 93:3925-3930.

TABLES AND FIGURES

Table 9. Slope, coefficient of determination of the standard curve (R^2), efficiency of amplification, and median cycle threshold (Ct) of quantitative reverse transcription-PCR

Gene	Slope[1]	(R^2)	Efficiency[2]	Median Ct[3]	mRNA abundance[4] (%)
SAHH	-3.24	1.00	2.04	17.40	17.86
CP	-3.21	1.00	2.05	18.01	12.19
SOD1	-3.13	1.00	2.09	18.15	11.89
APOB	-3.23	1.00	2.04	18.16	10.98
HMGCS2	-3.18	1.00	2.06	18.36	10.04
SAA2	-3.27	0.99	2.02	18.77	5.61
BHMT	-3.36	0.99	1.98	18.93	5.61
ACOX1	-3.24	1.00	2.04	19.05	5.39
MAT1A	-3.25	1.00	2.03	19.42	4.14
PCK1	-3.22	1.00	2.05	19.70	3.28
SOD2	-3.13	0.99	2.09	19.77	3.12
HP	-3.26	1.00	2.02	20.50	1.78
GPX1	-3.06	1.00	2.12	20.80	1.47
STAT3	-3.35	0.98	1.99	21.44	1.00
CBS	-3.26	0.99	2.03	21.52	0.92
MTTP	-3.12	0.99	2.09	22.07	0.60
PPARA	-3.21	1.00	2.05	22.33	0.49
BBOX1	-3.10	1.00	2.10	22.49	0.42
RXRA	-3.36	0.99	1.98	22.69	0.42
PEMT	-3.23	0.99	2.04	22.70	0.39
PC	-3.59	1.00	1.90	23.00	0.38
IGF1	-3.33	0.99	2.00	22.78	0.36
STAT5B	-3.14	0.99	2.08	22.96	0.31
PDK4	-3.09	1.00	2.11	23.28	0.23
SOCS2	-3.00	0.99	2.15	23.39	0.21
NFKB1	-3.10	1.00	2.10	23.81	0.16
CSAD	-3.27	1.00	2.02	24.34	0.13
BHMT2	-2.93	0.99	2.19	24.01	0.12
ANGPTL4	-3.20	0.99	2.05	24.91	0.07
TMLHE	-3.29	0.99	2.01	25.29	0.07
GSR	-3.12	1.00	2.09	25.13	0.06
GCLC	-3.12	0.99	2.09	25.26	0.06
CTH	-3.12	1.00	2.09	25.44	0.05
FGF21	-3.24	0.99	2.04	25.26	0.05
SLC22A5	-3.08	0.99	2.11	25.63	0.04
GSS	-3.12	1.00	2.09	25.89	0.03

Table 9. (cont.)

Gene	Slope[1]	(R^2)	Efficiency[2]	Median Ct[3]	mRNA abundance[4] (%)
MTR	-3.30	0.98	2.01	26.32	0.03
DNMT3A	-2.96	0.99	2.17	27.14	0.01
DNMT1	-2.97	0.99	2.17	27.48	0.01

[1]Slope of the 7-point standard curve.
[2]Efficiency of amplification [$E=10^{(-1/slope)}$].
[3]Ct = median cycle threshold which is defined as the number of cycles required for the fluorescent signal to cross the threshold (i. e. exceed background level); and the amount of target nucleic acid in the sample is inversely correlated to Ct cycles (i. e., the greater the amount of target nucleic acid the lower the Ct cycles will be required).
[4]mRNA abundance is calculated as percentage of ($1/E^{\Delta Ct}$) specific gene in the sum ($1/E^{\Delta Ct}$) all genes.

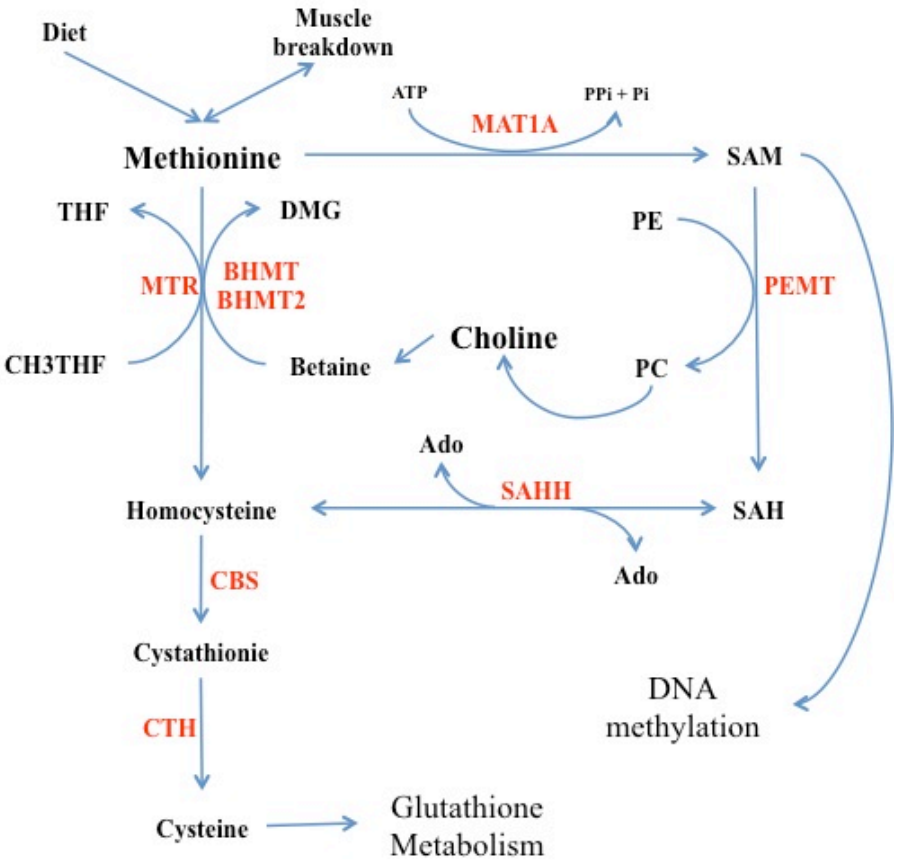

Figure 12. Methionine cycle. Key genes encoding enzymes analyzed: methionine adenosyltransferase 1A (*MATIA*), phosphatidylethanolamine methyltransferase (*PEMT*), S-adenosylhomocysteine hydrolase (*SAHH*), betaine homocysteine methyltransferase (*BHMT* and *BHMT2*), 5-methyltetrahydrofolate-homocysteine methyltransferase (*MTR*), cystathionine β-synthase (*CBS*), cystathionine β-lyase (*CTH*). SAM = S-adenosylmethionine, PE= phosphatidylethanolamine, PC = phosphatidylcholine, SAH = S-adenosylhomocysteine, Ado=adenosyl, THF = tetrahydrofolate, CH3THF = 5-methyl-tetrahydrofolate, DMG = dimethyl-glycine.

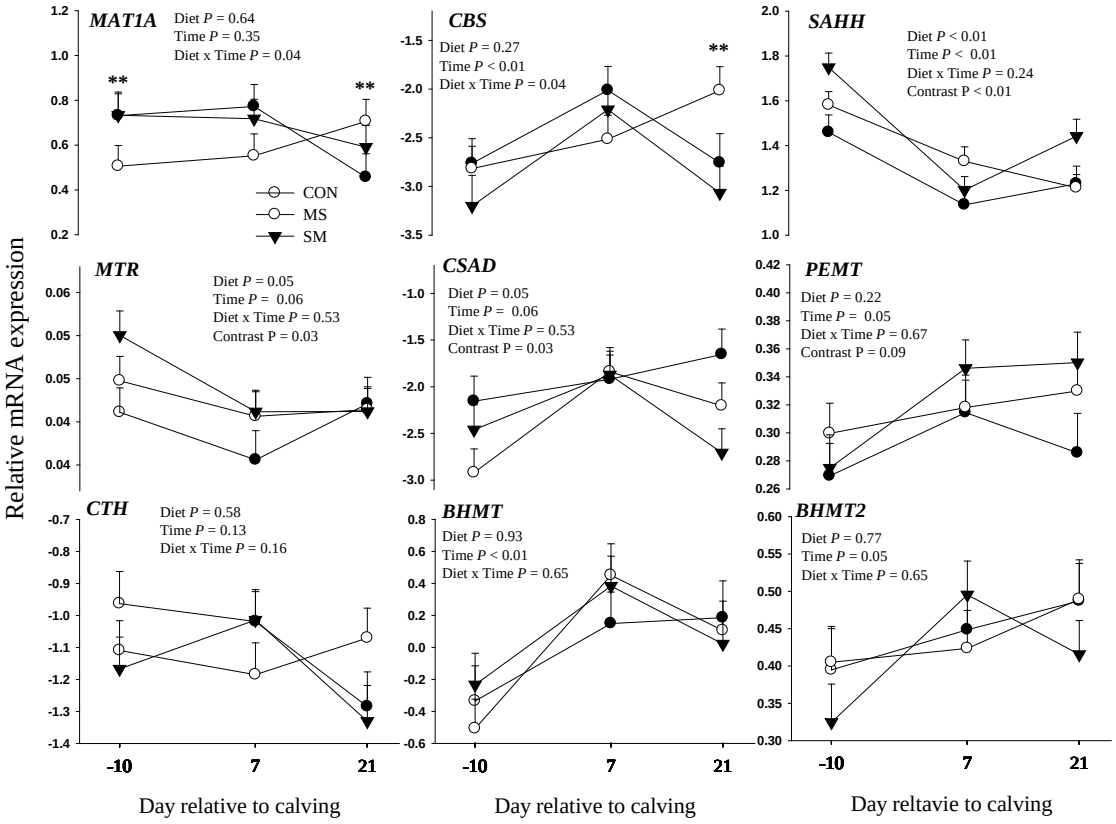

Figure 13. Expression of genes related to the Met cycle in cows supplemented with MS or SM at rate of 0.19% or 0.07% of DM feed throughout the peripartal period. The *P* values for main effect of treatment (Diet) and time and interaction of treatment and time (Diet×Time) are shown in figure. Mean separation between diets ($P < 0.05$) was evaluated via contrast: CON versus SM (***). Values are means, with standard errors represented by vertical bars.

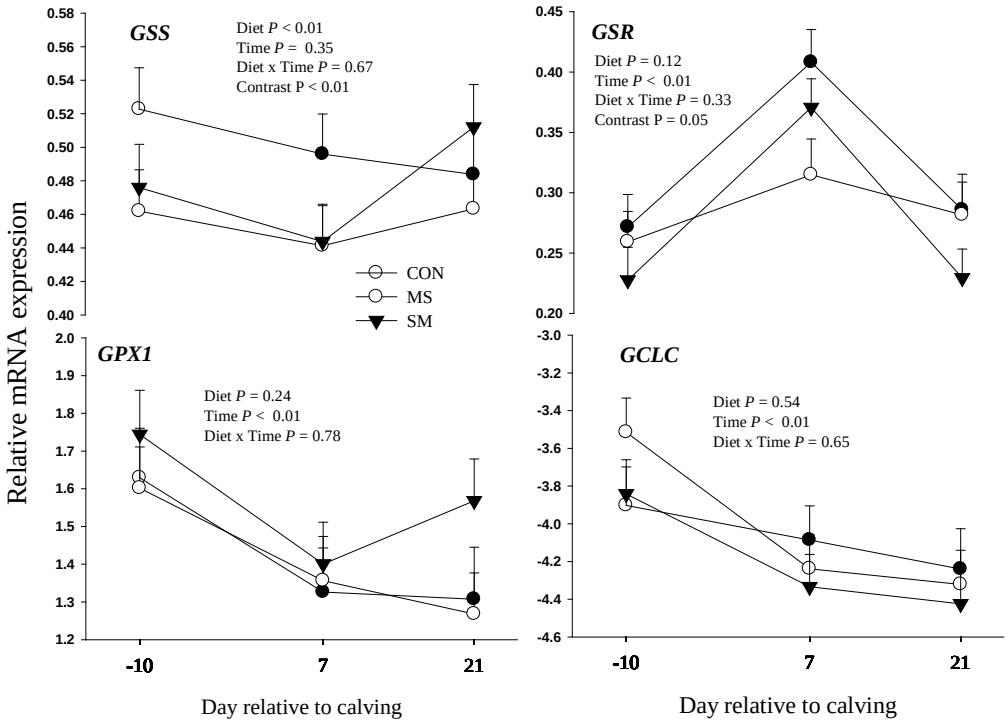

Figure 14. Expression of genes related to the glutathione cycle in cows supplemented with MS or SM at rate of 0.19% or 0.07% of DM feed throughout the peripartal period. The *P* values for main effect of treatment (Diet) and time and interaction of treatment and time (Diet×Time) are shown in figure. Values are means, with standard errors represented by vertical bars.

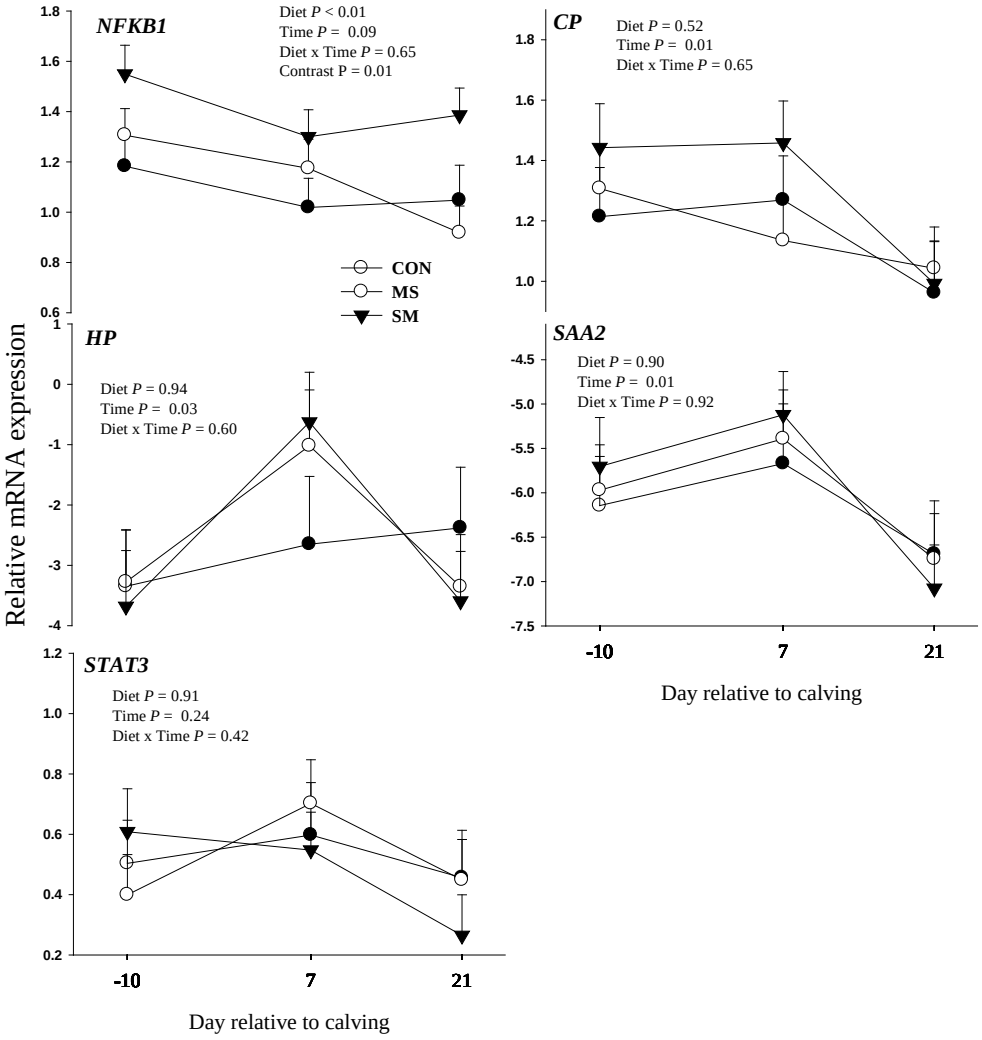

Figure 15. Expression of genes related to the inflammation in cows supplemented with MS or SM at rate of 0.19% or 0.07% of DM feed throughout the peripartal period. The *P* values for main effect of treatment (Diet) and time and interaction of treatment and time (Diet×Time) are shown in figure. Values are means, with standard errors represented by vertical bars.

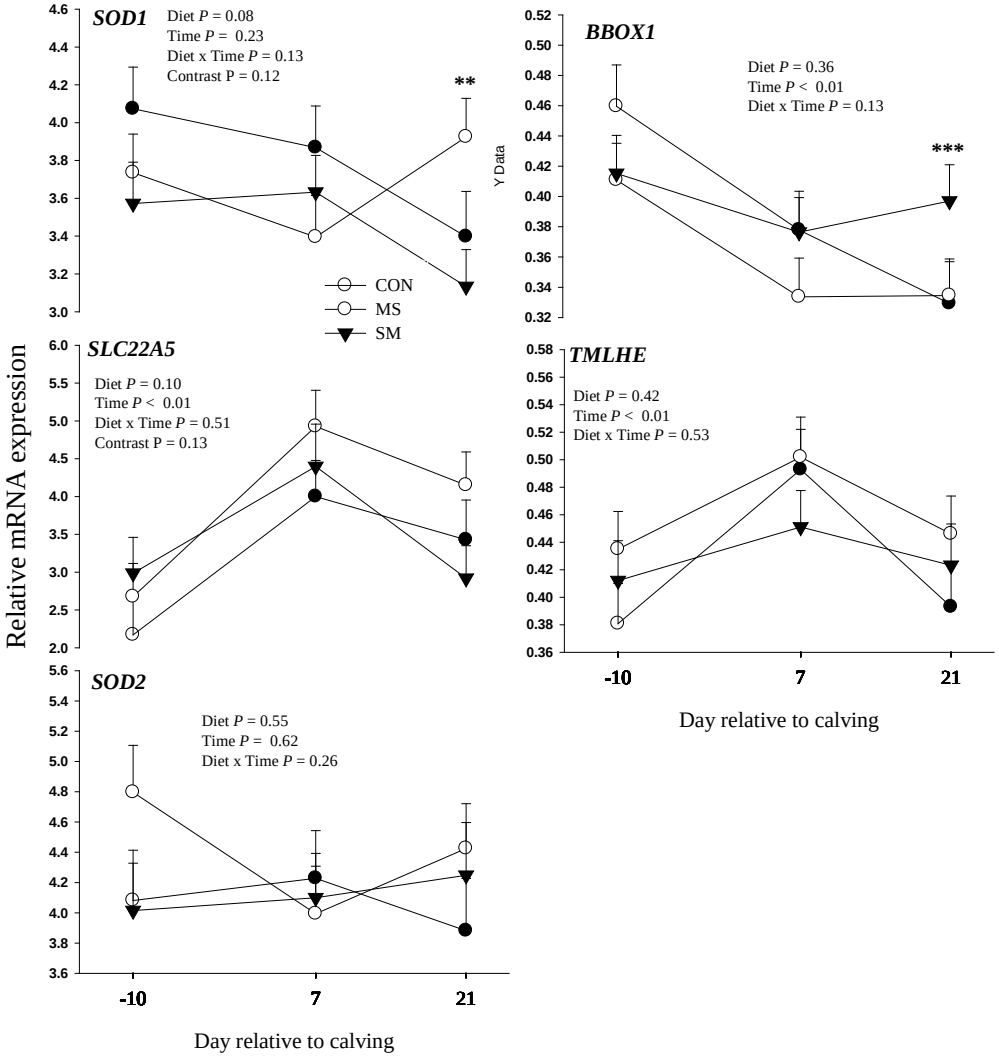

Figure 16. Expression of genes related to the oxidative stress in cows supplemented with MS or SM at rate of 0.19% or 0.07% of DM feed throughout the peripartal period. The *P* values for main effect of treatment (Diet) and time and interaction of treatment and time (Diet×Time) are shown in figure. Mean separation between diets ($P < 0.05$) was evaluated via contrast: CON versus SM (***). Values are means, with standard errors represented by vertical bars.

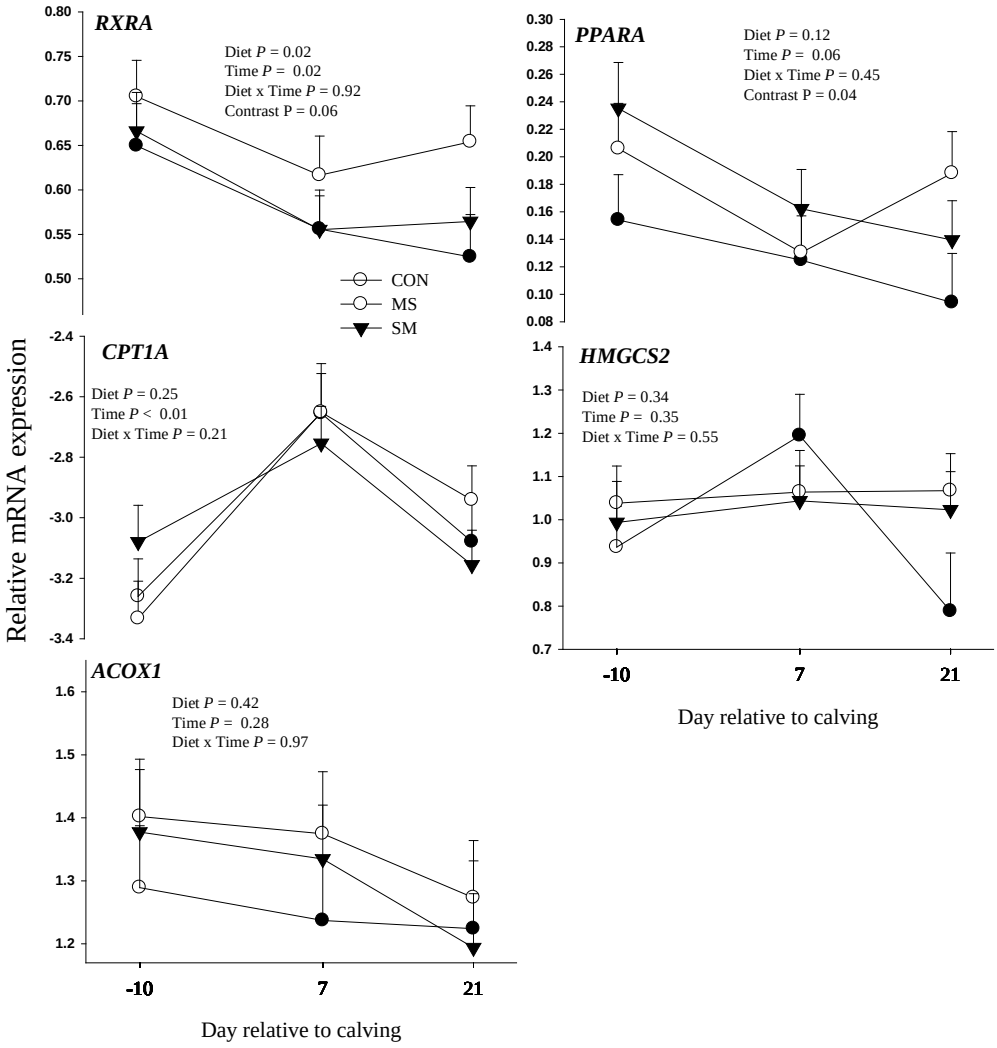

Figure 17. Expression of genes related to the fatty acid metabolism in cows supplemented with MS or SM at rate of 0.19% or 0.07% of DM feed throughout the peripartal period. The *P* values for main effect of treatment (Diet) and time and interaction of treatment and time (Diet×Time) are shown in figure. Mean separation between diets ($P < 0.05$) was evaluated via contrast: CON versus SM (***). Values are means, with standard errors represented by vertical bars.

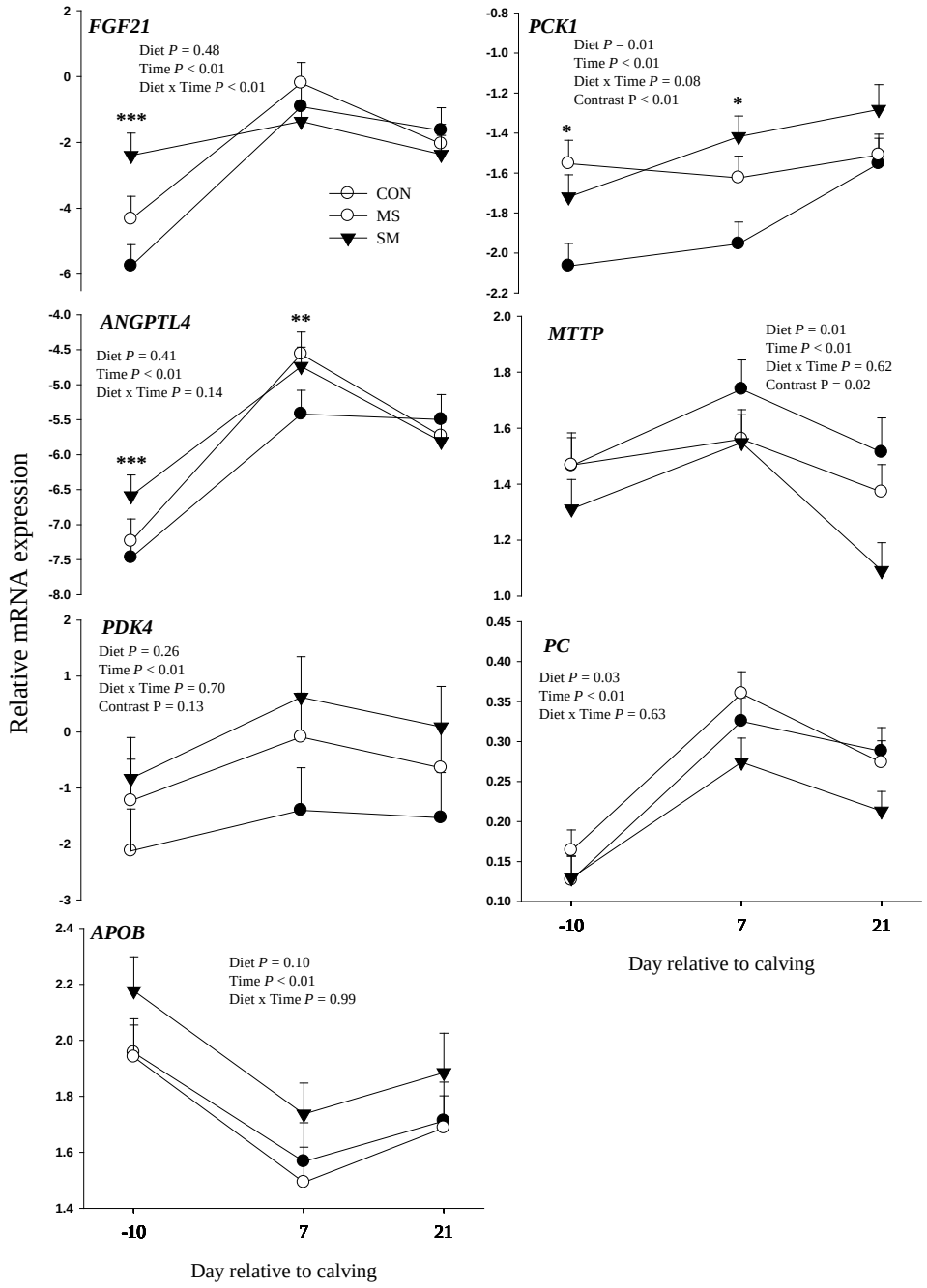

Figure 18. Expression of genes related to the hepatokines (*FGF21* and *ANGPTL4*), gluconeogenesis (*PDK4*, *PCK1*, and *PC*), and VLDL synthesis/export (*MTTP* and *APOB*) in cows supplemented with MS or SM at rate of 0.19% or 0.07% of DM feed throughout the peripartal period. The *P* values for main effect of treatment (Diet) and time and interaction of treatment and time (Diet×Time) are shown in figure. Mean separation between diets ($P < 0.05$) was evaluated via contrast: CON versus MS + SM (*). Values are means, with standard errors represented by vertical bars.

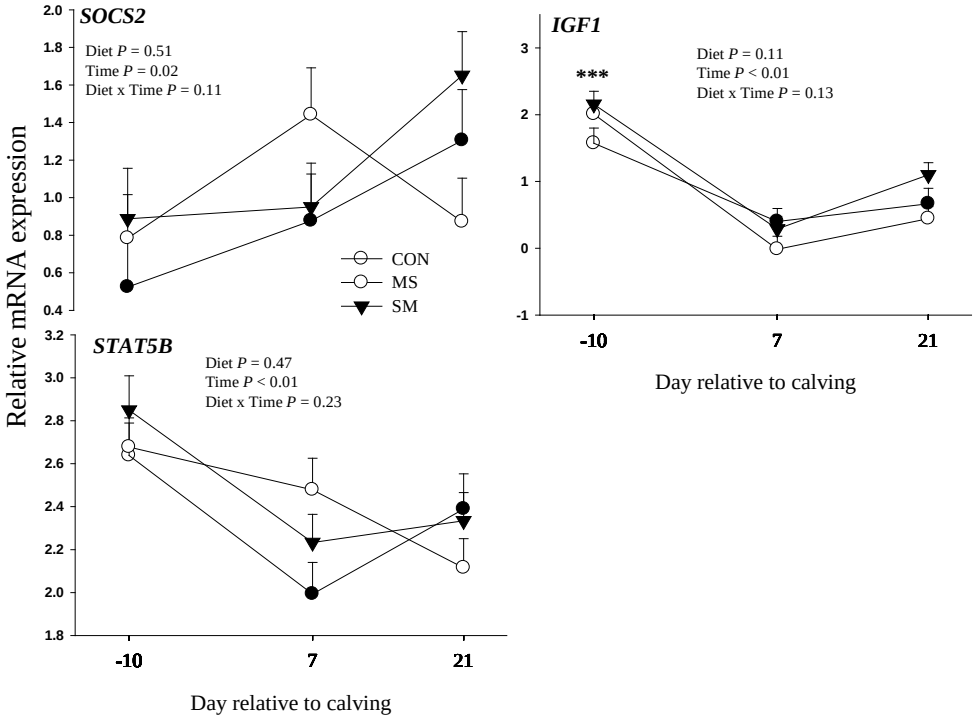

Figure 19. Expression of genes related to the growth hormone (GH)-insulin-like growth factor 1 (*IGF1*) axis in cows supplemented with MS or SM at rate of 0.19% or 0.07% of DM feed throughout the peripartal period. The *P* values for main effect of treatment (Diet) and time and interaction of treatment and time (Diet×Time) are shown in figure. Mean separation between diets ($P < 0.05$) was evaluated via contrast: CON versus MS (**) and CON versus SM (***). Values are means, with standard errors represented by vertical bars.

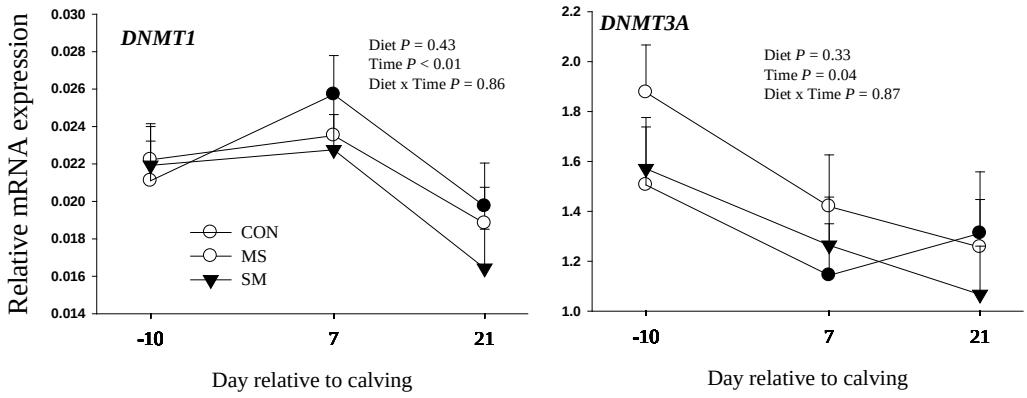

Figure 20. Expression of genes related to the DNA methylation in cows supplemented with MS or SM at rate of 0.19% or 0.07% of DM feed throughout the peripartal period. The *P* values for main effect of treatment (Diet) and time and interaction of treatment and time (Diet×Time) are shown in figure. Values are means, with standard errors represented by vertical bars.

CHAPTER 5

PERIPARTAL SUPPLEMENTATION OF RUMEN-PROTECTED METHIONINE MODIFIES AA GENE NETWORKS IN DAIRY COWS

INTRODUCTION

The transition period is considered the most important phase during the lactation cycle (Drackley, 1999). 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 metabolic disorders (Drackley, 1999; Ingvartsen, 2006). Energy consumption may be a determinant factor for the success of the transition period; there is evidence that dairy cows can easily consume more energy than required during these periods (Dann et al., 2006). Therefore, it is important to avoid overconditioning of cows during gestation, which eventually reduce excessive lipid mobilization after calving (Ingvartsen, 2006). However, as negative energy balance (NEB) postpartum is universal for cows in the same way lipid mobilization is ubiquitous during this time. In this context, it is possible that alterations in management or complications at calving might trigger a chain-reaction effect of hormonal and metabolic alterations that eventually predispose cows to infectious diseases and metabolic disorders.

Methionine (Met) supplementation of cows during lactation generally improves cow performance (Armentano et al., 1997; Rulquin and Delaby, 1997; Chen et al., 2011). Although, similar effects have been observed when supplementing Met to peripartal cows (Phillips et al., 2003; Socha et al., 2005; Ordway et al., 2009), there is still a lack of data in this regard. Rumen-protected Met is expected to spare EAA once metabolized,

consequently it can serve as a methyl donor, backbone for gluconeogenesis, and also stimulate the clearance of TAG from liver via assembly and secretion of VLDL. Here we evaluate these effects, specifically in hepatic tissue, through a microarray platform to uncover transcriptional adaptations in bovine liver response to Met supplementation in the form of Smartamine M or MetaSmart during the transition period.

MATERIALS AND METHODS

Experimental Design and dietary treatments

The Animal Care and Use Committee of the University of Illinois approved all procedures conducted in this study. Details of the experimental design have been published previously (Osorio et al., 2013). Twenty-five Holstein cows entering their second or greater lactation were enrolled in the study and were fed experimental treatments consisting of a basal control diet (CO, n = 14) with no Met supplementation, CO plus MS (n = 12) at a rate of 0.19% of DM, and CO plus SM (n = 13) at a rate of 0.07% of DM (Osorio et al., 2013). All cows received the same far-off diet (1.24 Mcal/kg DM, 14.3% CP) from -50 to -21 d before expected calving, a close-up diet (1.54 Mcal/kg DM, 15% CP) from -21d to expected calving, and a fresh-cow diet from calving (1.75 Mcal/kg DM, 17.5% CP) through 30 DIM. Methionine supplements were top-dressed from -21 to 30 DIM. A subset of cows (CO, n = 8; MS, n = 8; SM, n = 8) was used to evaluate hepatic transcriptomic profile through a microarray analysis.

Animal management

The Institutional Animal Care and Use Committee (IACUC) of the University of Illinois approved all protocols for this study (protocol no. 09214). Details of the experimental design have been published previously (Osorio et al., 2013). Briefly, prior to calving all cows were individually fed once daily at 0630 h using an individual gate system (American Calan, Northwood, NH). Cows were housed in a ventilated enclosed barn during the dry period and had access to sand-bedded free stalls until 3 d before expected parturition, when they were moved to individual maternity pens bedded with straw until parturition. After parturition, cows were housed in a tie-stall barn and were fed a common lactation diet once daily (Table 2) and milked 3 × daily. Refusals were recorded daily before feeding and intake was calculate base on previous day amount of TMR offered. After 30 d postpartum cows returned to the farm herd. Feed offered was adjusted daily to achieve 5 to 10% refusal.

Sample collection

Liver was sampled via puncture biopsy (Dann et al., 2006) from cows under local anesthesia at approximately 0730 h on d −10, 7 to 11, and 21 d relative to parturition. Tissue specimens were stored in liquid N_2 until RNA extraction (Graugnard et al., 2010).

RNA extraction

RNA samples were extracted from frozen tissue using established protocols in our laboratory (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,

Corning, NY, USA) with 1 µl of Linear Acrylamine (Ambion® Cat. No. 9520, Austin, TX, USA) as a co-precipitant, and 5 ml of ice-cold Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA) to proceed with RNA extraction. This extraction procedure also utilizes acid-phenol chloroform (Ambion® Cat. No. 9720, Austin, TX, USA), which removes residual DNA. Any residual genomic DNA was removed from RNA with DNase using RNeasy Mini Kit columns (Qiagen, Hilden, Germany). RNA concentration was measured using a Nano-Drop ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). The purity of RNA (A_{260}/A_{280}) for all samples was above 1.81.

Microarrays procedure

An annotated bovine oligonucleotide microarray containing >10,000 unique elements were used in this study (Loor et al., 2007). The cDNA was obtained by RT-PCR in a 30 µL reaction adding 10 µg RNA, 2 µL of random hexamer primers (3 mg/ml; Invitrogen Corp., CA) and 1 µg oligo dT18 (Operon Biotechnologies, AL), and DNase-RNase-free water to a volume of 17.78 µL. The mixture was incubated at 65°C for 5 min and kept on ice for 3 min. To the mixture 12.2 µL solution composed of 6 µL 5X First-Strand Buffer, 3 µL 0.1 M DTT, 0.6 µL 100 mMdNTP mix (Invitrogen Corp., CA), 0.12 µL of 50 mM 5-(3-aminoallyl)-dUTP (Ambion, CA), 2 µL (100 U) of SuperScriptTM III RT (Invitrogen Corp., CA), and 0.5 µL of RNase Inhibitor (Promega, WI) was added. The reaction was performed at 23°C for 1 min and 46°C for 9 h. The cDNA obtained was then treated with 10 µL 1M NaOH, and incubated for 15 min at 65°C to remove residual RNA. The solution was neutralized by adding 10 µL 1M HCl. The unincorporated 5-(3-

aminoallyl)-dUTP and free amines were removed using a Qiagen PCR Purification Kit (Qiagen, Germany). Clean cDNA was dried and resuspended in 4.5 µL 0.1 M Na2CO3 buffer (pH 9.0) and 4.5 µL of AmershamCyDye™ fluorescent dyes diluted in 60 µL of DMSO (Cy3 or Cy5; GE Healthcare, USA). Binding of Cy dyes with 5-(3-aminoallyl)-dUTP incorporated into cDNA was obtained by incubation at room temperature for 1 h. The unbound dyes are removed using a Qiagen PCR Purification Kit (Qiagen, Germany) and clean labelledcDNA was measured by means of a NanoDrop ND-1000 spectrophotometer (www.nanodrop.com). Sample and reference were then vacuum-dried in the dark.

Hybridizations

Hybridizations were performed in a dye-swap reference design. The reference was prepared by pooling RNA from bovine muscle, liver, and adipose tissues already available in our tissue bank. Prior to hybridization, slides were re-hydrated, placed in an UV cross-linker, washed with 0.2% SDS solution, thoroughly rinsed with purified water to remove un-bound oligonucleotide, and pre-hybridized using a solution containing 1% albumin, 5 × SCC, and 0.1% SDS at 42 C° for ≥ 45 min with the aim of decreasing background. After pre-hybridization, slides were rinsed with abundant purified water and immersed in isopropanol for ~10s and spin-dried. Dried slides were immediately hybridized in a dye-swap-reference design (i.e. each sample is labeled twice with each of the two dyes and hybridized in each slide with the reference labeled with the opposite dye). Labeled cDNA of the sample was re-hydrated with 80µL of hybridization buffer #1 (Ambion) and mixed thoroughly. This solution was used to re-suspend the reference

sample labeled with the opposite dye and mixed thoroughly in order to obtain a homogenous solution of the two-labeled cDNA. Before hybridization, the labeled cDNA resuspension of the sample + reference was incubated at 90-95 C for ca. 3 min to allow for cDNA denaturation to increase the efficiency of binding of oligos onto the slide.

Hybridizations were carried out using humidified slide chambers (Corning) with cover slips (LifterSlip, Thermo Scientific) at 42 C° for ca. 40 hours in the dark. After hybridization, slides were removed from the chamber and washed for 5 min by agitation 3 times with wash buffers in the following order: 1×SSC and 0.2% SDS solution preheated at 42°C, 0.1× SSC and 0.2% SDS, solution, and 0.1× SSC solution. Lastly, slides were spin-dried and inserted into a 50 mL tube prior to gassing with Argon to preserve dye from bleaching. Arrays were scanned with a ScanArray 4000 (GSI-Lumonics, Billerica, MA) dual-laser confocal scanner and images processed and edited using GenePix 6.0 (Axon Instruments). Array quality was assessed using an in-house parser written in Perl language as previously described (Loor et al., 2007). Spots on the slide are considered "good" if the median intensity was ≥3 standard deviation above median background for each channel (i.e., dye). Spots are flagged "present" when both dyes pass the criteria, "marginal" if only one dye passes the criteria, or "absent" when both dyes fail to pass the criteria. Statistical analysis was conducted on oligos that are flagged as "present" and "marginal".

Data mining

Data from a total of 130 microarrays were 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, SAS Inst. Inc., Cary, NC). Fixed effects were treatments (CO, MS, and SM), day (-10, 7, 21) 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 $P < 0.05$. Computational analysis was performed by using a recently developed approach and online databases.

Functional Enrichment Analysis

The whole annotated microarray was used as the reference dataset (i.e., background) for enrichment analysis. Furthermore, we have provided functional analysis using the novel proposed Dynamic Impact Approach (DIA) (Bionaz et al., 2012). The DIA is a functional analysis tool that allows visualization of the impact and the direction of the impact of DEG from several annotation databases including Gene Ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The DIA is described in detail in Bionaz et al. (2012); briefly, the impact and the direction of the impact (flux) for KEGG pathways were calculated for only those terms that were represented by at least 30% in the microarray compared to the whole annotated bovine genome.

RESULTS

In this experiment, repeated measures analysis with a cutoff FDR $P < 0.10$ and $P < 0.05$ was used for generating the list of differentially expressed genes (DEG) in all the comparisons mentioned. In order to analyze the gene expression data, we used the dynamic impact approach (DIA), a computational analysis of pathways adequately

represented in our bovine microarray using the KEGG database. Adequately represented pathways were represented > 30% (genes in pathways of whole bovine genome in array/gene in pathways whole bovine genome) (Bionaz et al., 2012). By following this criterion, 215 out of a total 230 *Bos taurus* KEGG pathways were represented in at least one within-diet or one within-time comparison in our array.

Differentially expressed genes (DEGs) and their overall pattern

The microarray analysis of data with above mentioned cutoff values showed a significant effect of diet × day interaction for the bovine liver transcriptome. A total of 18 comparisons were used for comprehensive analysis of data. Within-diet comparisons (same day with comparison of three diets), we analyzed 9 comparison including -10 d MS *vs.* CON, 7 d MS *vs.* CON, 21 d MS *vs.* CON, -10 SM *vs.* CON, 7 d SM *vs.* CON, 21 d SM *vs.* CON, -10 SM *vs.* MS, 7 d SM *vs.* MS, and 21 d SM *vs.* MS (Table 10). Within-time comparisons (same diet with comparison of three time points), we analyzed 9 comparisons including; CON 7 d *vs.* -10 d, CON 21 d *vs.* 7 d, CON 21 d *vs.* -10 d, MS 7 d *vs.* -10 d, MS 21 d *vs.* 7 d, MS 21 d *vs.* -10 d, SM 7 d *vs.* -10 d, SM 21 d *vs.* 7 d, SM 21 *vs.* -10 d (Table 11).

Table 10 and Table 11 show the total number of DEG in each comparison of diet and time respectively. From 75-82% of the DEGs were annotated to bovine ENTREZ gene ID and were used for further computational gene expression analysis. We observed a greater number of DEG within-diet comparison at 21 d in SM *vs.* MS (total 731; up-regulated: 400, down-regulated: 331), at 7 d in CON *vs.* MS (total 583; up-regulated: 342, down-regulate: 241), and at 21 d in CON *vs.* SM (total 508; up-regulated: 185, down-

regulated: 323) (Table 10). Similarly, the greater number of DEGs within-time comparisons was observed in SM at 7 d *vs.* -10 d (total 1190; up-regulated: 612, down-regulated: 578), in SM at 21 d *vs.* 7 d (total 1085; up-regulated: 461, down-regulated: 624), and in CON at 7 d *vs.* -10 d (total 934; up-regulated: 430, down-regulated: 504) (Table 11). Overall, a greater number of DEGs were observed within-time comparisons.

Impact of DEGs on KEGG pathways using DIA

Within-diet comparisons

An overall summary result of all major categories from KEGG analysis using DIA within-diet comparisons is shown in Figure 21. We observed that 'Metabolism' was the most impacted category within-diet comparisons, and the diet × time interaction of SM vs. MS at 21 d was the most impacted among all measured comparisons. The major category 'Metabolism' is further categorized into 11 sub-categories including amino acid metabolism (AAM) and metabolism of other amino acids (MOAA) which were impacted in all 9 comparisons. The overall effect of MS *vs.* CON was greater 7 d postpartum with a greater impact and inhibition of MOAA, in contrast this effect was least impacted at -10 d, with MOAA the most impacted and inhibited. The effect of SM *vs.* CON was greater at 21 d with the metabolism of terpenoids and polyketides as the major sub-category impacted and inhibited. In contrast, impact of SM *vs.* CON was lower at -10 d with the AAM as major impacted and activated pathway. The greatest impacted sub-category was MOAA, in turn the highest impact and inhibition of this sub-category was observed in the effect SM *vs.* MS at -10 d. In contrast, MOAA was highly activated by effect SM *vs.* MS

at 21 d postpartum. Additionally, MOAA was activated by SM *vs.* CON at -10 and 7 d, while MS *vs.* CON inhibited MOAA at the same time points. In addition to 'Metabolism', membrane transport under 'Environmental Information Processing' was impacted, similarly circulatory and sensory system under 'Organismal Systems'. Membrane transport was activated by SM *vs.* CON and SM *vs.* MS at 7 d, while MS *vs.* CON inhibited it at -10 d. The effect SM *vs.* MS at 21 d postpartum activated the circulatory sensory systems.

The pathways contained within the sub-categories of AAM and MOAA were sorted from all 9 comparisons within-diet effects and are shown in Figure 22. The cyanoamino acid metabolism and taurine and hypotaurine metabolism were the main impacted pathways within MOAA, which in turn were overall the 1st and 3rd most impacted pathways, respectively (Figure 23). Additionally, glutathione metabolism under MOAA and Cys and methionine metabolism AAM were among the 10 most impacted pathways (Figure 23). While MS *vs.* CON at -10 d inhibited both cyanoamino acid metabolism and taurine and hypotaurine metabolism, the effect SM *vs.* CON activate this pathways at the same time point. Although, effect SM *vs.* CON maintained an activation of cyanoamino acid metabolism and taurine and hypotaurine metabolism at 7 d similar to -10 d, MS *vs.* CON maintained an inhibitory effect in cyanoamino acid metabolism while activating the taurine and hypotaurine metabolism (Figure 22). Both MS and SM *vs.* CON inhibited taurine and hypotaurine as well as cyanoamino acid metabolisms at 21 d postpartum. Although, the glutathione metabolism was highly impacted, the within-diet comparisons showed a negligible impact and direction of such of impact, especially in the effects MS *vs.* CON and SM *vs.* CON. The effect of SM *vs.* CON at -10 d activated the

lysine biosynthesis, while inhibiting the valine, leucine, and isoleucine biosynthesis pathway.

Within-time comparisons

There was a strong effect of time within the same diet as shown in Figure 24. Similar to the within-diet comparisons in Figure 21, the category 'Metabolism' was the most impacted across the time of parturition. From these results we observed profound changes in metabolic pathways but especially in MOAA across diet and time comparisons. These changes resulted in activation of MOAA pathways from prepartum to postpartum in all treatments. Supplementing Met as MS and SM activate most sub-categories under 'Metabolism' at 7 d *vs.* -10 d with the exception metabolism of cofactors and vitamins, xenobiotics biodegradation and metabolism, and lipid metabolism in MS, while glycan biosynthesis and metabolism was inhibited in SM. In contrast to similar patterns between MS and SM at 7 d *vs.* -10 d, all metabolic sub-categories were activated within MS cows at 21 d *vs.* 7 d, while SM cows have an inhibition of all these sub-categories. In fact, overall impact of SM in 'Metabolites' was greater at 7 d *vs.* -10 d, while MS had a greater impact at 21 d *vs.* -10 d. Within the previous time comparisons energy metabolism was the most impacted sub-category in SM at 7 d *vs.* -10 d, in contrast MOAA was the most impacted in MS at 21 d *vs.* -10 d. Overall CON diet had similar impact pattern as SM, with greater impact at 7 d *vs.* -10 d comparison, and within this effect MOAA was the most impacted sub-category.

Similar to within-diet comparisons, membrane transport under 'Environmental Information Processing' was considerably impacted, where both SM and MS have a

significant impact and activation of membrane transport at 7 d *vs.* -10 d and 21 d *vs.* -10 d. Most sub-categories under 'Organismal System' were impacted and activated specifically in SM at 7 d *vs.* -10 d. The previous effect was accompanied with a greater impact and activation of most sub-categories under 'Genetic Information and Processing' in SM at 7 d *vs.* -10 d.

Further analysis of pathways under the AAM and MOAA sub-categories among 9 comparisons within-time revealed an evident overall activation of cyanoamino acid metabolism across time comparisons, with the exception of 21 d *vs.* 7 in CON diet (Figure 25). Additional, activation of cyanoamino acid metabolism was less pronounced at 21 d vs. 7 d and 21 d vs. -10 d in SM. Supplementation of SM produced activation of taurine and hypotaurine metabolism at 7 d *vs.* -10 d, in contrast CON inhibited this pathway. However, this pathway was activated at 21 d *vs.* 7 d across all dietary treatments. A similar patter was observed in cysteine (Cys) and Met metabolism across dietary treatments, where this pathway was activated at 7 d *vs.* -10 follow by inhibition at 21 d *vs.* 7 d, however this pathway was overall activated from -10 d to 21 d postpartum. In contrast to a negligible impact and flux observed in glutathione metabolism observed in the within-diet comparisons (Figure 22), the within-time comparisons revealed a significant impact and flux on this pathway (Figure 25). Glutathione metabolism was inhibited by MS while activated by SM and CON at 7 d *vs.* -10 d, in contrast SM inhibited it while MS and CON activated at 21 d *vs.* 7 d. Overall glutathione metabolism was activated by MS and CON from -10 d to 21 d, while SM inhibited.

DISCUSSION

The inability of postpartal cows to consume sufficient protein to meet mammary and extra-mammary AA requirements, including a significant demand for hepatic gluconeogenesis, will likely promote active mobilization of tissue protein during the first 2 wk of lactation (Bell et al., 2000). Therefore, supplementation of AA such as Met in the forms of MS and SM improves milk production at least in part due to increases of postpartal DMI while reducing the loss of BCS (Osorio et al., 2013). Postpartal energy requirements for milk production coupled with insufficient DMI will promote negative energy balance (NEB). Previously we observed that Met supplementation (SM or MS) led to a faster recovery from NEB toward a positive energy balance during the first 4 wk postpartum (Osorio et al., 2013). Our group has previously determined the effect of the transition period adaptations under either restricted or overfeeding energy prepartum in bovine liver using a microarray platform in quantitative gene expression (Loor et al., 2005; Loor et al., 2006). To our knowledge, this is the first study to evaluate the effect of peripartal Met supplementation on hepatic gene expression of dairy cows using microarray techniques.

In this experiment, the category ‘Metabolism’ of KEGG pathways was highly impacted in both within-diet and within-time comparisons. Metabolism is a general category that is comprised of 11 sub-categories including 1.1) carbohydrate metabolism, 1.2) energy metabolism, 1.3) lipid metabolism, 1.4) nucleotide metabolism, 1.5) amino acid metabolism, 1.6) metabolism of other amino acids, 1.7) glycan biosynthesis and metabolism, 1.8) metabolism of cofactors and vitamins, 1.9) metabolism of terpenoids and polyketides, 1.10) biosynthesis of other secondary metabolites, and 1.11) xenobiotics

biodegradation and metabolism (Kanehisa et al., 2006). The greater impact in the metabolism category was evident, especially in MOAA, which is obvious because nutrients can act as modulators of gene expression and play major role to control gene regulation in different cellular responses and biological processes (Bionaz et al., 2012). In fact, Met can exert gene expression modulation as being substrate for s-adenosylmethionine (SAM) synthesis; in turn SAM is important methyl-donor that is involve in DNA methylation, and consequently regulating gene expression.

Supplementation of Met in the forms of MS and SM not only impacted MOAA and AAM sub-categories, but also modified the profile of impact and flux between the MS and SM *vs.* CON diets as well as within-time comparisons. Additionally, the MOAA sub-category was highly impacted in cows fed CON diet, which underlines the importance of AA metabolism in the adaptations that occur during the transition period. This is in agreement with Bell et al. (2000),where it was estimated that during the first week of lactation cows will undergo negative metabolizable protein balance up to 600 g/d, and consequently high-yield cows will mobilize up to 1000 g of tissue protein/d. Hence, high impact and activation of MOAA was observed from -10 d to 7 d postpartum in SM and CON and to a lesser activation in MS. The latter effect in MS could be associated to inhibition of pathways for selenoamino acid metabolism and glutathione metabolism under MOAA. The inhibition of glutathione metabolism from -10 d to 7 d is consistent with a 63.3% reduction in liver glutathione concentration in MS, while a 39.3% and 36.3% were observed in MS and CON, respectively (Osorio et al., 2012). It is possible that MS cows might have relied on other antioxidant mechanisms during this period, since the oxygen radicals absorbance capacity (ORAC) was greater (12.6 vs.

11.9) in both MS and SM vs. CON. Although taurine and hypotaurine metabolism was not represented in MS diet at 7 d *vs.* -10 d comparison, this was activated in SM but inhibited in CON. This is important, since taurine is derived from Cys, which in turn is an important product of the Met cycle through homocysteine (Champe et al., 2008). This suggests that CON favored re-methylation of homocysteine to regenerate Met in order to compensate for lower availability of this AA. Also, SM cows might have benefited from antioxidant effects of taurine previously observed in human neutrophils (Green et al., 1991).

The importance of Cys as a hub between taurine and hypotaurine metabolism, glutathione metabolism, and cyanoamino acid metabolism might have a key role in the activation or inhibition of these pathways, however the specific mechanism by which Met in the form of MS or SM produced such alterations between these and CON remains to be completely elucidated. For instance, MS and SM produce an activation of cyanoamino acid metabolism at 21 d vs. 7 d, while this was inhibited in CON cows. This difference in cyanoamino acid metabolism result might be associated to inhibition of Cys and Met metabolism at 21 d vs. 7 d, and shifting inhibition of cyanoamino acid metabolism for activation of taurine and hypotaurine metabolism in CON cows. Although there is a lack of knowledge on the extent and role of this pathway within the context of the peripartal period, this was the most impacted pathway in our experiment. Hence, the study of this particular pathway during the peripartal period might shed new findings in the physiological adaptations, not only in terms of AA metabolism but also its interactions with other metabolisms such as lipid and carbohydrate.

CONCLUSIONS

Hepatic transcriptome analysis for this experiment indicated that Met supplementation during the peripartal period of dairy cows exerts profound changes on liver function. The main biological alterations occurred in metabolism several AA that can potentially determine the fate of other biological functions such as lipid and carbohydrate metabolism. The fact that gene networks associated with AA metabolism in CON cows was highly impacted, underlines the importance of AA metabolism in the adaptations that occur during the transition period. Further microarray data interpretation with accurate biological reasoning within the context of the peripartal period can help us uncover meaningful and novel mechanism to improve health and promote high performance in dairy cows.

REFERENCES

Armentano, L. E., S. J. Bertics, and G. A. Ducharme. 1997. Response of lactating cows to methionine or methionine plus lysine added to high protein diets based on alfalfa and heated soybeans. J Dairy Sci. 80:1194-1199.

Bell, A. W., W. S. Burhans, and T. R. Overton. 2000. Protein nutrition in late pregnancy, maternal protein reserves and lactation performance in dairy cows. Proc Nutr Soc. 59:119-126.

Bionaz, M., K. Periasamy, S. L. Rodriguez-Zas, W. L. Hurley, and J. J. Loor. 2012. A novel dynamic impact approach (DIA) for functional analysis of time-course omics studies: validation using the bovine mammary transcriptome. PLoS One. 7:e32455.

Champe, P. C., R. A. Harvey, and D. R. Ferrier. 2008. Biochemistry. 4th ed. Lippincott's illustrated reviews. Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia.

Chen, Z. H., G. A. Broderick, N. D. Luchini, B. K. Sloan, and E. Devillard. 2011. Effect of feeding different sources of rumen-protected methionine on milk production and N-utilization in lactating dairy cows. J Dairy Sci. 94:1978-1988.

Dann, H. M., N. B. Litherland, J. P. Underwood, M. Bionaz, A. D'Angelo, J. W. McFadden, and J. K. Drackley. 2006. Diets during far-off and close-up dry periods affect periparturient metabolism and lactation in multiparous cows. J Dairy Sci. 89:3563-3577.

Drackley, J. K. 1999. ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: the final frontier? J Dairy Sci. 82:2259-2273.

Graugnard, D. E., L. L. Berger, D. B. Faulkner, and J. J. Loor. 2010. High-starch diets induce precocious adipogenic gene network up-regulation in longissimus lumborum of early-weaned Angus cattle. Br J Nutr. 103:953-963.

Green, T. R., J. H. Fellman, A. L. Eicher, and K. L. Pratt. 1991. Antioxidant role and subcellular location of hypotaurine and taurine in human neutrophils. Biochim Biophys Acta. 1073:91-97.

Ingvartsen, K. L. 2006. Feeding- and management-related diseases in the transition cow. Anim Feed Sci and Tech. 126:175-213.

Kanehisa, M., S. Goto, M. Hattori, K. F. Aoki-Kinoshita, M. Itoh, S. Kawashima, T. Katayama, M. Araki, and M. Hirakawa. 2006. From genomics to chemical genomics: new developments in KEGG. Nucleic Acids Res. 34:D354-357.

Loor, J. J., H. M. Dann, R. E. Everts, R. Oliveira, C. A. Green, N. A. Guretzky, S. L. Rodriguez-Zas, H. A. Lewin, and J. K. Drackley. 2005. Temporal gene expression

profiling of liver from periparturient dairy cows reveals complex adaptive mechanisms in hepatic function. Physiol Genomics. 23:217-226.

Loor, J. J., H. M. Dann, N. A. Guretzky, R. E. Everts, R. Oliveira, C. A. Green, N. B. Litherland, S. L. Rodriguez-Zas, H. A. Lewin, and J. K. Drackley. 2006. Plane of nutrition prepartum alters hepatic gene expression and function in dairy cows as assessed by longitudinal transcript and metabolic profiling. Physiol Genomics. 27:29-41.

Loor, J. J., R. E. Everts, M. Bionaz, H. M. Dann, D. E. Morin, R. Oliveira, S. L. Rodriguez-Zas, J. K. Drackley, and H. A. Lewin. 2007. Nutrition-induced ketosis alters metabolic and signaling gene networks in liver of periparturient dairy cows. Physiol Genomics. 32:105-116.

Ordway, R. S., S. E. Boucher, N. L. Whitehouse, C. G. Schwab, and B. K. Sloan. 2009. Effects of providing two forms of supplemental methionine to periparturient Holstein dairy cows on feed intake and lactational performance. J Dairy Sci. 92:5154-5166.

Osorio, J. S., P. Ji, J. K. Drackley, D. Luchini, and J. J. Loor. 2013. Supplemental Smartamine M or MetaSmart during the transition period benefits postpartal cow performance and blood neutrophil function. J Dairy Sci. 96:6248-6263.

Osorio, J. S., E. Trevisi, P. Ji, D. Luchini, J. K. Drackley, G. Bertoni, and J. J. Loor. 2012. Immunometabolic indices in dairy cows supplemented with Smartamine M or MetaSmar during the peripartal period. . J Dairy Sci. 95. (Suppl. 2), T298

Phillips, G. J., T. L. Citron, J. S. Sage, K. A. Cummins, M. J. Cecava, and J. P. McNamara. 2003. Adaptations in body muscle and fat in transition dairy cattle fed differing amounts of protein and methionine hydroxy analog. J Dairy Sci. 86:3634-3647.

Rulquin, H. and L. Delaby. 1997. Effects of the energy balance of dairy cows on lactational responses to rumen-protected methionine. J Dairy Sci. 80:2513-2522.

Socha, M. T., D. E. Putnam, B. D. Garthwaite, N. L. Whitehouse, N. A. Kierstead, C. G. Schwab, G. A. Ducharme, and J. C. Robert. 2005. Improving intestinal amino acid supply of pre- and postpartum dairy cows with rumen-protected methionine and lysine. J Dairy Sci. 88:1113-1126.

TABLES AND FIGURES

Table 10. Differentially expressed genes (DEG) within-diet comparisons ($P < 0.05$, FDR < 0.10) in hepatic tissue of cows (n = 8/treatment) fed a control diet (CON), CON diet plus MetaSmart (MS), and CON diet plus Smartamine (SM) from -21 d to 30 d relative to calving.

Comparison	Day	DEG	DEG annotated to Bovine gene ID	Up-regulated	Down-regulated
CON vs. MS	-10	491	367	116	251
CON vs. SM	-10	388	317	150	167
SM vs. MS	-10	604	470	190	280
CON vs. MS	7	766	583	342	241
CON vs. SM	7	642	483	278	205
SM vs. MS	7	637	504	249	255
CON vs. MS	21	359	283	77	206
CON vs. SM	21	636	508	185	323
SM vs. MS	21	914	731	400	331

Table 11. Differentially expressed genes (DEG) within-time comparisons ($P < 0.05$, FDR < 0.10) in hepatic tissue of cows (n = 8/treatment) fed a control diet (CON), CON diet plus MetaSmart (MS), and CON diet plus Smartamine (SM) from -21 d to 30 d relative to calving.

Comparison	Day	DEG	DEG annotated to Bovine gene ID	Up-regulated	Down-regulated
Day 7 vs. -10	CON	1209	934	430	504
Day 21 vs. -10	CON	797	622	340	282
Day 21 vs. 7	CON	996	771	449	322
Day 7 vs. -10	MS	999	787	473	314
Day 21 vs. -10	MS	1018	800	422	378
Day 21 vs. 7	MS	1035	811	358	453
Day 7 vs. -10	SM	1518	1190	612	578
Day 21 vs. -10	SM	926	740	320	420
Day 21 vs. 7	SM	1362	1085	461	624

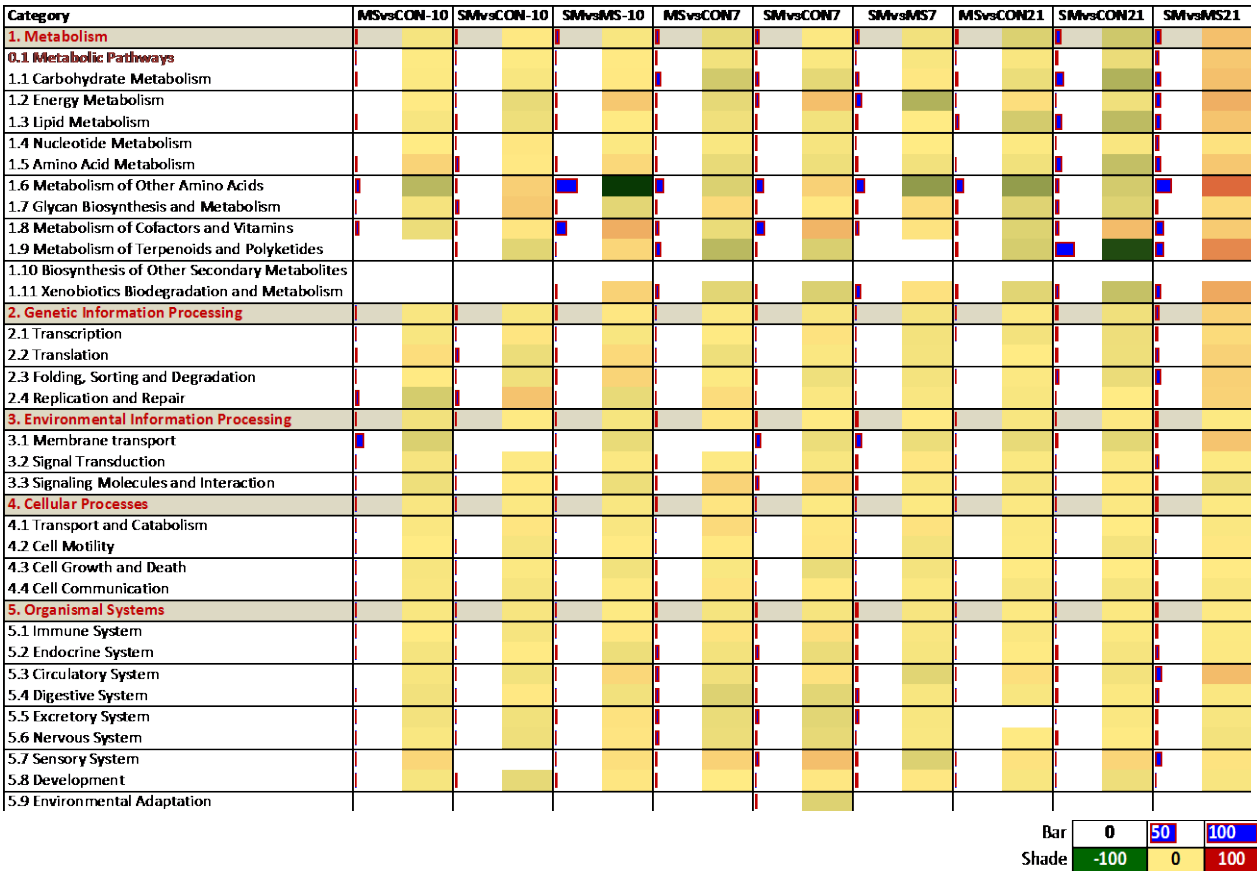

Figure 21. Impact and flux of major KEGG categories with pathways (by DIA) within-diet comparisons ($P < 0.05$, FDR < 0.10) in hepatic tissue of cows (n = 8/treatment) fed a control diet (CON), CON diet plus MetaSmart (MS), CON diet plus Smartamine (SM) from -21 d to 30 d relative to calving. Reported are the total impact (blue horizontal bars; larger the bars higher the impact) and the direction of the impact (or flux; green shade denotes inhibition and red shade denotes activation). MS *vs.* CON is denote by MSvsCON, SM *vs.* CON is denoted by SMvsCON, and SM *vs.* MS by SMvsMS at respective days -10, 7 and 21 d relative to calving.

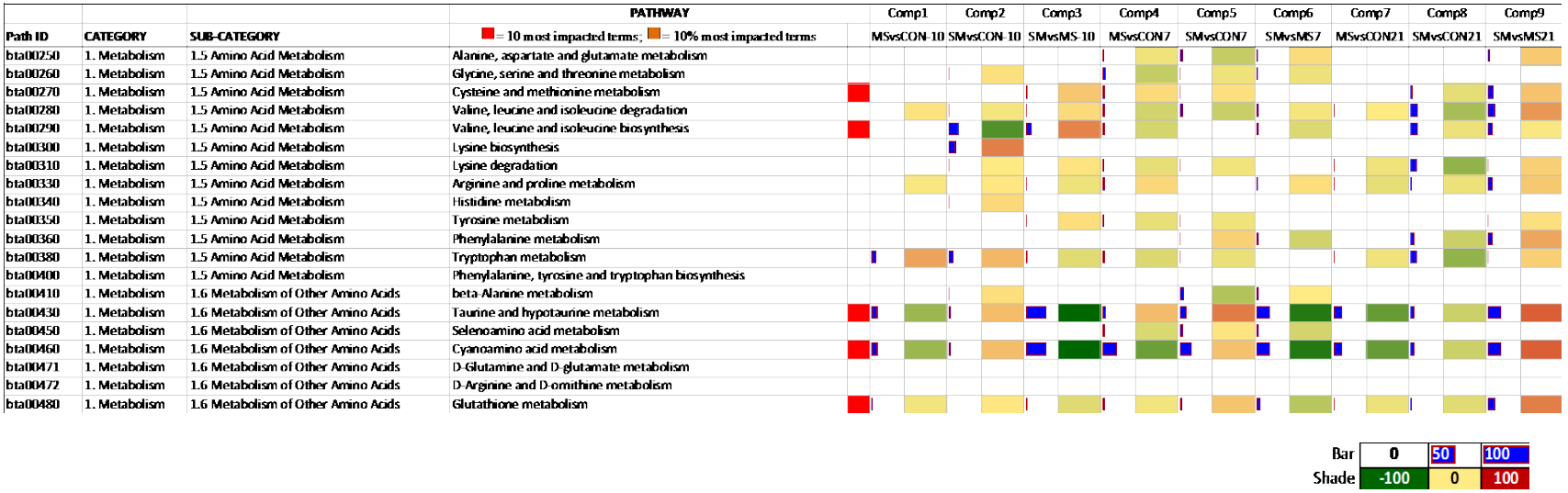

Figure 22. Impact and flux of major KEGG categories, sub-categories and pathways (by DIA) within-diet comparisons ($P < 0.05$, FDR < 0.10) in hepatic tissue of cows (n = 8/treatment) fed a control diet (CON), CON diet plus MetaSmart (MS), CON diet plus Smartamine (SM) from -21 d to 30 d relative to calving. Reported are the total impact (blue horizontal bars; larger the bars higher the impact) and the direction of the impact (or flux; green shade denotes inhibition and red shade denotes activation). MS *vs.* CON is denote by MSvsCON, SM *vs.* CON is denoted by SMvsCON, and SM *vs.* MS by SMvsMS at respective days -10, 7 and 21 d relative to calving.

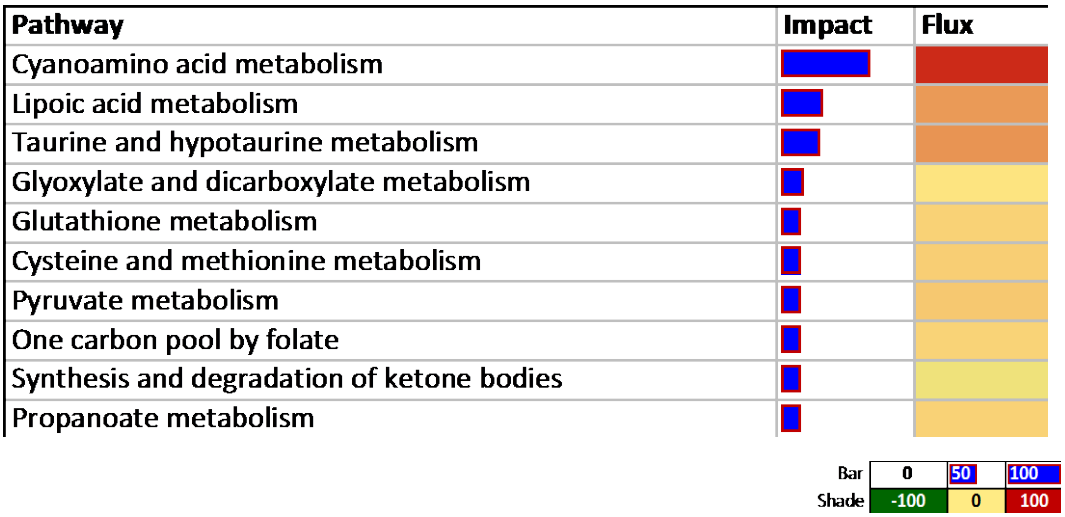

Figure 23. Impact and flux of top-10 most impacted KEGG pathways (by DIA) across diet and time comparisons (FDR $P = 0.10$, $P < 0.05$) in hepatic tissue of cows (n = 8/treatment) fed a control diet (CON), CON diet plus MetaSmart (MS), CON diet plus Smartamine (SM) from -21 d to 30 d relative to calving. Reported are the total impact (blue horizontal bars; larger the bars higher the impact) and the direction of the impact (or flux; green shade denotes inhibition and red shade denotes activation).

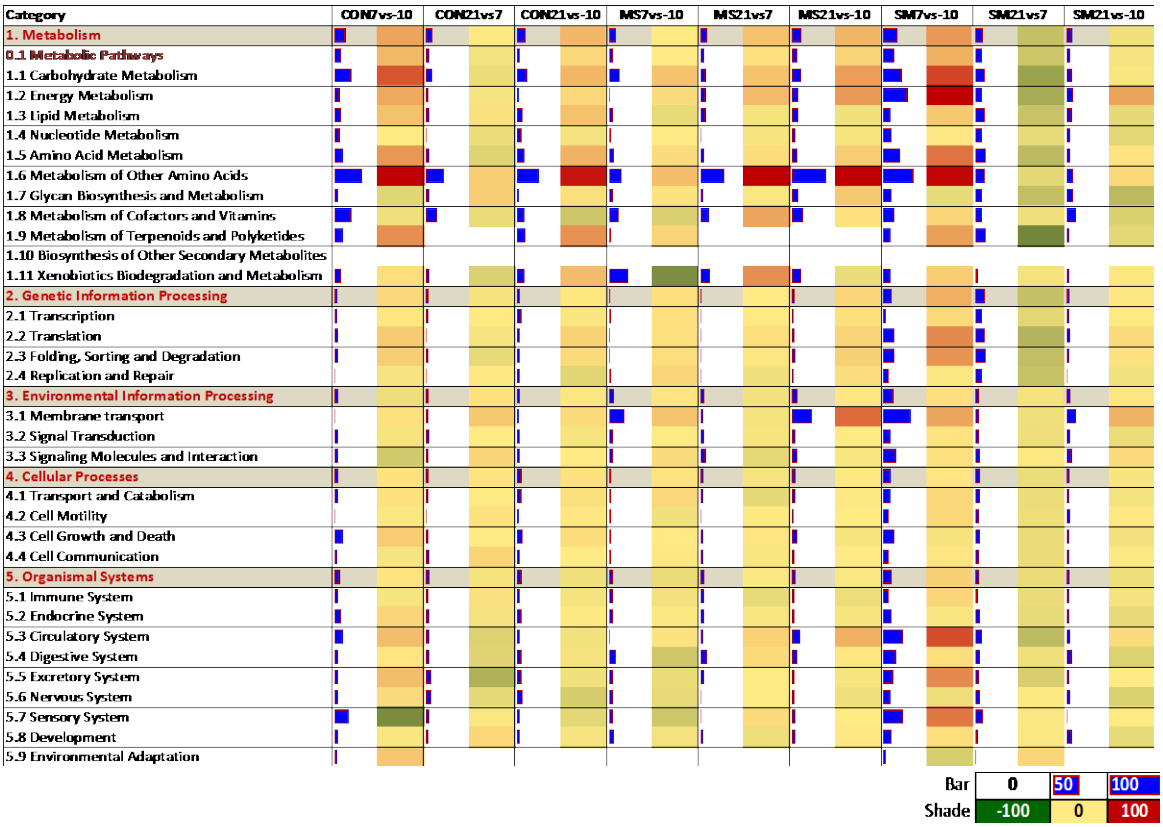

Figure 24. Impact and flux of major KEGG categories with pathways (by DIA) within-time comparisons ($P < 0.05$, FDR < 0.10) in hepatic tissue of cows (n = 8/treatment) fed a control diet (CON), CON diet plus MetaSmart (MS), CON diet plus Smartamine (SM) from -21 d to 30 d relative to calving. Reported are the total impact (blue horizontal bars; larger the bars higher the impact) and the direction of the impact (or flux; green shade denotes inhibition and red shade denotes activation). Control is denoted by CON, MetaSmart by MS, and Smartamine by SM at respective comparisons of days -10, 7 and 21 d relative to calving.

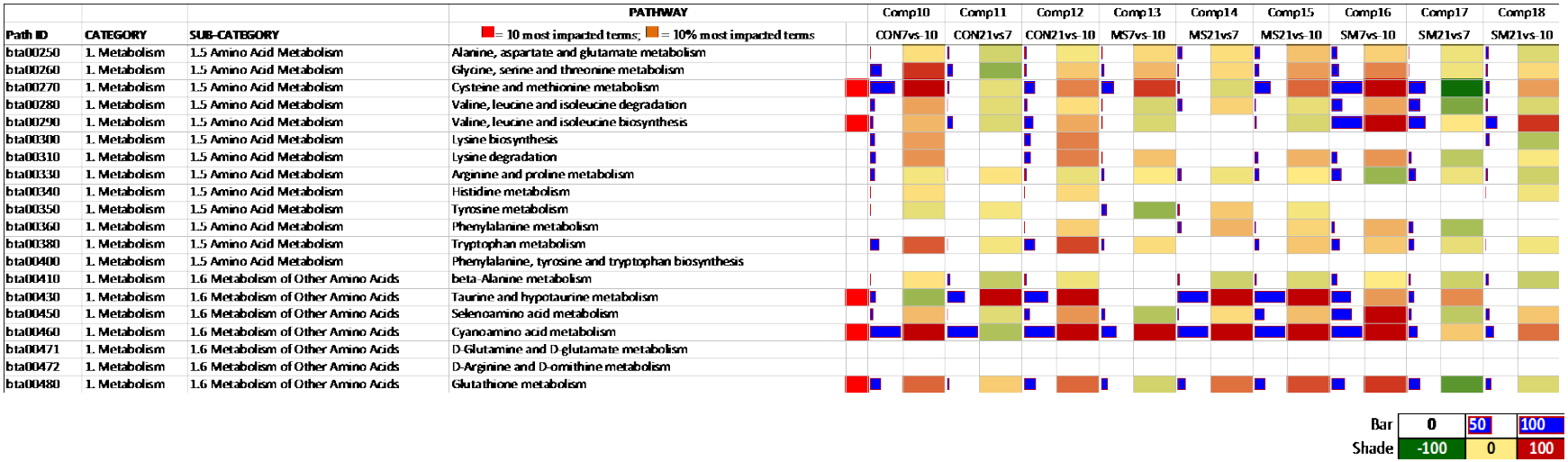

Figure 25. Impact and flux of major KEGG categories, sub-categories, and pathways (by DIA) within-time comparisons ($P < 0.05$, FDR < 0.10) in hepatic tissue of cows (n = 8/treatment) fed a control diet (CON), CON diet plus MetaSmart (MS), CON diet plus Smartamine (SM) from -21 d to 30 d relative to calving. Reported are the total impact (blue horizontal bars; larger the bars higher the impact) and the direction of the impact (or flux; green shade denotes inhibition and red shade denotes activation). Control is denoted by CON, MetaSmart by MS, and Smartamine by SM at respective comparisons of days -10, 7 and 21 d relative to calving.

CHAPTER 6

SUMMARY AND CONCLUSIONS

The overall objective of this dissertation was to evaluate the performance parameters, blood and liver biomarkers, and hepatic transcriptomic alterations in diary cows supplemented with methionine (Met) in the forms of Smartamine (SM) or MetaSmart (MS) during the peripartal period. Our general hypothesis was that supplementing rumen-protected Met to peripartal dairy cows will improve DMI, milk yield and components, and energy balance by enhancing liver function and antioxidant capacity, and decreasing the inflammatory response, and that in turn these effects are controlled at the molecular level by cross-talk of gene expression.

Supplementation of MS or SM can improve milk production at least in part by increasing voluntary DMI and perhaps by optimizing the use of body lipid reserves as observed in chapter 2. In fact, the overall milk protein and milk fat was positively affected by Met supplementation. Improvements in production were accompanied by a faster recovery to a positive energy balance, coupled with a lower predisposition to develop ketosis in Met-supplemented cows, which indicates that overall health was at least not compromised and perhaps improved.

Results from chapter 3 indicate that supplementing Met during the peripartal period promotes favorable alterations of inflammatory and oxidative stress status of cows. The greater serum albumin in response to Met-supplementation may indicate enhanced liver function and improved AA status. Overall blood and liver biomarkers

analyzed indicated that improved postpartal performance when feeding SM and MS was due partly to a better immunometabolic status.

Relative mRNA expression of targeted genes associated with biological processes of interest such as the Met cycle, inflammation, and oxidative stress, among others, was evaluated in chapter 4. Results indicate that feeding MS or SM to cows during the peripartal period can profoundly affect the hepatic transcriptomics of Met metabolism, *PPARA* activation, hepatokines synthesis, and gluconeogenesis.

To better understand the molecular effects of peripartal Met-supplementation on hepatic metabolism in Holstein dairy cows, a microarray platform with advanced computational and bioinformatics techniques were used in chapter 5. Hepatic transcriptome analysis revealed a high impact on metabolism especially in pathways AA metabolism.

In general, supplementation of rumen-protected Met during the peripartal period resulted in significant improvements in postpartal performance and health status from an immunometabolic standpoint as well as significant hepatic transcriptomics alterations. Our findings presented in this dissertation not only indicate that supplementing rumen-protected Met is beneficial for peripartal dairy cows, but also has re-established the importance of maintaining an adequate protein balance as previous literature suggested. Our results may not only be restricted to the effects of a single AA as Met, but also may indicate that maintaining an adequate balance among AA as indicated by an optimal Lys:Met will promote beneficial effects in peripartal dairy cows. The implications observed in this research should drive greater attention in industry by taking more consideration to AA when balancing rations for peripartal cows. Results in this

dissertation also may prompt new avenues of research by promoting the study of peripartal AA metabolism in a more comprehensive manner, taking advantage of new molecular techniques with the application of bioinformatics as a new approach to better understand the molecular basis of physiological effects.