PHYSIOLOGICAL AND MOLECULAR MECHANISMS ASSOCIATED WITH PERFORMANCE, IMMUNOMETABOLIC STATUS, AND LIVER FUNCTION IN TRANSITION DAIRY COWS FED RUMEN PROTECTED METHIONINE OR CHOLINE

BY

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DISSERTATION

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ABSTRACT

The onset of lactation in dairy cows is characterized by high output of methylated compounds in milk when sources of methyl group are in short supply. Methionine (MET) and choline (CHOL) are key methyl donors and their availability during this time may be limiting for milk production, hepatic lipid metabolism, and immune function. Supplementing rumen-protected MET and CHOL may improve overall performance and health of transition cows. Physiological and molecular mechanisms for methyl donor supplementation benefits are unknown. Objectives were to evaluate performance, immunometabolic status, liver function, and the underlying mechanisms in response to MET and CHOL supplementation. Eighty-one multiparous Holstein cows were used in a randomized complete block design with 2×2 factorial arrangement of MET (Smartamine M, Adisseo NA) and CHOL (ReaShure, Balchem Inc.) level (with or without). Treatments were control (CON), no MET or CHO; CON+MET (SMA); CON+CHO (REA); and CON+MET+CHO (MIX). From −50 d to −21 d before expected calving, all cows received the same diet (1.40 Mcal/kg DM). From −21 d to calving, cows received the same close up diet (1.52 Mcal/kg DM) and were assigned randomly to treatments (CON, SMA, REA, or MIX). From calving to 30 DIM, cows were on the same postpartal diet (1.71 Mcal/kg DM) and continued to receive the same treatments through 30 DIM. MET supplementation was adjusted daily at 0.08% DM of diet and CHOL was supplemented at 60 g/cow/d. Blood (-10, 4, 8, 20, and 30 d) and liver (-10, 7, 21, and 30 d) samples were harvested for biomarker and molecular analyses. Incidence of clinical ketosis and retained placenta tended to be lower in MET-supplemented cows. MET supplementation led to greater DMI during close-up ($P = 0.01$) and first 30 d postpartum ($P = 0.02$). Milk yield ($P = 0.03$) and milk protein % ($P < 0.01$) also were greater in MET- compared with CHOL-supplemented cows. The greater overall

plasma albumin concentration (P = 0.04), blood neutrophil phagocytosis capacity (P = 0.01), and neutrophil oxidative burst (P = 0.03) in MET-supplemented cows underscored a better liver function and immune status. In addition, the greater concentrations of hepatic reduced and total (P = 0.01) glutathione indicated a lower degree of oxidative stress in cows fed MET compared with CHOL. MET-supplemented cows had greater (0.38 vs. 0.27; SEM = 0.05; P = 0.02) methionine adenosyltransferase 1A (*MAT1A*) and phosphatidylethanolamine methyltransferase (*PEMT*) expression (0.74 vs. 0.58; SEM = 0.08; P = 0.05). Greater (0.93 vs. 0.74; SEM = 0.05; P = 0.01) *S*-adenosylhomocysteine hydrolase (*SAHH*) and *CBS* (1.16 vs. 1.02; SEM = 0.07; P = 0.04) as well as lower (23.4 vs. 29.7 nmol product h^{-1} mg $protein^{-1}$; SEM = 2.9; P = 0.04) MTR activity were also detected in MET but not CHOL-supplemented cows. Although greater (1.07 vs. 0.93; SEM = 0.05; P = 0.01) expression of betaine aldehyde dehydrogenase (*BADH*) was observed in response to CHOL supplementation, expression of (*BHMT*) and *MTR* and BHMT enzyme activity did not change (P > 0.05). Furthermore, overall MTR enzyme activity was lower (23.5 vs. 29.6 nmol product h^{-1} mg $protein^{-1}$; SEM = 2.9; P = 0.05) in CHOL cows. In terms of AA profile, MET supplementation led to greater (P < 0.01) circulating methionine and proportion of methionine in the essential AA pool, total AA, and total sulfur-containing compounds. Lysine in total AA also tends to be greater (P = 0.08) in these cows, indicating a better overall AA profile. Sulfur-containing compounds (cystathionine, cystine, homocystine, and taurine) also were greater (P < 0.05) in MET-supplemented cows, indicating an enriched sulfur-containing compound pool due to enhanced transsulfuration activity. Circulating essential AA (P = 0.06) and total AA (P = 0.03) concentrations were greater in MET cows due to greater lysine, arginine, tryptophan, threonine, proline, asparagine, alanine, and citrulline. In contrast, tryptophan and cystine were greater. Plasma 3-methylhistidine concentration was lower (P =

0.02) in response to CHOL, suggesting less tissue protein mobilization in these cows. Overall, results indicate that MET supplementation improves performance of transition cows through a combination of better immunometabolic status, plasma AA profile, and a reduction in oxidative stress; insufficient regeneration of MET may be one reason for the lack of effect with supplemental CHOL.

Key words: Methionine, choline, transition cow, inflammation, oxidative stress

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TABLE OF CONTENTS

CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

Modern dairy cows have been selected over generations to maximize milk yield, thereby creating a nutrient highway from the daily ration to sustain milk production (Tamminga et al., 1997). From a metabolic, physiologic, and immunologic standpoint the periparturient or transition period, defined as 3 weeks before to 3 weeks after parturition, is the most stressful period in the lactation cycle (Drackley, 1999). During this time, dairy cows experience the highest incidence of displaced abomasum, retained placenta, and ketosis. Although many cows are able to meet the adaptive changes, health problem such as displacement of abomasum, retained placenta, ketosis, and fatty liver, occur to nearly one in two cows underscores the fragility of the system during the transition period (Loor et al., 2013a).

In fact, requirements for energy, protein as well as many minerals double overnight as cows go through parturition and commence lactation. As the nutrient demand is unable to be met through feed intake alone due to the rate of dry matter intake is slower than output for milk production, the onset of lactation in dairy cows is characterized by a severe negative balance of energy, metabolizable protein (MP), and limiting AA. These negative changes render dairy cows in a state of immune-suppression, which leads to increased susceptibility to metabolic disorders (Drackley, 1999, Ingvartsen, 2006). Therefore, energy consumption and availability of MP and limiting AA (e.g. methionine) may be determinant factors for the success of the transition period.

Owing to the fact that methyl donors are required for the synthesis of key compounds such as phosphatidylcholine and carnitine in tissues (Pinotti et al., 2002), a negative methyl

donor balance also may be an important challenge for the transition dairy cow. In fact, milk from dairy cows is high in methylated compounds and levels of these compounds secreted into milk are maintained even at the cost of depleting liver tissue reserves (Pinotti et al., 2002). Due to extensive microbial degradation in the rumen, dietary availability of key methyl donors [(e.g., Met (MET) and choline (CHOL)] is limited (Sharma and Erdman, 1989, Girard and Matte, 2005). Consequently, the increased mobilization of body protein in dairy cows during this period may serve, at least in part, to compensate for the shortfall in methyl groups required by mammary gland and liver (Komaragiri and Erdman, 1997). Supplementing rumen-protected methyl donors may help fulfill the daily methyl group requirement, and possibly improve the overall production and health of dairy cows during the transition period (Zom et al., 2011, Osorio et al., 2013, Osorio et al., 2014b).

Therefore, our general hypothesis was that supplementing rumen-protected MET and/or CHOL to transition dairy cows improve overall health and lactation performance by improving liver function and alleviating inflammation and oxidative stress. These effects are controlled at the molecular level by cross-talk of gene expression, enzyme activity, and substrate availability.

Transition period

The transition period is the most challenging stage in the lactation cycle when most metabolic disorders and infectious diseases occur (Drackley, 1999). The importance of the transition from late pregnancy to lactation in health, productivity, and profitability has been underscored by intense interest in nutrition and management of dairy cows over the past 20 years (Drackley, 1999).

From parturition through peak lactation, mobilization of body reserves, e.g. skeletal muscle or adipose tissue, is often observed and occurs in response to the metabolic burden elicited by the onset of lactation, i.e. the need of the mammary gland to uptake glucose and other nutrients in support of milk component synthesis. Although considered "normal" adaptations, a period of stress accompanies the well-established physiological adaptations starting during the peripartal period through peak lactation. An important driver of the metabolic stress induced by the sudden onset of milk production is the lag in dry matter intake (DMI) that is not achieved until after peak lactation (Drackley, 1999). In light of such changes, the metabolism of dairy cows changes drastically which contribute greatly to the incidences of metabolic diseases occurred in the peripartal period. A summary of key metabolic changes and adaptations are presented below.

Protein and AA metabolism

The amount of MP flowing to the intestine from both dietary and microbial source are often diminished by lower DMI around calving. The increased demand for fetal development prepartum and the inability of cows to consume sufficient protein, greatly contributes to negative protein/amino acid balance, especially when the onset of lactation greatly increases the mammary gland demand for AA (Bell et al., 2000). It has been estimated that during the first week of lactation cows are 600g/d in negative MP balance. The inability of dairy cows to produce sufficient MP to meet mammary and extra-mammary AA requirement during the first 2 weeks of lactation promotes active mobilization of tissue protein (Bell et al., 2000, van der Drift et al., 2012). In fact, continuous synthesis and breakdown of proteins characterizes the process of body protein metabolism (Bergen, 2008). Previous studies with dairy cattle proposed that AA

profiles in serum are affected by a multitude of events such as skeletal muscle catabolism (Bell et al., 2000, Korhonen et al., 2002), dietary AA profile (Polan et al., 1991), liver gluconeogenesis, and gastrointestinal absorption (Meijer et al., 1995a). More recent data from a large number of studies revealed that the uptake of total non-essential amino acids by the liver was substantially greater in the peripaturient period (Kuhla et al., 2011, Larsen and Kristensen, 2013). It has also been suggested that the liver may take up more AA from the circulation during the periparturient period for production of APP (Kurpad, 2006).

The importance of balancing AA has been denoted by Schwab (2012). Lysine and Met have been identified as the two most limiting AA for lactating dairy cows in North America (NRC, 2001b). Thus, by supplementing the most limiting AA such as Lys and Met, AA requirements are being met with lower concentrations of dietary protein.

Lipid Metabolism

After calving, the total energy intake by dairy cows are usually less than energy requirements, even in healthy cows (Bell et al., 2000). To make up for the negative energy balance, long chain fatty acids are mobilized from adipose tissue and released to the circulation as non-esterified fatty acids (NEFA). As a result, NEFA concentration in blood commonly increase after calving and reflects the degree of adipose tissue mobilization (Pullen et al., 1990).

As liver is the most important site for removal of NEFA from circulation, over mobilization of lipids from adipose tissue leads to increased liver NEFA uptake and increased triglycerides (TAG) accumulation (Drackley, 1999). Subsequently, 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). The low capacity of ruminant liver to synthesize and secrete very-low density lipoproteins (VLDL) for TAG export from liver renders dairy cows susceptible to increased TAG accumulation (Kleppe et al., 1988). Therefore, excessive liver NEFA uptake give rise to fatty liver. Production of ketone bodies also increases in negative energy balance and carbohydrate insufficiency in the liver after calving, which can result in ketosis. It has been reported that ad libitum feeding of high-energy diets during dry period increased esterification and decreased oxidation capacity in liver at 1 d post-calving, which favors deposition of TAG in liver (Litherland et al., 2011).

Factors regulating disposition of NEFA between oxidation and esterification in liver of dairy cows remains to be investigated. Studies in nonruminants have suggested that the primary site of control seems to be at the entry of NEFA into the mitochondria, under the regulation of the enzyme carnitine palmitoyltransferase (CPT-1). However, little has changed both for activity of CPT-1 and its sensitivity to the key inhibitor molecule malonyl-CoA during the transition period (Dann et al., 2006). The peroxisomal fatty acid–oxidation system is an auxiliary pathway for fatty acid oxidation and increases in peroxisomal fatty acid–oxidation capacity in the liver may help the liver to cope with the large influx of NEFA from body fat mobilization (Grum et al., 1996, Grum et al., 2002).

Fat infiltration impairs the liver's ability to detoxify ammonia (Strang et al., 1998) as well as endotoxin (Andersen et al., 1996). Severe fatty liver disrupt normal liver functions, which can result in fatty liver syndrome, or clinical fatty liver (Morrow, 1976). Although fat infiltration per se does not lead to liver failure (Rehage, 1996), it may be a contributing factor at least in some circumstances. Therefore, maintaining liver function is crucial for dairy cows to ensure a smooth transition.

Methionine Metabolism

Methionine in vivo is mainly metabolized through the methionine cycle and the transsulfuration pathway, which share the first three steps (Finkelstein, 2003). The first step of methionine metabolism converts methionine to S-adenosyl Methionine (SAM). This step is catalyzed by methionine adenosyltransferase (MAT) and activates the methyl group on the sulfur atom of SAM. The activated methyl group on SAM is used for methylating numerous kinds of molecules including other methyl group donors such as choline. Upon cleavage of activated methyl group from the sulfur atom, SAM becomes S-adenosyl Homocysteine (SAH), which is subsequently converted to homocysteine. After the initial 3 common steps of the methionine cycle, homocysteine can either irreversibly enter the transsulfuration pathway to form other sulfur containing amino acids or converted back to methionine by accepting a methyl group from 5-methylenetetrahydrofolate or from betaine (Finkelstein, 2003). Although the methionine cycle is present in every tissue, transsulfuration only occurs in the liver, kidney, small intestine, and pancreas (Finkelstein, 1998). It has been estimated that 60% of homocysteine is metabolized by transsulfuration in the liver (Ulrey et al., 2005). Additionally, the liver is the only organ that possesses the isoenzyme of MAT that catalyzes the synthesis of SAM in response to increased availability of methionine (Finkelstein, 2003).

The importance of Met as one of the most-limiting AA for milk yield and components can also be associated with Met helping alleviate the increased demand for methylated compounds with the onset of lactation (Preynat et al., 2009a). Increased Met bioavailability in cows supplemented with rumen-protected Met (Graulet et al., 2005) is likely to increase entry of Met into the 1-carbon metabolism cycle in liver where it is initially converted into S-

adenosylmethionine (SAM), the major biological methyl-donor (Martinov et al., 2010b). Part of the mechanism elicited by supplemental Met in postpartal dairy cows is driven by changes in gene expression for key enzymes in the Met cycle. An end-result of such effect is more availability of enzyme protein that could help enhance the flux through the pathway leading to synthesis of several important intermediate metabolites (e.g. SAM, glutathione, taurine, PC).

Choline Metabolism

Dietary choline is absorbed from the small intestine and can be accumulated by all tissues of the body (Sweet et al., 2001). Upon uptake by various tissues, choline can be acetylated, oxidized or phosphorylated (Zeisel, 1990). Only a small portion of dietary choline is acetylated into neurotransmitter acetylcholine. Choline acetyltransferase in the terminals of cholinergic neurons are responsible for the reaction (White and Cavallito, 1970). Major choline metabolism takes place in the liver, and of all the possible fates of choline, formulating PC is the major pathway of dietary choline, and phosphorylation of choline is the first step for PC synthesis (Pelech and Vance, 1984). However, when synthesis of PC is saturated, choline can be irreversibly oxidized to betaine (Zeisel, 2006). Betaine can then participate in the methionine cycle and produce methionine by donating methyl groups acquired from choline to homocysteine (Zeisel, 1990). As for in vivo synthesis, choline can only be derived from de novo synthesis of PC through sequential methylation of phosphatidylethanolamine using the methyl group from SAM (Vance et al., 1997).

It is well established that choline plays an important role in liver lipid transport (Zeisel, 1990). Rats challenged with diets deficient in choline and methionine develops fatty liver and

such effect can be reversed upon resupplementation of choline (Hebbard and George, 2011). As VLDL is responsible for exporting triglycerides out of the liver and PC is an indispensable component for VLDL, choline deficiency leads to decreased synthesis of VLDL and thus triglycerides are accumulated in the cytosol of liver cells, which eventually induce the onset of fatty liver (Cole et al., 2012).

Interrelated Metabolism of Methionine and Choline

The direct interrelated metabolism of methionine and choline happens in the de novo synthesis of choline. During de novo synthesis of PC, phosphatidylethanolamine has to be sequentially methylated. As the global methyl donor, SAM is responsible for providing the methyl groups for sequential methylation and thus interacts with choline (Vance et al., 1997).

In the methionine cycle, choline and methionine metabolism interact when converting homocysteine to methionine (Wong and Thompson, 1972b). Upon receiving a methyl group from betaine or 5-methylenetetrahydrofolate, methionine is regenerated from homocysteine. Although betaine acts as the direct methyl group donor for remethylation of homocysteine, betaine acquired the methyl group from choline through irreversible oxidation (Li and Vance, 2008a). Therefore, choline is connected to methionine metabolism through betaine. As major methyl donors, both choline and methionine contribute to the methyl group pool of one carbon metabolism. Deficiency in one or more of the methyl group donors result in compensatory changes in others (Selhub et al., 1991). For example, feeding choline deficient diets to rodents not only decreased tissue concentrations of choline, but methionine level in tissue is also decreased (Zeisel et al., 1989). Although the irreversible reaction from choline to betaine makes

it impossible for choline to be formed directly from methionine through the methionine cycle, the compensatory changes in methionine may be beneficial for sparing choline for other important functions of choline, such as synthesis of VLDL. In terms of transition dairy cows, it has been debated whether supplementing one methyl donor is enough to satisfy the requirement of another. In the case of MET and CHO, it has yet to be determined the quantity of MET utilized to generate required CHO and vice versa during the peripartal period. More importantly, how much the flux from MET to CHO and vice versa serve to alleviate immunometabolic stress in peripartal dairy cow remains unknown.

Methionine cycle enzyme assays and kinetics

Kinetic studies on key enzymes in the Methionine cycle in rats revealed that the Km of CBS for Hcy is estimated at 4.8 mmol/L and for serine at 2.0 to 3.0 mmol/L (Taoka et al., 1999). MTR and BHMT, which use Hcy as substrate for remethylation back to methionine, has an estimated Km of 1.7 μmol/L and 106 μmol/L, respectively (Yamada et al., 1997). Therefore, at low concentrations of Hcy, remethylation of Hcy to methionine would be favored whereas at high concentrations, the transulfuration pathway would be activated to remove excess Hcy. These results suggest an important role of measuring key enzyme activities in the methionine cycle as an indication of flux and, more specifically, remethylation or transulfuration pathway especially in response to deficiency or over consumption of methyl group-containing nutrients.

To our knowledge, kinetics of the methionine cycle enzymes in ruminants are unknown. However, several studies have determined the developmental activity of these enzymes in sheep (Radcliffe and Egan, 1974, Xue and Snoswell, 1986) and growing steers in response to abomasal

methionine supplementation (Lambert et al., 2002). It is important to note that compared with rats, sheep have lowered activity of key enzymes in the one carbon metabolism pathway. In response to abomasal supplementation of methionine, MTR activity was reduced but not CBS. To our knowledge, activity of key enzymes in one carbon metabolism have not been determined in dairy cows.

Assessing oxidative stress status with pro- and anti-oxidants

Oxidative stress is defined as the serious imbalance between oxidants and antioxidants, and has been proven to exert pleiotropic act-ions in a large number of tissues and cells in monogastric animals (Sies, 1985). In the context of the transition dairy cows, hepatic uptake of oxygen increased significantly around parturition for production of energy through oxidation (Reynolds, 2003). Consequently, production of reactive oxygen species (ROS), including oxygen ions, free radicals and lipid hydroperoxides, were greatly augmented. Although cellular antioxidants such as glutathione were capable of neutralizing ROS and prevent cellular damage, depletion of such antioxidants lead to ROS accumulation which give rise to substantial tissue damage and induce inflammatory responses (Sordillo and Aitken, 2009). As a result, oxidative stress contribute greatly to dysfunctional host immune and inflammatory response upon increased metabolic stress e.g. the transition period (Sordillo et al., 2009). Therefore, oxidative stress is associated with the increase in susceptibility of transition dairy cows to various health disorders (Bernabucci et al., 2005, Castillo et al., 2005), suggesting the imbalance between ROS production and neutralizing capacity of antioxidant in tissues and blood is the driving force of oxidative stress-related damage.

Pro-oxidants as biomarkers of oxidative stress

The term ”pro-oxidant” refers to all reactive, free radical-containing molecules capable of inducing oxidative stress either by generation of ROS or by inhibiting antioxidant systems (Rahal et al., 2014). The increase in pro-oxidant levels is usually associated with increased risk of oxidative stress and therefore may be used as biomarkers. Because ROS concentration is an indicator of free radical production (Miller et al., 1993, Sugino, 2006), and when in excess can compromise cell function through damaging cellular lipid, protein, and DNA, it has been widely adopted as a biomarker for oxidative stress in non-ruminants (Fukui et al., 2011, Leufkens et al., 2012, Jansen et al., 2013) and ruminants (Bernabucci et al., 2002, 2005, Celi et al., 2010). In fact, the level of ROS is now considered as the gold standard for assessing total oxidative status (Celi, 2010). Bionaz et al. (2007) observed a correlation between paraoxonase (PON) and ROM during the first 2 weeks of lactation, indicating that lower PON levels led to a reduction of anti-oxidative protection during the early postpartum period (Bionaz et al., 2007). The fact that ROM level has been reported to decrease close to calving and was positively associated with NEFA and BHBA suggest it could be a comparative biomarker for oxidative stress at least during the transition period (Bernabucci et al., 2005).

Other pro-oxidants, oxidized protein products, and biomarkers of lipid peroxidation also have been considered as useful biomarkers of oxidative stress (Georgieva, 2005). However, the complexity of the assays required for their measurement have limited their use as oxidative stress biomarkers in the transition period.

Antioxidants as biomarkers of oxidative stress

Antioxidants are defined as substances with the ability to significantly inhibit or delay the oxidative process at low concentrations. Endogenous and exogenous antioxidants are used to protect tissues or cells by neutralizing free radicals and maintaining redox balance (Sies, 1991, Valko et al., 2007). Enzymatic and non-enzymatic molecules within the cytoplasm and various cell organelles constitute the two major parts of endogenous antioxidant defenses (Celi, 2010).

In humans, several ubiquitous primary antioxidant enzymes of significance, including superoxide dismutase (**SOD**), catalase, glutathione peroxidase (**GSH-Px**), malondialdehyde (**MDA**) and glutathione reductase (**GR**) are well known biomarkers of oxidative stress (Saikat and Raja, 2011). In dairy cows, the activity and concentration of these enzymes were also reported to be altered around parturition. For instance, GSH-Px activity was greater after calving (Sordillo et al., 2007), and erythrocyte SOD activity increased before calving and it decreased to prepartum levels one week after parturition (Bernabucci et al., 2005). However, the accumulation of lipid peroxides despite elevated GSH-Px suggested that elevated GSH-Px may not be sufficient to prevent oxidative damage. Therefore, caution must be taken when interpreting the results of enzyme biomarkers in the context of oxidative stress.

By directly interacting with ROS and free radicals, small molecular-weight non-enzymatic antioxidants (e.g., GSH, taurine, NADPH, thioredoxin, vitamins E and C, and trace metals, such as selenium) also work as scavengers (Young and Woodside, 2001). Of these redox pairs, the GSH-to-GSSG ratio is thought to be one of most abundant redox buffering systems in mammalian species (Ďuračková, 2007). As the most abundant non-protein thiol in the cell, glutathione (**GSH**) is a powerful antioxidant that is highly-abundant in the cytosol (1-11 mM), nuclei (3-15 mM), as well as mitochondria (5-11 mM) (Saikat and Raja, 2011). The critical role of GSH in maintaining the redox state of proteins essential for DNA repair and expression in the

nucleus, detoxifying foreign compounds in the cytosol, and protecting mitochondrial DNA damage are well-established (de la Asuncion et al., 1996, Saikat and Raja, 2011). In fact, GSH has been used as a biomarker in various oxidative stress-related diseases (Romeu et al., 2010, Vetrani et al., 2013, Saharan and Mandal, 2014).

In the context of the transition period, dairy cows are likely to endure oxidative stress and changes in GSH levels are expected. Plasma thiol levels (representing GSH) have been reported to peak at -4 d relative to calving and decrease to baseline levels after parturition (Bernabucci et al., 2005). Using assays specific for measuring GSH, it was reported that oxidized GSH (GSSG) concentration in blood increased while the GSH:GSSG ratio was markedly decreased at calving (Sordillo et al., 2007). In the liver, GSH concentration in the transition period was also shown to decrease after parturition and did not return to prepartum levels at least until 21 d postpartum (Osorio et al., 2014b). Considering the amplifying effect from both the decrease in GSH and increase in GSSG upon oxidative stress, measuring both GSH and GSSG level and calculating the GSH: GSSG ratio may be a more sensitive biomarker of oxidative stress. If measuring both GSH and GSSG is not an option, in light of these results, changes in GSH from prepartum to postpartum could still serve as a suitable biomarker for evaluating oxidative stress in the transition dairy cow.

Taurine (2-aminoethylsulphonic acid) is a semi-essential sulfur containing amino acid present in almost all tissues. Although taurine accounts for only 3% of the free amino acid pool in plasma, it accounts for 25%, 50%, 53%, and 19%, respectively, of this pool in liver, kidney, muscle, and brain (Brosnan and Brosnan, 2006). Apart for its well-known role in bile formation, osmoregulation, and neuromodulation (Ripps and Shen, 2012), taurine also acts as a cytoprotetant through alleviating oxidative stress. Mammalian neutrophils and monocytes are

high in taurine, which binds to excess hypochlorous acid produced from oxidative burst and protects cells from oxidative stress damage (Schaffer et al., 2009). Although taurine is not capable of scavenging ROS directly, it enhances other cellular antioxidant functions such as GSH and inhibits ROS production by stabilizing the electron transport chain in mitochondria (Schaffer et al., 2009, Jong et al., 2012).

Due to the limitation of reliable ELISA kits for measuring taurine concentration, data on the concentration or change in concentration of taurine during the peripartal period are not available. However, considering taurine is a precursor of GSH and can help maintain GSH levels even during oxidative stress (Roy and Sil, 2012), a higher taurine concentration may be indicative of an elevated state of oxidative stress during the transition period.

Liver function during the transition period: links with oxidative stress and inflammation

It has been recognized in the last decade that the peripartal dairy cow experiences a state of reduced liver function coupled with increased inflammation and oxidative stress (Bionaz et al., 2007, Trevisi et al., 2012a). 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 to liver cell damage (e.g. lysis and necrosis).

The peripartal inflammatory response is characterized by an increase in the production of positive acute-phase proteins (posAPP) such as haptoglobin and serum amyloid A (SAA), and a concomitant decrease in the production of negative APP (negAPP) such as albumin (Bertoni et

al., 2008). 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 fact that albumin is classified as a negAPP implies that hepatic production (the main site in the body) is commonly reduced during the onset of inflammation (Bertoni et al., 2008). Concentrations of ceruloplasmin and SAA are likely to increase during inflammatory episodes such as those occurring in the peripartal period (Ceciliani et al., 2012b).

During the transition period cows will normally experience an increase in adipose tissue lipolysis due to changes in hormones such as insulin (decrease) and growth hormone (increase), and consequently blood non-esterified fatty acid (NEFA) concentrations increase. Once NEFAs reach the liver these can be oxidized to provide energy to the liver, partially oxidized to produce ketone bodies, or esterified to triglyceride (TAG). A major organelle within hepatocytes where NEFA oxidation takes place is the mitochondria, and carnitine is essential for transport of NEFA from cytosol into mitochondria for subsequent β-oxidation (Drackley, 1999). Carnitine is derived endogenously from trimethyllysine (TML), in turn TML supply is driven by turnover of proteins containing Lys and Met, where Lys serves as the backbone while Met is the methyl donor for TML synthesis (Carlson et al., 2007).

Immune function and immune response

It is well known that the transition dairy cow is regarded as an immune-compromised animal, characterized by inflammatory conditions such as infectious disease and metabolic disorders that often result in pro-inflammatory cytokine release (Mallard et al., 1998, Bertoni et al., 2008). The decreased ability of the immune system to respond to infectious challenges is

likely responsible for the high incidences of environmental mastitis as well as metritis. Although the reasons for the impaired immune functions are not well understood, it has been suggested that a negative energy or protein balance may be a major contributing factor (Goff and Horst, 1997). In particular, inadequate supply of metabolizable protein has been related to impaired function of the immune system (Houdijk et al., 2001). It has also been shown that supplementation of rumen-protected methionine reduces oxidative stress and benefits blood neutrophil function (Osorio et al., 2013). These findings correlated well with the common observation that cows that seem to be the most stressed by nutrition and environmental factors are the most likely to have health problems during the transition period.

Phagocytosis and Oxidative Burst

Phagocytosis is defined as the engulfment and degradation of large solid particles, usually over 0.5 μm in diameter, by phagocytic cells. As the first line of defense from pathogens, phagocytosis is evolutionary conserved and an essential part of innate immunity. Upon activation, phagocytes remove not only invading pathogens but also apoptotic cells or cell debris through internalizing these particles (Gaipl et al., 2004). Following internalization, these specialized leukocytes can facilitate host immunity by signaling to lymphocytes and promote inflammatory cytokine production via antigen presentation to T-lymphocytes (Pfeifer et al., 1993). Owing to their capability of rapidly and efficiently ingesting invading pathogens at the sites of inflammation, neutrophils and macrophages are referred to as professional phagocytes. Although monocytes have lower phagocytic capabilities, they were also included as professional phagocytes as precursor of macrophages (Rabinovitch, 1995).

When exposed to stimuli such as invading microorganisms, oxygen uptake increased markedly in professional phagocytes. Although the magnitude of oxygen consumption is dependent on the stimulus used, the uptake of oxygen is usually more than 50-fold higher compared with the unstimulated state. Subsequently, large quantities of superoxide and hydrogen peroxide were generated and meanwhile large amounts of glucose were metabolized through hexose monophosphate shunt. These reactive oxygen species as well as lysosomal enzyme released by activated leukocytes assume the bactericidal role. Such series of changes in phagocytes are known as respiratory/oxidative burst. Similar to phagocytes, products from oxidative burst activation are also well known important mediators of host reactions. For instance, products derived from NADPH oxidase are key components of microbicidal system. The pathogen killing ability of macrophages is also closely correlated with oxygen metabolism (Murray and Cohn, 1980).

As direct measurements of immune response, phagocytosis and oxidative burst capabilities of professional phagocytes can be quantified upon pathogen challenge. In fact, commercial kits are available for determination of phagocytic and oxidative burst capabilities in leukocytes. Results from multiple studies have shown that the ability of professional phagocytes to conduct phagocytosis and oxidative burst declines with age (Horn et al., 2014) or exposure to stress factors such as hyperoxia (Wang et al., 2015) and oxidative stress (Amer and Fibach, 2005). Considering the oxidative stress status during the peripartal period reviewed above, bovine leukocytes are likely to have compromised phagocytic and oxidative burst capabilities.

Hepatic oxidation theory

Non-ruminant research have suggested that elevated hepatic oxidation and generation of ATP can terminate a meal through signals from liver to brain via afferents in the vagus nerve (Langhans et al., 1985, Tordoff et al., 1991, Berthoud, 2004). Such control of food intake is referred to as Hepatic Oxidation Theory (Allen et al., 2009). As reviewed above, dairy cows enter a lipolytic state around parturition which was characterized by increased hepatic NEFA uptake and oxidation as well as a shortage of gluconeogenic precursors. As a result, insufficient intermediates for the TCA cycle lead to accumulation of acetyl-CoA. In agreement with hepatic oxidation theory, propionate infusion decreased dry matter intake by 20% in lactating dairy cows (Stocks and Allen, 2012). As propionate infusion provided the TCA cycle intermediates, the hypophagic effect of propionate infusion likely originates from oxidation of accumulated acetyl-CoA, which leads to a dramatic increase in ATP production. Similarly, increasing hepatic fatty acid oxidation also lead to decreased feed intake in early lactating dairy cows (Allen et al., 2009). Such results support a negative relationship between hepatic oxidation and dry matter intake in early lactating dairy cows. Considering hepatic oxidation lead to intracellular acetyl-CoA build up, monitoring the hepatic acetyl-CoA pool may help elucidate the efficacy of dry matter intake during the transition period.

Short-chain acyl CoA: links with AA catabolism

It is well known that AA catabolism normally account for only a small portion of the body's energy production. However, increased catabolism of AA through oxidation can be expected in times of severe negative energy balance e.g. the transition period. Although different AAs have distinct catabolic pathways, their catabolism converge to form only five products, all of which enter the TCA cycle where they were diverted to gluconeogenesis, ketogenesis or

complete oxidation to CO_2. In fact, ten AAs ultimately broke down to yield acetyl-CoA, four into succinyl-CoA, two into fumarate, and two into oxaloacetate. Therefore, 14 out of 20 AAs enter the TCA cycle for oxidation through short-chain acyl CoA. It is also noteworthy that isoleucine, threonine, methionine, and valine are precursors of propionyl-CoA and methylmalonyl-CoA, which subsequently enter the TCA cycle via succinyl-CoA. This metabolic pathway has high flux level in ruminants for gluconeogenesis due to large amounts of propionate formed during ruminal fermentation (Aschenbach et al., 2010). Considering the critical roles of acetyl-CoA, succinyl-CoA, propionyl-CoA, and methylmalonyl-CoA in energy metabolism, monitoring hepatic short chain acyl-CoAs may provide insight into AA catabolism related energy metabolism during the transition period.

Link between liver functionality index (LFI) and AA concentrations in transition period

Using changes in plasma concentrations of albumin, cholesterol, and bilirubin, the liver functionality index (LFI) characterizes the extent of the inflammatory response and helps to predict its likely consequences on health and well-being of the cow (Bertoni and Trevisi, 2013b). A low LFI (**LLFI**) value is indicative of a pronounced inflammatory response, suggestive of a more difficult transition from gestation to lactation while a high LFI (**HLFI**) is suggestive of a smooth transition (Trevisi et al., 2012b). Previous work utilizing blood and milk samples has confirmed the usefulness of this approach to assess the severity of the immune and inflammatory status (Trevisi et al., 2010a).

In terms of AA during the transition period, it is well known that the needs of the fetus pre-partum and the needs of the mammary gland post-partum increase greatly the demand for

AA but the supplies of metabolizable protein and essential AA (**EAA**) are limiting around calving (Bell et al., 2000). Therefore, "better" AA plasma profiles could serve as indicators of a successful adaptation during the transition period. Recent results have established that HLFI cows have greater plasma concentrations of Val, Thr, Ile, EAA, and branched-chain AA compared with LLFI cows without marked differences in dry matter intake (Zhou Z, 2016). Because there are very few published data on AA profiles around parturition, these data seem to indicate a biologically important linkage between liver function and systemic AA concentrations. Therefore, in the medium-to-long term additional research on AA and their biological roles beyond milk synthesis could help better define their potential use as biomarkers of health and disease.

Uncover adaptations during the transition period with omics approach

Omics is a suffix signifying the measurement of entire level of biological molecules, which encompasses a variety of high-throughput technologies to study the genome, proteome, and metabolome of the experimental unit. Along with bioinformatics, omics enhance understanding of the complex biological interactions in cells and tissues at the gene, protein, and metabolite level (Loor et al., 2015). Although the field of omics is ever expanding, transcriptomics, epigenomics, proteomics, and metabolomics are well-established research tools for nutritional research.

In terms of dairy cows, results from both our group and others have confirmed the alterations on the transcriptome level (Ji et al., 2012, Osorio et al., 2014a, Li et al., 2016) as well as metabolome (Sun et al., 2015) level in response to nutritional interventions. For instance,

microarray analysis from our group revealed a greater overall subclinical inflammatory-like condition in feed-restricted cows compared with overfed cows (Shahzad et al., 2014). Metabolomics profiling of rumen fluid, milk, serum, and urine from lactating cows fed high- vs. low-quality diets suggest higher N loss and low N utilization efficiency in response to low quality diet (Sun et al., 2015).

As mentioned above, high producing dairy cows undergo complex adaptations as the cow approaches parturition and enters lactation. Although nutritional interventions such as peripartal methionine supplementation was shown to help achieve a successful transition (Osorio et al., 2013), integration of functional genomics technology with measurement of metabolism may help uncover the underlying key mechanisms.

Summary

The transition period is considered the most challenging phase during the lactation cycle (Drackley, 1999). The increased nutritional requirements and decreased DMI greatly contribute to the well-known metabolism related disorders during this period. Apart from the negative energy balance, negative metabolizable protein balance, and negative methyl donor balance have also been shown to closely associate with oxidative stress, inflammation, and compromised liver function in transition dairy cows.

Rumen-protected methionine is expected to improve transition dairy cow performance as it can spare essential AAs, stimulate sulfur containing antioxidant synthesis, and provide backbone for gluconeogenesis. Rumen-protected choline has also been suggested to alleviate fatty liver during the transition period by enhancing TAG export. However, peripartal methyl

donor supplementation in the form of methionine and choline have yielded inconsistent effects on transition dairy cow performance and health (Erdman and Sharma, 1991, Hartwell et al., 2000, Pinotti et al., 2003, Piepenbrink et al., 2004, Guretzky et al., 2006, Ordway et al., 2009, Preynat et al., 2009b, Osorio et al., 2013). In addition, as methyl donors, methionine and choline metabolism are closely interrelated, the flux from methionine to choline and vice versa during the transition period remains unknown.

Our general hypothesis was that supplementing rumen-protected methionine and/or choline to peripartal dairy cows will improve overall lactation performance by enhancing liver function and antioxidant capacity, and decreasing the inflammatory response. Such effects were realized through changes in transcriptome and metabolome. The overall objective of this dissertation was to evaluate the performance parameters, blood and liver biomarkers, and transcriptomics and metabolomics alterations on dairy cows supplemented with methionine and choline during the transition period.

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CHAPTER 2

BETTER POSTPARTAL PERFORMANCE IN DAIRY COWS SUPPLEMENTED WITH RUMEN-PROTECTED METHIONINE THAN CHOLINE DURING THE PERIPARTAL PERIOD

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INTRODUCTION

During the transition from pregnancy to lactation ("transition period"), dairy cattle enter a period of negative energy and MP balance as a result of increased metabolic demand by the mammary gland and the low DMI (Drackley, 1999). Owing to the fact that methyl donors are required for the synthesis of key compounds such as phosphatidylcholine (**PC**) and carnitine in tissues (Pinotti et al., 2002), a negative methyl donor balance also may be an important challenge for the transition dairy cow. In fact, milk from dairy cows is high in methylated compounds and levels of these compounds secreted into milk are maintained even at the cost of depleting liver tissue reserves (Pinotti et al., 2002).

Due to extensive microbial degradation in the rumen, dietary availability of key methyl donors [(e.g., Met (**MET**) and choline (**CHOL**)] is limited (Sharma and Erdman, 1989, Girard and Matte, 2005). Consequently, the increased mobilization of body protein in dairy cows during this period may serve, at least in part, to compensate for the shortfall in methyl groups required by mammary gland and liver (Komaragiri and Erdman, 1997). Supplementing rumen-protected methyl donors may help fulfill the daily methyl group requirement, and possibly improve the overall production and health of dairy cows during the transition period (Zom et al., 2011, Osorio et al., 2013, Osorio et al., 2014c).

Both MET and CHOL are key methyl donors in mammals and their availability is important for various biological functions. For instance, MET together with Lys are the two most-limiting AA for milk synthesis in lactating cows (NRC, 2001b). Being the only essential sulfur-containing AA, MET acts as the precursor for other sulfur-containing AA such as Cys, homocysteine and taurine (Brosnan and Brosnan, 2006). It has been estimated in lactating goats that as much as 28% of absorbed MET could be used for CHOL synthesis (Emmanuel and Kennelly, 1984). Hence, it is thought that rumen-protected CHOL supplementation could spare MET to help cows achieve better overall performance (Hartwell et al., 2000, Pinotti, 2012). Current recommendations for duodenal supply of Lys and MET to maximize milk protein content and yield in established lactation are 7.2 and 2.4% of MP, respectively (NRC, 2001b). More recent work demonstrated a benefit of supplementing rumen-protected MET to achieve a Lys:Met ratio close to 2.8:1 during the peripartal period in terms of production performance (Osorio et al., 2013).

Oxidative stress status during the peripartal period also was influenced by increased MET availability due to the fact that it serves as a substrate for glutathione synthesis, the most-abundant natural antioxidant produced within cells (Martinov et al., 2010a, Osorio et al., 2014c). Furthermore, as a lipotropic agent, MET is directly involved in very low density lipoprotein (**VLDL**) synthesis via the generation of S-adenosylmethionine (**SAM**), the most important methyl donor (Martinov et al., 2010a). In turn, SAM can be used to methylate phosphatidylethanolamine (**PE**) to generate PC, which is essential for VLDL synthesis (Auboiron et al., 1995). In the context of VLDL synthesis and liver lipid metabolism, CHOL-containing nutrients (mainly in the form of PC) are indispensable for the synthesis and release of chylomicrons and VLDL (Pinotti et al., 2002). Thus, supplementation of rumen-protected MET

and/or CHOL (Zom et al., 2011) may increase hepatic triacylglycerol (**TAG**) export and consequently decrease lipidosis.

To date, the reported effects of rumen-protected MET and/or CHOL supplementation on dairy cow performance have been inconsistent. Although previous studies from our group and others have observed beneficial effects of MET (Chen et al., 2011, Osorio et al., 2013) or CHOL (Pinotti et al., 2003, Zom et al., 2011) supplementation, other studies did not detect significant improvements on peripartal production performance with MET (Socha et al., 2005, Ordway et al., 2009, Preynat et al., 2009b) or CHOL (Guretzky et al., 2006, Leiva et al., 2015) supplementation. In particular, data demonstrating whether CHOL alone or in combination with MET provide equal or different benefits to cows are limited. Our general hypothesis was that supplementation of rumen protected MET or CHOL improves overall health and lactation performance during the peripartal period. The objective of this study was to evaluate the impact of feeding a commercially-available rumen protected MET or CHOL products alone and in combination on transition cow performance and health.

MATERIALS AND METHODS

Experimental Design and Dietary Treatments

All procedures for this study (protocol 13023) were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Illinois. The experiment was conducted as randomized, complete, unbalanced block design with 2×2 factorial arrangement of MET (Smartamine M, Adisseo NA) and CHOL (ReaShure, Balchem Inc) inclusion (with or without). A total of 88 cows were blocked according to expected calving date. Each block had 12 cows (except for the last block). Cows within each block were balanced for parity, previous lactation

milk yield, and BCS before the close-up. A complete data set was obtained from 81 cows receiving a basal control (**CON**) diet (n = 20) with no MET or CHOL supplementation; CON plus MET (**SMA**, n = 21) at a rate of 0.08% of DM; CON+CHOL (**REA**, n = 20) at 60 g/d; or CON+MET+CHOL (**MIX**, n = 20). Dosage of MET was based on Osorio et al. (2013), whereas CHOL was supplied following manufacturer's recommendations. All cows received the same far-off diet (1.40 Mcal NE_L/kg of DM, 10.2% RDP, and 4.1% RUP) from -50 to -23 d before expected calving, the same close-up diet (1.52 Mcal NE_L/kg of DM, 9.1% RDP, and 5.4% RUP) from -21d to expected calving, and the same lactation diet from calving (1.71 Mcal NE_L/kg of DM, 9.7% RDP, and 7.5% RUP) through 30 DIM (Table 2.1).

The MET and CHOL supplements were both top-dressed from -21 ± 2 to 30 DIM once daily at the AM feeding using approximately 50 g of ground corn as carrier. The TMR DM for the close-up and lactation diets was measured weekly for estimation of daily TMR DM offered. Supplementation of SMA (0.08% DM of TMR offered) was calculated daily for each cow. Smartamine M was supplied as small beads containing a minimum of 75% DL-Met, physically protected by a pH sensitive coating, which is considered to have a Met bioavailability of 80% (Graulet et al., 2005); therefore, per 10 g of Smartamine, the cows received 6 g of metabolizable MET. The ReaShure supplement is reported to contain 28.8% choline chloride and is protected by microencapsulation. The product is considered to have CHOL bioavailability of 72% (Benoit et al., 2009); therefore, per 60 g of ReaShure, the cows received 12.4 g of metabolizable choline chloride. To our knowledge, neither Smartamine M nor ReaShure have specific characteristics that may affect palatability of diets.

Animal Management

All cows were enrolled in the experiment from late July 2013 to mid-October 2014, with an average temperature of 12.2 ± 11.1 °C (Illinois State Water Survey; http://www.isws.illinois.edu/atmos/statecli/cuweather/index.htm). Dry cows were housed in a ventilated enclosed barn during the dry period and fed individually once daily at 0630 h using an individual gate system (American Calan Inc., Northwood, NH). Cows 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. On average, cows remained in the maternity pen for 3.69 ± 3.61 days. After parturition, cows were housed in a tie-stall barn and were fed a common lactation diet once daily in the AM (Table 2.1) and milked 3 times daily at approximately 6:00, 14:00, and 22:00. At 30 DIM, cows returned to the farm herd. Feed offered was adjusted daily to achieve 10% refusals.

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 2 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. Milk composition was analyzed, while ECM and energy balance (**EB**) were calculated from calving to 30 DIM.

General cow housing and health-care conform to approved standard operating procedures for the University of Illinois Dairy cattle research Unit. Attending veterinarians from the University of Illinois Veterinary College of Medicine conducted diagnosis and, when needed, performed treatment for displaced abomasum (**DA**), ketosis (via urine test), endometritis, and other health problems. Per IACUC guidelines, cows only were removed from the experiment when failing to recover from a clinical disorder after treatment or surgery (e.g. feed intake less

than 80% of pre-diagnosis within 2 d after DA surgery). A total of 2, 0, 2, and 3 cows were removed from the study in the CON, SMA, REA and MIX groups, respectively (Table 2.2). All data from the cows removed were not included in the statistical analysis.

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; http://dairyone.com/wp-content/uploads/2014/02/Forage-Lab-Analytical-Procedures.pdf). The values for NE_L, RUP, RDP, Lys, and Met also were predicted using the NRC (2001) model with actual DMI, BW, BCS, milk production, and milk composition as inputs. Analytical values from individual feed ingredients (alfalfa silage, alfalfa hay, brewer grains, corn silage, cottonseeds, expeller soybean meal, wheat straw) were used to adjust the default values in the model. Consecutive morning, midday, 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, MUN, and SCC by mid-infrared procedures (Chemists, 1995) in a commercial laboratory (Dairy Lab Services, Dubuque, IA). Based on milk sample analysis, the ECM (at 3.5% fat) and FCM was calculated daily as follows: ECM = [12.82 × fat yield (kg)] + [7.13 × protein yield (kg)] + 0.323 × milk yield (kg)], FCM = (0.4324 × kg of milk yield) + (16.216 × kg of milk fat) (Hutjens, 2010).

The EB was calculated for each cow using equations from NRC (2001). Net energy intake (NE_I) was determined using daily DMI multiplied by NE_L density of the diet. Net energy

of maintenance (NE_M) was calculated as $BW^{0.75} \times 0.080$. Requirements of NE_L were calculated as $NE_L = (0.0929 \times \text{fat\%} + 0.0547 \times \text{protein \%} + 0.0395 \times \text{lactose \%}) \times \text{milk yield}$. The net energy requirement for pregnancy (NE_P; Mcal/d) was calculated as $NE_P = [(0.00318 \times \text{day of gestation} - 0.0352) \times \text{calf birth weight}/ 45)]/0.218$. The equation used to calculate prepartal EB (EB_{PRE}; Mcal/d) was $EB_{PRE} = NE_I - (NE_M + NE_P)$ and EB_{PRE} (as % of requirements) = $[NE_I/(NE_M + NE_P)] \times 100$. The equation used to calculate postpartal EB ($EB\text{-}_{POST}$) was EB_{POST} (Mcal/d) = $NE_I - (NE_M + NE_L)$ and EB_{POST} (as % of requirements) = $[NE_I/(NE_M + NE_L)] \times 100$.

Blood Collection and Analyses

Blood was sampled from the coccygeal vein on -10 d relative to expected calving date and on 4, 8, 20, and 30 d relative to actual calving date before the morning feeding. Samples were collected into evacuated 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 2,000 × *g* for 30 min at 4 °C. Aliquots of serum and plasma were frozen (-20 °C) until further analysis. Concentrations of NEFA, BHBA, and glucose were analyzed by methods described in Osorio et al. (2013). Plasma insulin was assayed by a double-antibody radioimmunoassay that uses a primary antiserum to bovine insulin (Gutierrez et al., 2013).

Liver Tissue Composition

Liver was sampled via puncture biopsy (Dann et al., 2005) from cows under local anesthesia at approximately 0800 h on d -10, 7, 20, and 30 d relative to parturition. Liver was frozen immediately in liquid N and stored until further analysis for concentration of TAG. A

total of 50 mg of tissue was first homogenized in 1.5 mL of PBS/10 mM EDTA using a hand-held homogenizer (Tissue-Tearor, Biospec Products). Subsequently, 200 µL of GPBS-142 EDTA along with 3 mL of isopropanol-hexane-water (80:20:2 vol/vol) were added to each sample, the tube was covered with aluminum foil, and the mixture was incubated for 30 min at room temperature. One milliliter of hexane-diethyl ether (1:1) was then added to each sample followed by vortexing and incubating for 10 min at room temperature (protected from light). One milliliter of water was added to each sample to separate the lipid phase and the mixture was vortexed. Samples were incubated covered with aluminum foil for ~20 min at room temperature. The organic phase was then aspirated and placed into glass vials, prior to evaporation under a stream of N gas. An 8-point TAG standard was prepared with Infinity TG reagent (catalog#10010509, Cayman Chemicals). Each 150 µL sample was mixed with 540 µL of Infinity TG reagent prior to vortexing. A total of 160 µL of this sample mixture was pipetted into a flat-bottom 96-well plastic microplate. The plate was incubated for 15 min at 37 °C prior to determining absorbance at 540 nm using a microplate reader. Concentration of TAG was calculated from the standard curve.

Statistical Analysis

Data were analyzed using PROC MIXED of SAS (SAS Institute Inc., Cary, NC) according to the following model:

$$y_{ijklm} = \mu + b_i + M_j + C_k + MC_{jk} + T_l + TM_{jl} + TC_{kl} + TMC_{jkl} + A_{m:ijk} + \varepsilon_{ijklm}$$

where Y_{ijklm} is the dependent, continuous variable; μ is the overall mean; b_i is the random effect of the ith block; M_j is the fixed effect of MET (j = with or without); C_k is the fixed effect of CHOL (k = with or without);T_l is the fixed effect of time (day or week) of the experiment;

$A_{m:ijk}$ is the random effect of the *m*th animal (cow) nested within block×MET×CHOL; ε_{ijklm} is the residual error. The covariates of parity (2nd vs. 3rd lactation and greater), previous 305-d milk yield, and BCS before the close-up period were kept in the model for all variables when significant ($P < 0.05$). For the analysis of prepartal DMI, the average DMI of each cow during the last week of the far-off period were used as covariate in the model. Blood metabolites and liver composition were analyzed at various time points that were not equally spaced with a heterogeneous variance over time. Therefore, the first order ante-dependence covariance structure ANTE (1) was used for repeated measures. Health data, except for mastitis and metritis with a single case each, were analyzed with PROC GLIMMIX (distribution = binary and link = logit) of SAS. Statistical differences were declared significant at $P \leq 0.05$ and tendencies at $P \leq 0.15$.

RESULTS

Health

Health-related problems that occurred during the experiment and causes for the removal of cows are summarized in Table 2.2. Seven cows, none belonging to SMA, were removed from the experiment due to health-related problems. Failure to recover from surgery to correct DA was the major contributor (n = 6) to the removal of cows. Out of the 6 cows excluded due to DA, 3 cows from CON, REA, and MIX also were diagnosed with ketosis. Among the 81 cows completing the study, ketosis had the highest occurrence (n = 20) while mastitis and metritis only occurred once. Among the 3 main health-related problems, incidence of ketosis (7 vs. 13) and retained placenta (3 vs. 9) tended ($P = 0.07$) to be lower in MET-supplemented cows. It should be noted that incidence of ketosis in 3 cows, 2 fed the CON diet and the other fed MIX, occurred

along with DA. Similarly, 3 other ketotic cows, 2 fed the CON diet and one REA, also were diagnosed with retained placenta.

Ingredient and Nutrient Composition of Diets

The ingredient composition of the diets is presented in Table 2.1. The nutrient composition (Table 2.3) was determined by analyzing each individual feed ingredient for its chemical composition and then entering the feed analysis results into the NRC (2001) model. Analyzed chemical composition of diets is presented in Suppl. Table 2.1.

Prepartal DMI, BW, and BCS

As expected, DMI of cows in all treatments (Fig. 2.1E) gradually decreased ($P < 0.01$) from -21 d to calving. A significant ($P = 0.02$) increase in prepartal DMI was detected due to the main effect of MET (Fig. 2.1A); no such effect ($P = 0.28$) was detected for CHOL (Fig. 2.1C). Analysis of prepartal DMI as percentage of BW (Table 2.4) also revealed a tendency for an increase in response to MET ($P = 0.13$) compared with CHOL ($P = 0.27$). This response appeared mostly associated with differences in DMI than BW because no differences ($P > 0.15$) were detected in prepartal BW and BCS (Table 2.4). Similarly, the tendency ($P = 0.12$) for an increase in EB with MET rather than CHOL also could be explained by differences in DMI prepartum. Prepartal BW and EB were the only variables for which parity explained some of the variation. Visual inspection indicated that all cows consumed the MET or CHOL supplement top-dressed on the TMR, thus, differences in DMI did not seem to be associated with product palatability or smell.

Postpartal DMI, BW, and BCS

Similar to prepartum, postpartal DMI was greater ($P = 0.02$) in cows supplemented with MET (Fig. 2.1B) while DMI was similar ($P = 0.79$) with or without CHOL supplementation (Fig. 2.1D). The BW postpartum did not differ ($P > 0.15$) among treatments (Table 2.4); however, there was greater DMI as a percentage of BW when cows were fed MET ($P = 0.05$) compared with CHOL ($P = 0.93$). Although no main effect of MET or CHOL was detected for BCS, there was an interaction ($P = 0.09$) of CHOL × Time for postpartal BCS (Table 2.4). Postpartal EB did not differ ($P > 0.15$) among treatments (Table 2.4).

Milk Production and Composition

Main effects and interactions for milk composition and milk production variables are presented in Table 2.5. The MET supplementation resulted in greater ($P < 0.01$) milk protein percentage while CHOL had no effect ($P = 0.19$). Milk fat, SCC, lactose, other solids, and MUN percentage were not affected ($P > 0.15$) by treatments. However, a tendency ($P = 0.13$) for higher total solids percentage was detected in response to MET supplementation.

Overall milk yield, ECM and FCM were greater ($P < 0.05$) with MET supplementation compared with CHOL ($P > 0.15$). The significant response in milk fat yield ($P = 0.02$) detected in cows fed MET was due to the higher milk production in MET-supplemented cows because no difference ($P = 0.91$) in milk fat percentage was detected among treatments. The higher ($P < 0.01$) milk protein yield in MET-supplemented cows was due to both greater ($P < 0.05$) milk protein percentage and milk production.

Blood Biomarkers and liver TAG composition

The main effects of MET, CHOL, and their interactions for blood and milk biomarkers as well as liver TAG composition are reported in Table 2.6. Significant ($P < 0.05$) main effects

were detected for glucose and insulin, which were greater in cows supplemented with CHOL (Fig 2.4D). Although no main effects of MET or CHOL were detected for NEFA:insulin, the glucose:insulin ratio was significantly lower ($P = 0.05$) in CHOL-supplemented cows (Table 2.6). Concentration of BHBA tended ($P = 0.12$) to be lower in response to CHOL supplementation, mainly due to lower concentration at 20 d postpartum (Fig 2.4H). Although there was no main effect of MET for NEFA or BHBA, a tendency ($P = 0.15$) for a MET×time interaction was detected for NEFA, which was associated with greater NEFA at 20 d in MET-supplemented cows (Fig 2.4E). Although liver TAG increased after calving ($P < 0.01$) for all treatments, a tendency ($P = 0.13$) for a CHOL×time interaction was detected because of greater concentration in CHOL-supplemented cows at 20 d postpartum (Table 2.6, Fig. 2.4J).

DISCUSSION

Ingredient, Nutrient Composition, and NRC Evaluation of Diets

The abrupt increase in MP demand and inability of cows to consume sufficient protein around parturition contribute to negative MP and AA balances, especially when the onset of lactation greatly increases the mammary gland demand for AA (Bell et al., 2000). Nutrient composition evaluation of prepartal and postpartal diets using the NRC (2001) and the mean chemical composition of feed ingredients throughout the experiment revealed different effects of MET and/or CHOL supplementation (Table 2.3). For instance, during the prepartal period, MET-supplemented diets (SMA and MIX) provided an average of 133 g/d more MP than diets without MET supplementation. Similarly, postpartum the average increase of MP was 288 g/d in cows fed MET-supplemented diets. This was due, at least in part, to a greater DMI in MET-supplemented cows both prepartum (14.3 kg/d) and postpartum (19.2 kg/d). In contrast,

supplementation of CHOL did not result in increased MP supply partly due to the failure to increase voluntary feed intake both prepartum and postpartum.

In accordance with results from a previous similar study, MP balance was positive prepartum and negative postpartum regardless of treatments (Osorio et al., 2013). Considering there was no difference in cow BW and calf birth weight (data not shown), it was expected that MET cows had a more positive MP balance prepartum. However, despite the greater estimated supply of MP in MET-supplemented cows, a more negative MP balance postpartum (an average of -88 g/d more) was detected in these cows. This was likely due to a greater (an average of 375 g/d more) MP requirement in MET cows to sustain the greater milk yield. Conversely, a lesser degree of imbalance of MP postpartum (an average of 18 g/d less) was detected in CHOL-supplemented cows (REA and MIX), which might have been the result of numerically lower (41.4 vs. 43.2 kg/d) milk yield.

Regardless of treatment, the estimated requirement of RUP increased by 11-fold from close up to the postpartal period (Table 2.3), which is similar to results reported previously (Osorio et al., 2013). Compared with unsupplemented cows, those fed MET had a lower estimated requirement for RUP prepartum (164 g/d vs. 187 g/d) as a result of a sparing effect from the MET supplementation. However, the postpartal requirement for RUP in MET cows was greater (an average of 315 g/d more) compared with cows without MET supplementation, which also was likely associated with the increased requirement to sustain the higher milk yield. In contrast, with or without CHOL, the estimated RUP requirement was similar both prepartum (179.5 g/d vs. 171.5 g/d) and postpartum (1938 g/d vs. 1968 g/d).

The desired duodenal supply of Lys and MET to maximize milk protein content and yield has been suggested to be 7.2% and 2.4% of MP, respectively, during established lactation (NRC,

2001b). In the present study, MET supplementation at the rate of 0.08% DM achieved a duodenal Lys:Met ratio of 2.80:1 prepartum and 2.71:1 postpartum, which was lower than the ideal Lys:Met of 3.0:1 for optimal milk protein production. In contrast, prepartum and postpartum Lys:Met ratio in response to CHOL supplementation were 3.61:1 and 3.54:1, respectively, which were higher than the ideal ratio (Table 2.3). Therefore, the greater milk yield and milk protein content detected in MET compared with CHOL might have been due, at least in part, to a lower ratio of Lys:Met ratio and closer to the ideal 3.0:1. It is well-known that MET and CHOL metabolism are interrelated (Stipanuk, 1986, Zeisel, 1992), and that CHOL can provide labile methyl groups for biosynthesis of MET after oxidation to betaine (Mato et al., 1994). Therefore, it is possible that CHOL supplementation alters the Lys:Met ratio by promoting the re-synthesis of MET. However, the production results in the present study do not support this assumption.

Effects on DMI, BW, and BCS

The effects of rumen-protected MET supplementation on DMI in previous studies has been inconsistent (Ordway et al., 2009, Chen et al., 2011, Osorio et al., 2013). For instance, Ordway et al. (2009) detected an increase in postpartal DMI with Metasmart (HMBi) supplementation but not SMA (Ordway et al., 2009). Socha et al. (2005) reported a tendency towards a decrease in DMI postpartum with 15 g/d SMA supplementation (Socha et al., 2005). In contrast, a recent study from Osorio et al. (2013) detected an increase in postpartal DMI as a percentage of BW when MET was supplemented in the form of SMA at a rate of 0.07% DM (Osorio et al., 2013). It has been suggested that the contrasting results in the literature may be due to the differences in level of MET supplementation, length of feeding, and/or stage of lactation (Osorio et al., 2013). In this regard, MET was top-dressed from -21 d to 30 d relative to

calving in the present study, which was similar to previous work from our group (Osorio et al., 2013). However, SMA was supplemented at a slightly higher rate (0.08% DM vs 0.07% DM), which probably contributed to the more pronounced effect of MET on DMI postpartum, suggesting that aiming at a lower than 3.0:1 Lys:Met during this stage of the lactation may be beneficial to the cow.

To our knowledge, this is the first report of greater DMI during the close-up dry period in response to rumen-protected MET supplementation. In fact, results from a previous study with cows consuming similar amounts of DM (14.1 kg/d vs. 13.8 kg/d) and dietary NE_L (21.2 Mcal/kg vs. 20.9 Mcal/kg) indicated that greater prepartum DMI was associated with detrimental metabolic responses postpartum (Douglas et al., 2006). Clearly, the greater DMI prepartum coupled with greater DMI postpartum and lower disease incidence in MET-supplemented cows suggest otherwise. Whether this response was due to the fine-tuned Met:Lys ratio through MET supplementation merits further investigation. In the case of CHOL supplementation, the lack of effect on DMI during the peripartal period is consistent with previous reports (Erdman and Sharma, 1991, Hartwell et al., 2000, Piepenbrink and Overton, 2003, Guretzky et al., 2006, Zom et al., 2011). In accordance with previous reports, MET or CHOL supplementation did not affect BW and BCS (Erdman and Sharma, 1991, Piepenbrink and Overton, 2003, Socha et al., 2005, Ordway et al., 2009).

Milk Production and Composition

Previous results both from our group (Osorio et al., 2013) and others (Overton et al., 1996, Piepenbrink et al., 2004) detected greater milk yield with MET or MET analog supplementation. Although other studies did not detect increases in milk yield with rumen-protected MET (Socha et al., 2005, Ordway et al., 2009, Preynat et al., 2009b) or MET analog

(Piepenbrink et al., 2004, Ordway et al., 2009) supplementation, an average increase of 3.8 kg/d in milk production in the first 30 d of lactation was detected in the present study in response to MET. Considering that MET has been identified as one of the two most-limiting AA for lactating cows (NRC, 2001b) and a greater DMI would increase daily protein intake, the milk yield response when supplementing MET to achieve a Lys:Met close to the suggested optimum was as expected.

Similar to previous reports (Ordway et al., 2009, Osorio et al., 2013), the greater milk protein percentage in response to MET supplementation underscores that milk protein early postpartum is affected in direct proportion to adequacy of MET in the MP (NRC, 2001b). Considering the increased milk yield in MET cows, the increase in milk protein yield was not surprising. As milk fat percentage was not affected by MET supplementation, the significant increase in milk fat yield was due to the increase in milk production. The tendency for greater total solids percentage in MET-supplemented cows likely was associated with the higher protein percentage in those cows.

The effect of CHOL supplementation on milk yield, ECM, and FCM in previous studies has been inconsistent. Although no significant effects on milk production and composition were reported in studies supplementing CHOL from -21 d to 42 d or longer (Hartwell et al., 2000, Guretzky et al., 2006), there also are studies reporting a tendency or significant increases in milk yield when CHOL was supplemented either during the peripartal period (Pinotti et al., 2003, Zahra et al., 2006, Elek et al., 2008) or from 5 to 21 wk of lactation (Erdman and Sharma, 1991). It has been suggested that CHOL would more likely have a positive effect in overconditioned cows because they are considered to be at a greater risk for hepatic accumulation of TAG (Guretzky et al., 2006). In agreement with this notion, Zahra et al. (2006) detected greater milk

production with supplemental CHOL specifically in cows with a BCS of 4 entering the close-up period, which was driven primarily by greater DMI (Zahra et al., 2006). Based on those results, it was suggested that in herds with over 1 in 5 cows (20%) entering the transition period at BCS ≥4, CHOL supplementation may result in a positive effect on milk production. In the present study, 11 cows (13.6%) entered the transition period with BCS ≥4, and were randomly assigned to one of the 4 treatments. Five cows received CHOL and the other 6 did not. On average, the 5 CHOL-fed cows produced 5.62 kg/d less milk compared with the other 6 cows (43.8 kg/d vs. 38.2 kg/d). Therefore, results from the present study do not seem to support the suggestion by Zahra et al. (2006). However, considering that only 11 cows were overconditioned in the present study, there is merit in further work with overconditioned cows and supplementation of CHOL and MET.

Energy Balance and Apparent Efficiency

Mean values of EB postpartum in the present study were between -7.8 and -10.7 Mcal/d (Table 2.4), which is similar to the range (-7.6 to -9.5 Mcal/d) reported previously by Osorio et al. (2013). Although Socha et al. (2005) and Ordway et al. (2009) reported less negative values for EB from calving to 105 and 140 d postpartum, the differences among studies are probably associated with the rate and length of MET supplementation (Socha et al., 2005, Ordway et al., 2009, Osorio et al., 2013).

Energy Balance Biomarkers and Liver TAG Content

Previous reports from both monogastric and ruminant studies have illustrated the importance of adequate lipotropic agents in the prevention of liver lipid accumulation (Cooke et al., 2007, Corbin and Zeisel, 2012). As lipotropic agents, MET and CHOL fed to ruminants may

help clear lipid from the liver through stimulating VLDL formation and export (Waterman and Schultz, 1972). Although some studies have detected lower liver TAG with CHOL supplementation (Cooke et al., 2007, Zom et al., 2011, Elek et al., 2013), other studies detected no differences in liver TAG (Hartwell et al., 2000, Piepenbrink and Overton, 2003). Similarly, rumen-protected MET in the form of SMA or analog (Piepenbrink et al., 2004, Osorio et al., 2013) either elicited no difference or increased liver TAG (Preynat et al., 2009b) in periparturient dairy cows. Therefore, the lack of main effect of MET or CHOL on liver TAG in the present study is not surprising (Table 2.6). In fact, the tendency for CHOL×time interaction due to greater liver TAG in CHOL-supplemented cows at 20 d postpartum indicates a lack of lipotrophic effect with CHOL.

The greater NEFA concentration in MET-supplemented cows at 20 d (MET×Time P = 0.15) was similar to results from previous research, where an interaction towards greater NEFA was detected in cows supplemented with Metasmart compared with controls (Osorio et al., 2013). However, whether this difference was due to greater NEFA release from adipose tissue or lower utilization by peripheral tissues is unclear (Piepenbrink et al., 2004). It is worth noting that despite the higher NEFA in MET-supplemented cows, liver TAG did not increase (Fig 2.4C and 4E). Therefore, it is reasonable to speculate that MET-supplemented cows were able to export TAG more efficiently in spite of the greater influx of NEFA from blood.

Blood insulin concentration in periparturient dairy cows was reported to increase in response to CHOL supplementation (Leiva et al., 2015), which agrees with the results in the present study. The glucose:insulin ratio is considered a crude index of tissue insulin sensitivity (Subiyatno et al., 1996), and has been previously used in dairy cattle receiving daily injections of the insulin-sensitizing drug 2,4-thiazolidinedione (Hosseini et al., 2015). In contrast with the lack

of CHOL effect on the glucose:insulin ratio in the study of Leiva et al. (2015), the significantly lower glucose:insulin in CHOL cows in the present study was indicative of lower insulin sensitivity. In agreement with this, dietary supplementation of CHOL (2.7 g/kg diet) increased plasma glucose concentration and induced insulin resistance in mice (Wu et al., 2013). Although the greater glucose and insulin concentration in response to CHOL supplementation in the present study seem to agree with the mouse data, the underlying mechanisms for these increases are not readily apparent.

Despite the fact that hepatic mRNA expression of pyruvate carboxylase and phosphoenolpyruvate carboxykinase early postpartum were not altered in response to CHOL (Hartwell et al., 2000), it is possible that changes in gluconeogenesis contributed to the glucose and insulin response detected in the present study. It is noteworthy that administration of choline chloride increased the number of pancreatic β-cells in non-ruminants (Sergeyeva, 1940). In agreement with this early finding, it was suggested more recently that choline may stimulate insulin secretion indirectly by enhancing synthesis and release of acetyl choline (Ilcol et al., 2003). Whether the same mechanism exists in ruminants merits further study.

Subclinical ketosis in the present study was defined as blood BHBA concentration between 1.4 to 3 mmol/L (Oetzel, 2004). Although the main effect of MET on BHBA concentration was not significant, in agreement with the results from previous studies (Piepenbrink et al., 2004, Osorio et al., 2013), the fact that fewer MET cows had clinical ketosis (Table 2.2; as judged from the urine ketone test) suggests that MET supplementation might play a role in reducing ketosis occurrence during the peripartal period. The CON, SMA, REA, and MIX groups had similar numbers of cows, i.e. 3, 4, 4, and 3 cows, respectively, with BHBA within the range of subclinical ketosis on d 4 and d 8 postpartum.

Previous studies reported no significant effect of CHOL on blood glucose or BHBA concentrations (Guretzky et al., 2006, Zahra et al., 2006, Zom et al., 2011). In the present study, the tendency for lower BHBA in response to CHOL supplementation agreed with the greater glucose concentration. Although speculative, the pattern of BHBA and glucose detected (Fig 2.4B) in CHOL-supplemented cows in the present study was associated with numerically lower negative EB (Table 2.4) as a result of lower milk production. The exact mechanisms for the lower milk yield in these cows that maintained greater blood glucose merits further study.

CONCLUSIONS

The greater DMI and greater milk production in MET-supplemented cows, coupled with a tendency for lower incidence of ketosis and retained placenta, underscore a smoother transition from pregnancy to lactation when an approximately 2.8:1 Lys:Met ratio was achieved through MET supplementation. The better prepartal DMI response with supplemental MET indicates that higher and sustained feed intake prior to calving might not always be detrimental to the postpartal metabolic response. Although rumen-protected CHOL has been suggested to improve cow performance through stimulating liver TAG export as well as sparing MET, CHOL supplementation failed to increase DMI, decrease liver TAG, milk yield, or optimize milk composition. The reasons for the lack of effects of CHOL are not readily apparent, but might be related to the cow BCS. Future research should concentrate on the interrelated metabolism of MET and CHOL to better understand unique and synergistic effects on cow performance.

TABLES AND FIGURES

Table 2.1. Ingredient composition of diets fed during far-off (-50 to -22 d relative to calving), close-up (-21 d to calving) and early lactation (calving to 30 d) periods.

	Diet		
Ingredient (% of DM)	Far-off	Close-up	Lactation
Alfalfa silage	12.00	8.34	5.07
Alfalfa hay	-	4.29	2.98
Corn silage	33.00	36.40	33.41
Wheat straw	36.00	15.63	2.98
Cottonseed	-	-	3.58
Wet brewers grains	-	4.29	9.09
Ground shelled corn	4.00	12.86	23.87
Soy hulls	2.00	4.29	4.18
Soybean meal, 48% CP	7.92	2.57	2.39
Expeller soybean meal[1]	-	2.57	5.97
Soychlor[2]	0.15	3.86	-
Blood meal, 85% CP	1.00	-	-
ProVAAl AADvantage[3]	-	0.86	1.50
Urea	0.45	0.30	0.18
Rumen-inert fat[4]	-	-	1.02
Limestone	1.30	1.29	1.31
Salt	0.32	0.30	0.30
Dicalcium phosphate	0.12	0.18	0.30
Magnesium oxide	0.21	0.08	0.12
Magnesium sulfate	0.91	0.99	-
Sodium bicarbonate	-	-	0.79
Potassium carbonate	-	-	0.30
Calcium sulfate	-	-	0.12
Mineral vitamin mix[5]	0.20	0.17	0.18
Vitamin A[6]	0.015	-	-
Vitamin D[7]	0.025	-	-
Vitamin E[8]	0.38	0.39	-
Biotin	-	0.35	0.35

[1]SoyPLUS (West Central Soy, Ralston, IA)
[2]By West Central Soy
[3]Perdue AgriBussiness (Salisbury, MD)
[4]Energy Booster 100 (Milk Specialties Global, Eden Prairie, MN)
[5]Contained a minimum of 5% Mg, 10% S, 7.5% K, 2.0% Fe, 3.0% Zn, 3.0% Mn, 5000 mg of Cu/kg, 250 mg of I/kg, 40 mg of Co/kg, 150 mg of Se/kg, 2200 kIU of vitamin A/kg, 660 kIU of vitamin D3/kg, and 7,700 IU of vitamin E/kg.
[6]Contained 30,000 kIU/kg
[7]Contained 5,009 kIU/kg

Table 2.1 (Continued)

[8]Contained 44,000 kIU/kg

Table 2.2. Frequency of occurrence of health problems in multiparous Holstein cows supplemented with rumen-protected MET (Smartamine M, Adisseo NA) or rumen-protected CHOL (ReaShure; Balchem Inc.) during the peripartal period.

	Diets[1]				*P* value		
Variable	CON	SMA	REA	MIX	MET	CHOL	MET×CHOL
Cows							
n[2]	20	21	20	20	--	--	--
Ketosis[3]	8	3	5	4	0.07	0.93	0.21
Displaced abomasum	2	0	3	1	0.99	0.99	0.99
Retained placenta[4]	4	2	5	1	0.07	0.76	0.50
Endometritis	0	0	0	1	--	--	--
Mastitis	0	1	0	0	--	--	--
Excluded cows							
n[5]	2	0	2	3			
Displaced abomasum	1	0	2	3			
Endometritis[6]	1	0	0	0			

[1]CON = control; SMA = rumen-protected methionine (0.08% of DMI); REA = rumen-protected choline (60 g/d); MIX = SMA+REA; MET = SMA+MIX; CHOL = REA+MIX.

[2]Actual number of cows completing the study.

[3]Defined as cows having moderate (~40 mg/dL) or large ketone concentrations (>80 mg/dL) in urine and treated by veterinarians with oral propylene glycol or intravenous dextrose.

[4]Defined as fetal membranes retained >24 h after calving.

[5]Actual number of cows excluded due to failure to recover after prolonged treatment or from surgery.

[6]Diagnosed by the attending veterinarian and defined as foul, watery or purulent orange-brown colored uterine discharge together with fever (T >39.5 °C) and decreased appetite.

Table 2.3. Nutrient composition and evaluation (NRC, 2001) of prepartal and postpartal diets fed to multiparous Holstein cows supplemented with rumen-protected MET (Smartamine M, Adisseo NA) or rumen-protected CHOL (ReaShure; Balchem Inc.) during the peripartal period[1].

	Prepartum					Postpartum			
		Close-up[2]							
Chemical component	Far off	CON	SMA	REA	MIX	CON	SMA	REA	MIX
NE_L (Mcal/kg of DM)	1.40	1.52	1.52	1.51	1.51	1.72	1.70	1.72	1.70
CP (% of DM)	14.3	14.6	14.6	14.4	14.4	17.2	17.3	17.2	17.3
RDP (% of DM)	10.2	9.2	9.2	9.1	9.0	9.7	9.6	9.7	9.6
RUP (% of DM)	4.1	5.3	5.4	5.3	5.4	7.4	7.7	7.4	7.7
NDF (% of DM)	51.1	42.0	41.9	41.7	41.7	33.9	33.9	33.7	33.6
ADF (% of DM)	35.4	28.3	28.3	28.2	28.1	21.4	21.4	21.3	21.2
RDP supplied (g/d)	1248	1180	1289	1231	1332	1679	1862	1662	1849
RDP balance (g/d)	138	-51	-61	-63	-79	-120	-141	-121	-144
RUP supplied (g/d)	507	680	763	714	798	1280	1481	1268	1474
RUP required (g/d)	92	184	159	190	169	1787	2149	1814	2062
RUP balance (g/d)	415	496	605	525	629	-507	-669	-546	-587
MP supplied (g/d)	1058	1255	1390	1314	1445	2090	2374	2070	2361
MP balance (g/d)	324	404	493	426	513	-434	-573	-467	-504
Lys:Met	3.89:1	3.62:1	2.81:1	3.61:1	2.79:1	3.54:1	2.71:1	3.54:1	2.71:1
Lys (% of MP)	7.24	6.74	6.66	6.72	6.63	6.33	6.24	6.33	6.24
MP-Lys (g)	77	85	93	88	96	132	148	131	147
Met (% of MP)	1.86	1.86	2.37	1.86	2.38	1.79	2.30	1.79	2.30
MP-Met (g)	20	23	33	24	34	37	55	37	54
NE_L allowable milk (kg/d)	-	-	-	-	-	27.3	30.0	26.3	30.6
MP allowable milk (kg/d)	-	-	-	-	-	31.5	33.5	29.8	33.3
NFC (% of DM)	25.0	32.2	32.2	32.2	32.2	37.8	37.8	37.7	37.7
EE (% of DM)	2.2	3.3	3.3	3.6	3.6	5.7	5.7	5.9	5.9
Ca (% of DM)	0.99	1.09	1.09	1.09	1.09	0.92	0.92	0.92	0.92
P (% of DM)	0.25	0.29	0.29	0.29	0.29	0.37	0.37	0.37	0.37
Mg (% of DM)	0.51	0.45	0.45	0.45	0.45	0.28	0.28	0.28	0.28
Cl (% of DM)	0.63	0.88	0.88	0.92	0.92	0.38	0.38	0.40	0.41
K (% of DM)	1.32	1.17	1.17	1.17	1.17	1.20	1.20	1.20	1.19
Na (% of DM)	0.15	0.15	0.15	0.15	0.15	0.37	0.37	0.37	0.37
S (% of DM)	0.40	0.31	0.32	0.31	0.32	0.22	0.24	0.22	0.24

[1]The NRC (2001) evaluation of diets was based on final averaged pre and postpartum DMI, production data, and feed analysis.

[2]CON = control; SMA = rumen protected methionine (0.08% of DMI); REA = rumen protected choline (60 g/d); MIX = SMA+REA. Composition of Smartamine and Reashure supplied by Adisseo NA (Alpharetta, GA) and Balchem Inc. (New Hampton, NY).

Table 2.4. Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected Met (Smartamine M, Adisseo NA), rumen-protected choline (ReaShure; Balchem Inc.), or both (MIX) on DMI, BW, and BCS.

	MET		CHOL			Treatments					*P*-value						
Item	With	Without	With	Without	SEM[1]	CON	SMA	REA	MIX	SEM	MET[2]	CHOL[3]	M×C[4]	Time	M×T[5]	C×T[6]	M×C×T
Prepartum																	
BW (kg)*	775.1	772.1	769.3	777.9	11.5	774.2	781.5	769.9	768.6	16.4	0.85	0.60	0.79	<0.01	0.18	0.28	0.18
BCS	3.53	3.50	3.48	3.55	0.05	3.54	3.56	3.46	3.51	0.07	0.60	0.35	0.87	0.50	0.52	0.48	0.66
DMI (kg/d)$	14.3[a]	13.2[b]	14.0	13.5	0.3	12.8	14.2	13.7	14.3	0.5	0.02	0.28	0.41	<0.01	0.98	0.81	0.49
DMI (% of BW)*$	1.84	1.74	1.83	1.76	0.05	1.71	1.81	1.78	1.88	0.07	0.13	0.27	0.98	<0.01	0.98	0.74	0.48
Energy balance (Mcal/d)*	6.20	4.76	5.86	5.10	0.66	4.63	5.56	4.88	6.84	0.94	0.12	0.40	0.57	<0.01	0.95	0.30	0.82
Postpartum																	
BW (kg)*	681.7	671.1	672.3	680.5	9.6	678.0	683.1	664.2	680.3	13.9	0.44	0.54	0.70	<0.01	0.44	0.62	0.63
BCS	3.19	3.10	3.13	3.16	0.06	3.11	3.21	3.09	3.17	0.08	0.26	0.71	0.95	<0.01	0.46	0.09	0.10
DMI (kg/d)#	19.2[a]	17.2[b]	18.1	18.3	0.6	17.6	19.0	16.8	19.4	0.8	0.02	0.79	0.49	<0.01	0.59	0.54	0.61
DMI (% of BW)#	2.87 [a]	2.60 [b]	2.73	2.74	0.10	2.67	2.81	2.53	2.92	0.14	0.05	0.93	0.37	<0.01	0.95	0.63	0.79
Energy balance (Mcal/d)	-9.25	-8.84	-8.84	-9.25	1.02	-7.84	-10.67	-9.83	-7.84	1.47	0.77	0.77	0.10	<0.01	0.84	0.97	0.40

[a,b]Mean values with different superscripts differ ($P < 0.05$).

[1]Greatest SEM.

[2]Overall effect of methionine supplementation.

[3]Overall effect of choline supplementation.

[4]Interaction of MET × CHOL.

[5]Interaction of MET × time.

[6]Interaction of CHOL × time.

*Parity included in the model as a covariate ($P < 0.05$). $Last week of far-off period DMI included in the model as a covariate ($P < 0.05$). #Previous 305 d milk yield included in the model as a covariate ($P < 0.05$).

Table 2.5. Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected Met (Smartamine M, Adisseo NA), rumen-protected choline (ReaShure; Balchem Inc.), or both (MIX) on milk production and composition.

	MET		CHOL			Treatments					*P*-value						
Parameter	With	Without	With	Without	SEM[1]	CON	SMA	REA	MIX	SEM	MET[2]	CHOL[3]	M×C[4]	Time	M×T[5]	C×T[6]	M×C×T
Milk composition (%)																	
Fat	3.72	3.74	3.78	3.68	0.10	3.61	3.75	3.87	3.70	0.15	0.91	0.47	0.28	<0.01	0.54	0.92	0.70
Protein[#]	3.32[a]	3.14[b]	3.27	3.19	0.04	3.07	3.31	3.22	3.32	0.06	<0.01	0.19	0.20	<0.01	0.56	0.68	0.07
SCC[7*]	1.86	1.82	1.85	1.82	0.06	1.87	1.77	1.76	1.94	0.09	0.64	0.75	0.12	<0.01	0.83	0.19	0.86
Lactose[*]	4.70	4.69	4.69	4.70	0.03	4.71	4.69	4.67	4.71	0.04	0.80	0.80	0.39	<0.01	0.85	0.55	0.28
Total solids	12.65	12.39	12.60	12.43	0.12	12.19	12.67	12.58	12.62	0.17	0.13	0.32	0.20	<0.01	0.18	0.97	0.52
Other solids[*]	5.62	5.60	5.61	5.62	0.03	5.62	5.61	5.59	5.63	0.04	0.59	0.90	0.46	<0.01	0.74	0.55	0.34
MUN[*]	12.85	12.90	12.99	12.77	0.28	12.65	12.89	13.15	12.82	0.40	0.91	0.58	0.47	0.46	0.93	0.55	0.21
Milk production																	
Milk yield[#]	44.2[a]	40.4[b]	41.4	43.2	1.2	41.8	44.6	39.1	43.7	1.6	0.02	0.27	0.60	<0.01	0.25	0.73	0.87
Milk fat yield*[#]	1.58[a]	1.45[b]	1.50	1.54	0.04	1.46	1.61	1.45	1.55	0.06	0.02	0.49	0.61	0.01	0.42	0.53	0.42
Milk protein yield	1.43[a]	1.25[b]	1.32	1.35	0.04	1.27	1.44	1.23	1.42	0.06	<0.01	0.61	0.86	<0.01	0.72	0.76	0.01
ECM[#]	44.6[a]	40.5[b]	41.9	43.1	1.0	41.0	45.3	40.0	43.9	1.5	0.01	0.40	0.89	<0.01	0.10	0.89	0.10
FCM[#]	44.6[a]	40.8[b]	42.1	43.4	1.0	41.3	45.4	40.3	43.8	1.4	0.01	0.36	0.84	<0.01	0.25	0.56	0.84
Efficiency																	
Milk:DMI	2.45	2.65	2.56	2.54	0.10	2.54	2.54	2.76	2.36	0.15	0.17	0.87	0.17	0.22	0.89	0.75	0.19
ECM:DMI	2.43	2.55	2.49	2.49	0.08	2.47	2.50	2.62	2.36	0.12	0.34	0.99	0.23	<0.01	0.04	0.42	0.25
FCM:DMI	2.44	2.58	2.51	2.51	0.09	2.51	2.51	2.65	2.36	0.13	0.26	0.99	0.25	<0.01	0.10	0.43	0.36

[a,b]Mean values with different superscripts differ ($P < 0.05$).

[1]Greatest SEM.

[2]Overall effect of methionine supplementation.

[3]Overall effect of choline supplementation.

[4]Interaction of MET × CHOL

[5]Interaction of MET × time.

Table 2.5 (Continued)

[6]Interaction of CHOL × time.

[7]SCC somatic cell count data were log transformed.

[*]Parity included in the model as a covariate ($P < 0.05$).

[#]Previous 305 d milk yield included in the model as a covariate ($P < 0.05$).

Table 2.6. Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected MET (Smartamine M, Adisseo NA), rumen-protected CHOL (ReaShure; Balchem Inc.), or both (MIX) on pre- and post-partum blood and liver biomarkers.

	MET		CHOL			Treatments					*P*-value						
Biomarker	With	Without	With	Without	SEM[1]	CON	SMA	REA	MIX	SEM	MET[2]	CHOL[3]	M×C[4]	Time	M×T[5]	C×T[6]	M×C×T
Blood																	
Glucose (mmol/L)	3.80	3.78	3.87[a]	3.71[b]	0.05	3.66	3.76	3.90	3.85	0.07	0.68	0.02	0.31	<0.01	0.27	0.84	0.75
NEFA (mmol/L)	0.62	0.61	0.60	0.64	0.04	0.63	0.64	0.59	0.60	0.06	0.87	0.53	0.99	<0.01	0.15	0.85	0.25
BHBA (mmol/L)	0.92	0.88	0.83	0.97	0.06	0.91	1.04	0.86	0.81	0.09	0.66	0.12	0.33	<0.01	0.54	0.24	0.51
Insulin (µg/L)	0.57	0.53	0.63	0.47	0.04	0.42	0.52	0.64	0.61	0.06	0.53	0.01	0.25	<0.01	0.56	0.21	0.26
Glucose:insulin	9.52	10.65	9.03	11.14	0.76	11.96	10.33	9.34	8.71	1.14	0.29	0.05	0.64	<0.01	0.49	0.61	0.25
NEFA:Insulin	1.78	1.70	1.60	1.88	0.24	1.97	1.80	1.43	1.77	0.35	0.79	0.39	0.44	<0.01	0.33	0.52	0.47
Liver																	
TAG (mg/g wet wt)	28.09	29.06	29.68	27.47	3.18	26.32	28.45	31.81	27.56	4.61	0.83	0.62	0.47	<0.01	0.95	0.13	0.16

[a,b]Mean values with different superscripts differ ($P < 0.05$).

[1]Greatest SEM.

[2]Overall effect of methionine supplementation.

[3]Overall effect of choline supplementation.

[4]Interaction of MET × CHOL.

[5]Interaction of MET × time.

[6]Interaction of CHOL × time.

Fig. 2.1

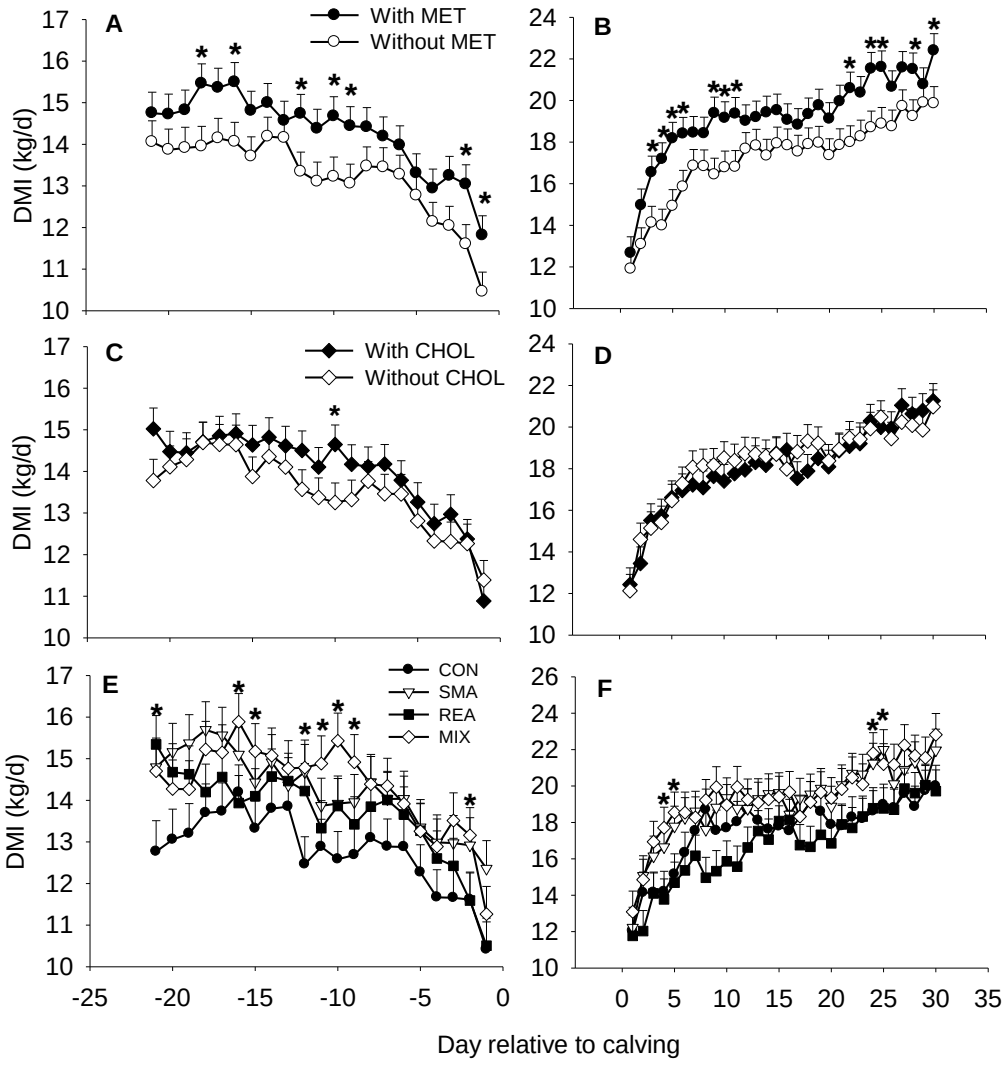
A
With MET
Without MET
B
C
With CHOL
Without CHOL
D
E
CON
SMA
REA
MIX
F
DMI (kg/d)
Day relative to calving

Fig. 2.2

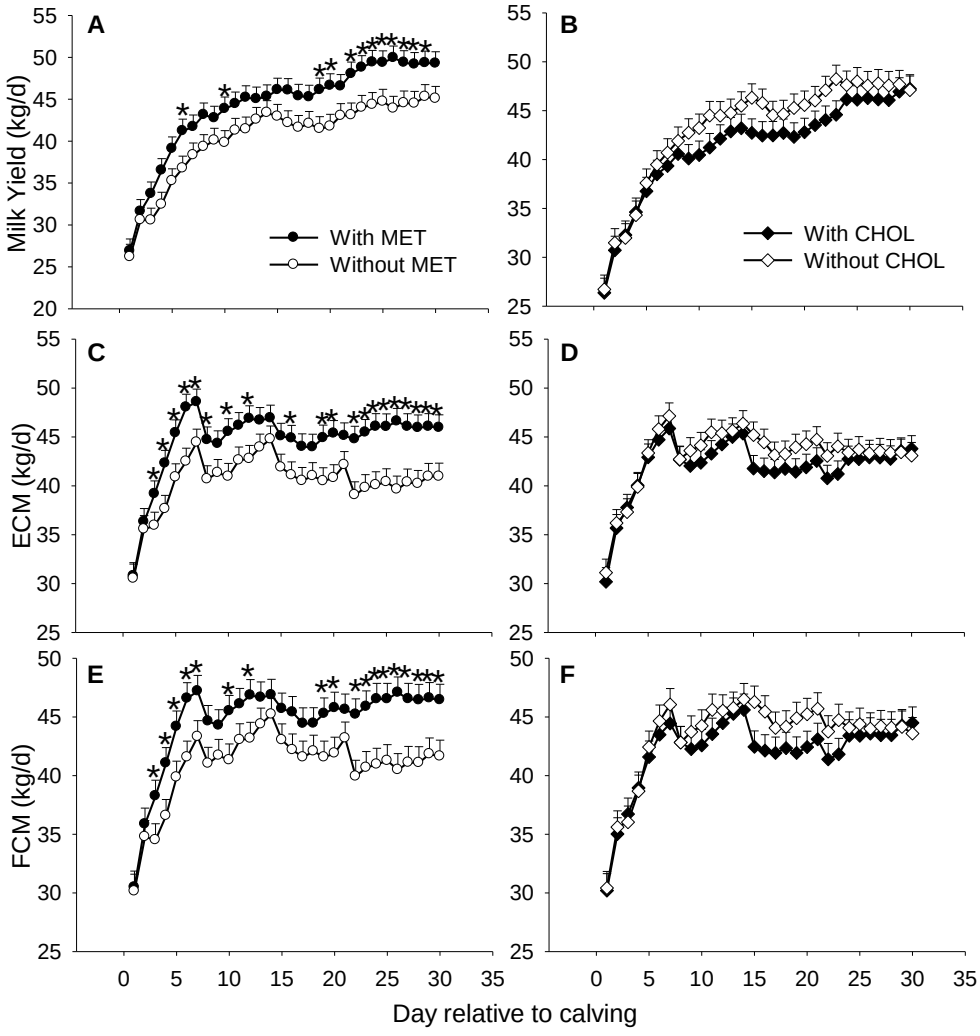
A
B
C
D
E
F
Milk Yield (kg/d)
ECM (kg/d)
FCM (kg/d)
With MET
Without MET
With CHOL
Without CHOL
Day relative to calving

Fig. 2.3

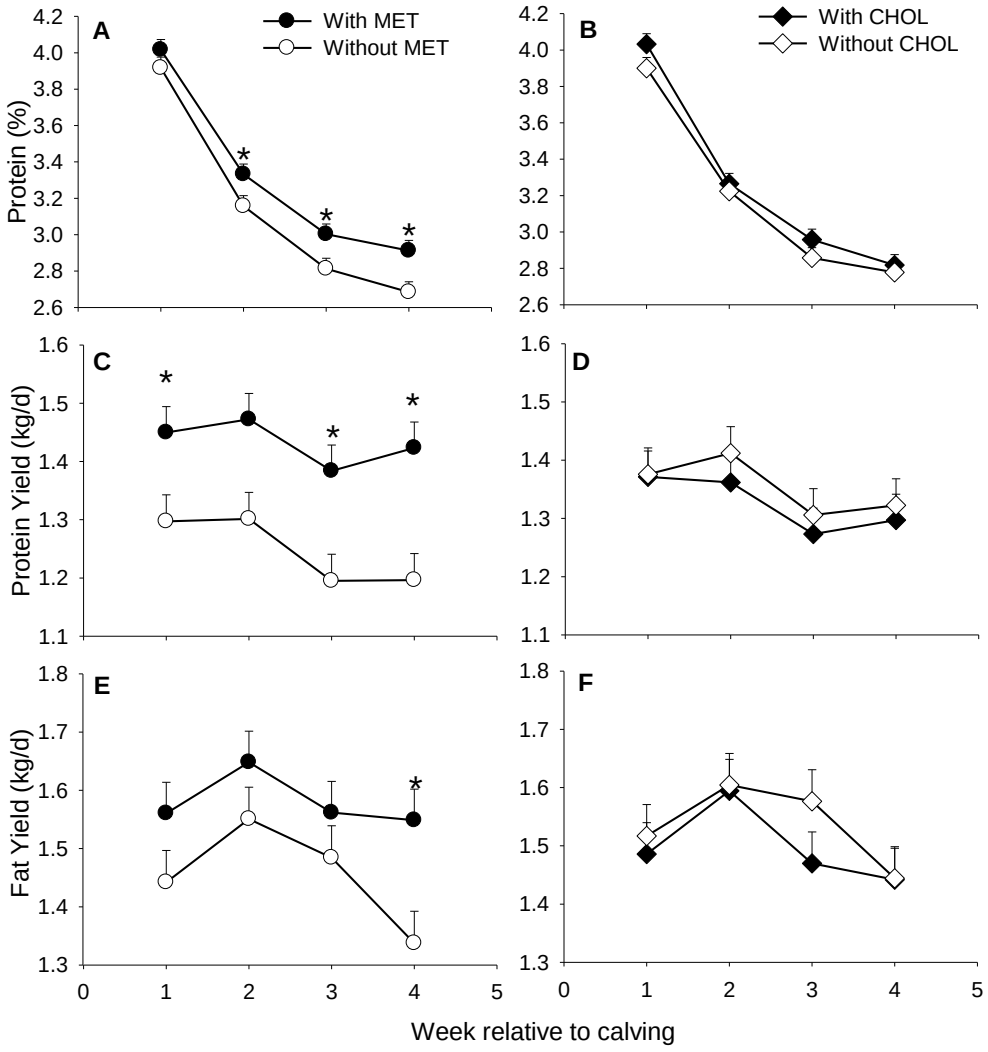
A
With MET
Without MET
B
With CHOL
Without CHOL
C
D
E
F
Protein (%)
Protein Yield (kg/d)
Fat Yield (kg/d)
Week relative to calving

Fig. 2.4

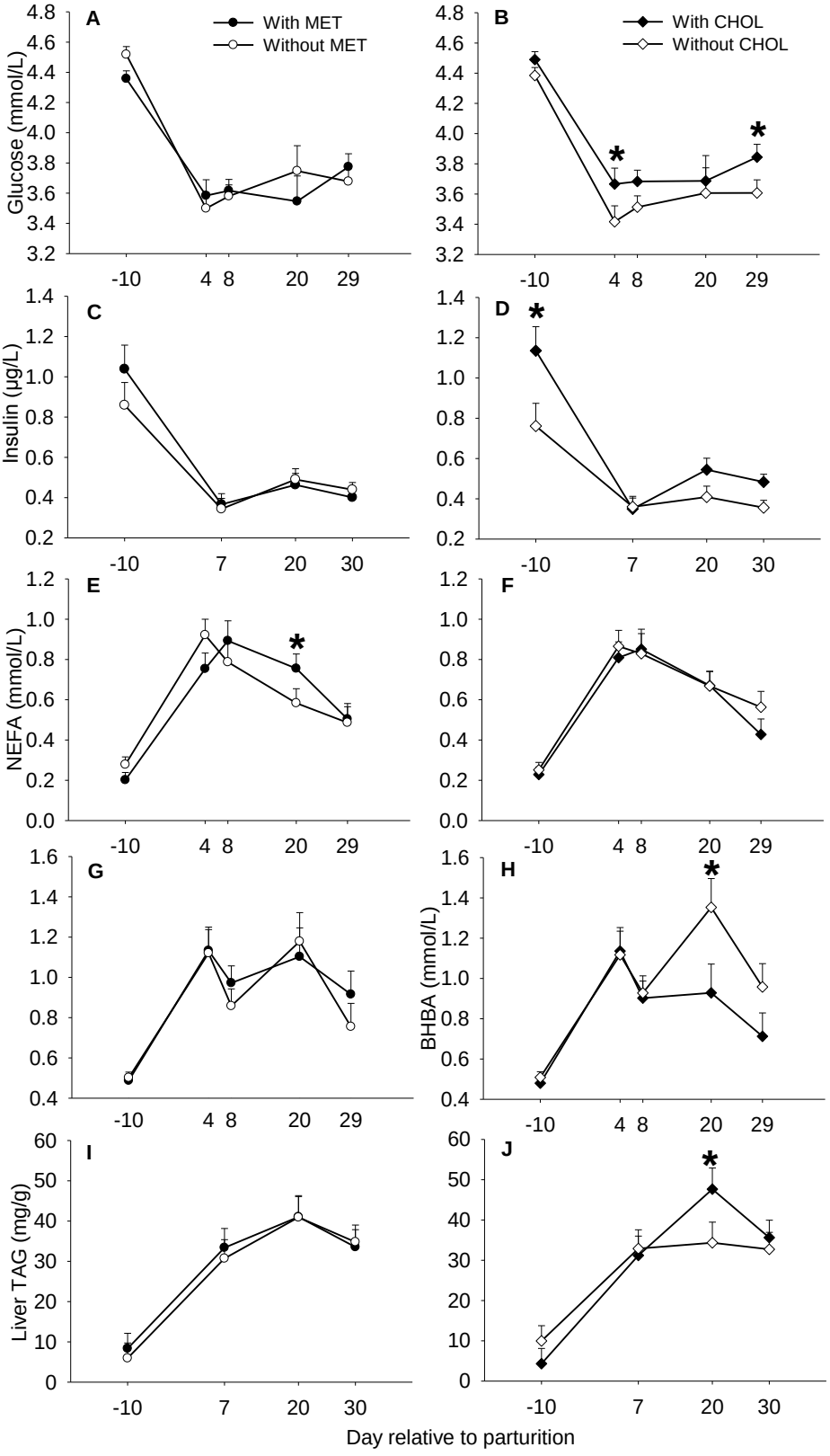
A
With MET
Without MET
B
With CHOL
Without CHOL
Glucose (mmol/L)
C
D
Insulin (µg/L)
E
F
NEFA (mmol/L)
G
H
BHBA (mmol/L)
I
J
Liver TAG (mg/g)
Day relative to parturition

Figure 2.1. Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected Met (Smartamine M, Adisseo NA), rumen-protected choline (ReaShure; Balchem Inc.), or both (MIX) on prepartal (A, C, E) and postpartal (B, D, F) DMI. CON = control; SMA = Smartamine M (0.08% of DMI); REA = ReaShure (60 g/d); MIX = SMA+REA; MET = SMA+MIX; CHOL = REA+MIX. Values are means, with standard errors represented by vertical bars. *MET×day, CHOL×day and MET×CHOL×day effects ($P < 0.05$) at a specific day.

Figure 2.2. Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected Met (Smartamine M, Adisseo NA), rumen-protected choline (ReaShure; Balchem Inc.), or both (MIX) on milk yield (A, B), ECM (C, D) and FCM (E, F). CON = control; SMA = Smartamine M (0.08% of DMI); REA = ReaShure (60 g/d); MIX = SMA+REA; MET = SMA+MIX; CHOL = REA+MIX. Values are means, with standard errors represented by vertical bars. *MET×day, CHOL×day and MET×CHOL×day effects ($P < 0.05$) at a specific day.

Figure 2.3. Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected Met (Smartamine M, Adisseo NA), rumen-protected choline (ReaShure; Balchem Inc.), or both (MIX) on milk protein percentage (A, B), milk protein yield (C, D) and milk fat yield (E, F). CON = control; SMA = Smartamine M (0.08% of DMI); REA = ReaShure (60 g/d); MIX = SMA+REA; MET = SMA+MIX; CHOL = REA+MIX. Values are means, with standard errors represented by vertical bars. *MET×week, CHOL×week and MET×CHOL×week effects ($P < 0.05$) at a specific week.

Figure 2.4. Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected Met (Smartamine M, Adisseo NA), rumen-protected choline (ReaShure; Balchem Inc.), or both (MIX) on blood glucose (A, B), NEFA (C, D) and liver TAG (E, F). CON = control; SMA = Smartamine M (0.08% of DMI); REA = ReaShure (60 g/d); MIX = SMA+REA; MET = SMA+MIX; CHOL = REA+MIX. Values are means, with standard errors represented by vertical bars. *MET×day, CHOL×day and MET×CHOL×day effects ($P < 0.05$) at a specific day.

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CHAPTER 3

RUMEN-PROTECTED METHIONINE COMPARED WITH RUMEN-PROTECTED CHOLINE IMPROVES IMMUNOMETABOLIC STATUS IN DAIRY COWS DURING THE PERIPARTAL PERIOD

- *Journal of dairy science, 2016, 99(11):8956-8969.*

INTRODUCTION

During the peripartal period, dairy cattle experience a state of negative energy and MP balance due to reduced DMI and increased nutrient requirements to support fetal growth and lactation (Drackley, 1999, Bell et al., 2000). Although some cows are able to adapt physiologically without being afflicted with metabolic and infectious diseases, the metabolic and immunologic challenges that occur during the peripartal period are important factors that limit the ability of most cows to achieve optimal performance and immunometabolic status (Drackley, 1999, Loor et al., 2013b).

Due to extensive microbial degradation in the rumen, dietary availability of key methyl donors [(e.g., methionine (**MET**) and choline (**CHOL**)] are limited (Sharma and Erdman, 1989, Girard and Matte, 2005). Therefore, a negative methyl donor balance also may be an important challenge for the peripartal dairy cow owing to the fact that the synthesis of key compounds such as phosphatidylcholine (**PC**) and carnitine in tissues requires methyl donors (Pinotti et al., 2002). The biological role of methyl donors goes beyond metabolism because they are important sources of the intracellular antioxidants glutathione (**GSH**) and taurine (Brosnan and Brosnan, 2006).

In the context of liver metabolism and function, endogenous synthesis of PC from supplemental MET and/or CHOL could play a role in the ability of the tissue to handle the incoming fatty acids produced from lipolysis of adipose tissue. It is well-established that excessive hepatic fatty acid infiltration (Drackley, 1999) can negatively impact the normal functions of the liver. In addition to hepatic fat infiltration, the peripartal period is characterized by an increase of reactive oxygen metabolite (**ROM**) production, the accumulation of which could deplete intracellular antioxidants such as GSH and give rise to oxidative stress that may cause substantial tissue damage (Bertoni et al., 2009, Sordillo and Aitken, 2009, Trevisi et al., 2012a). Increased oxidative stress likely leads to inflammation and could compromise the leukocyte responses during the peripartal period.

Biomarkers in plasma such as cholesterol and paraoxonase (**PON**), among others, have been used successfully to assess the degree of liver function around parturition (Bionaz et al., 2007, Bertoni et al., 2008, Loor et al., 2013b). Furthermore, because parturition also is characterized by inflammatory conditions (Bionaz et al., 2007), pro-inflammatory cytokines (e.g. IL-1β) (Trevisi et al., 2015) and the changes in concentrations of positive (e.g. haptoglobin) and negative acute-phase proteins (**APP**) offer a valuable tool to evaluate the functional welfare of the cow (Loor et al., 2013). Changes in inflammatory cytokines are closely linked with PMNL development and immunity-related activities (Burton et al., 2005), thus, coupling plasma biomarkers with measures of leukocyte function provide a more holistic view of the cow immune system. Immune dysfunction is a feature of the transition period and is characterized by impaired neutrophil trafficking, phagocytosis, and killing capacity (Kehrli et al., 1989, Goff and Horst, 1997), but also different ability of leukocytes to produce cytokines (Jahan et al., 2015). In the context of transition period management, the available data provide benchmarks that could be

used to assess relationships among liver function, performance, and fertility (Bertoni and Trevisi, 2013a).

Milk from dairy cows is high in methylated compounds and the levels secreted into milk are maintained even at the cost of depleting liver tissue reserves (Pinotti et al., 2002). Such effect would exert an even greater challenge on the cow soon after calving, and coupled with the needs of cells to synthesize sulfur-containing antioxidants the supplementation of MET (and potentially CHOL) may be beneficial. However, to date, data demonstrating whether CHOL alone or in combination with MET provide equal or different benefits to the immunometabolic status in transition cows are limited.

Our general hypothesis was that supplementation of rumen-protected MET or CHOL improves liver function and alleviates inflammation and oxidative stress during the peripartal period. Therefore, the objectives of the present study were to measure concentrations of biomarkers in plasma, liver tissue, and milk, and also PMNL function to assess the immunometabolic status of cows supplemented with MET or CHOL.

MATERIALS AND METHODS

Experimental Design and Dietary Treatments

All procedures for this study (protocol no. 13023) were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Illinois. Details of the experimental design have been described previously (Zhou et al., 2016b). Briefly, the experiment was conducted as a randomized complete block design with 2×2 factorial arrangement of MET (Smartamine M, Adisseo NA) and CHOL (ReaShure, Balchem Inc) level (with or without). Cows were blocked according to expected calving date. Each block has 12 cows (except for the

last block). Cows within each block were balanced for parity, previous lactation milk yield, and BCS before close-up diet. A total of 81 cows were used in a randomized complete block design with 2 × 2 factorial arrangement of MET and CHOL level (with or without). Treatments were control (**CON**, n = 20), with no MET or CHOL supplementation; Smartamine (**SMA**, n = 21), CON plus MET at a rate of 0.08% of DM; Reashure (**REA**, n = 20), CON+CHOL at 60 g/d; or Smartamine and Reashure (**MIX**, n = 20), CON+MET+CHOL. Dosage of MET was based on Osorio et al. (2013), whereas CHOL was supplemented following the manufacturer's recommendations. Per IACUC guidelines a subset of 48 multiparous cows (12 cows/treatment) were used for this portion of the study. All cows received the same far-off diet (1.40 Mcal/kg of DM, 10.2% RDP, and 4.1% RUP) from -50 to -22 d before expected calving, close-up diet (1.52 Mcal/kg of DM, 9.1% RDP, and 5.4% RUP) from -21d to expected calving, and lactation diet from calving (1.71 Mcal/kg of DM, 9.7% RDP, and 7.5% RUP) through 30 DIM.

The MET and CHOL supplements were both top-dressed from -21 ± 2 to 30 DIM once daily at the AM feeding using approximately 50 g of ground corn as carrier for all treatments. Supplementation of SMA (0.08% DM of TMR offered) was calculated daily for each cow. Smartamine M was supplied as small beads containing a minimum of 75% DL-Met, physically protected by a pH sensitive coating, which is considered to have a Met bioavailability of 80% (Graulet et al., 2005); therefore, per 10 g of SMA, the cows received 6 g of metabolizable MET. The REA supplement is reported to contain 28.8% choline chloride and is protected by microencapsulation. The product is considered to have CHOL bioavailability of 72% (Benoit et al., 2009); therefore, per 60 g of REA, the cows received 12.4 g of metabolizable choline chloride. To our knowledge, neither SMA nor REA have specific characteristics that may affect palatability of diets.

Animal Management

Dry cows were housed in a ventilated enclosed barn during the dry period and fed individually once daily at 0630 h using an individual gate system (American Calan Inc., Northwood, NH). Cows 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. On average, cows remained in the maternity pen for 3.69 ± 3.61 d. After parturition, cows were housed in a tie-stall barn and were fed a common lactation diet once daily and milked 3× daily at approximately 6:00, 14:00, and 22:00 through the end of the trial at 30 DIM. Feed offered was adjusted daily to achieve ~10% refusals.

Blood, Milk, and Liver Sample Collection and Biomarker Analyses

Blood was sampled for biomarker analysis from the coccygeal vein on -10 d relative to expected calving date and on 4, 8, 20, and 30 d relative to actual calving date before the morning feeding. An additional tube of plasma was collected on 1, 4, 14, and 28 d relative to calving for neutrophil and monocyte phagocytosis and oxidative burst analyses. Samples were collected into evacuated tubes (BD Vacutainer; BD and Co., Franklin Lakes, NJ) containing either clot activator or lithium heparin for serum and plasma, respectively.

Consecutive morning, midday, and evening milk samples were collected every Wednesday and Saturday until 30 DIM (Zhou et al., 2016b). Weekly composite milk samples were prepared in proportion to milk yield at each milking and frozen at -20 °C until analysis.

Liver was sampled via puncture biopsy (Dann et al., 2005) from cows under local anesthesia at approximately 0800 h on d -10, 7, 20, and 30 d relative to parturition. Liver was frozen immediately in liquid nitrogen and stored until analysis.

Serum or plasma was analyzed for creatinine, urea, bilirubin, AST/AGT, GGT, cholesterol, PON, albumin, ceruloplasmin, haptoglobin, myeloperoxidase (**MPO**), ROM, and ferric reducing ability of plasma (**FRAP**) using kits purchased from Instrumentation Laboratory (Lexington, MA) following the procedures described previously (Jacometo et al., 2015) using the clinical auto-analyzer (ILAB 600, Instrumentation Laboratory). Bovine IL-1β (Cat. No. ESS0027; Thermo Scientific, Rockford, IL) and IL-6 (Cat. No. ESS0029; Thermo Scientific, Rockford, IL) plasma concentration were determined using commercial kits. Plasma phosphatidylcholine (Cat. No.10009926; Cayman chemical, Ann Arbor, MI) and free CHOL (Cat. No. K615-100; Biovision Inc., Milpitas, CA) concentration was quantified using commercial kits. The concentration of free CHOL in milk was determined in weekly composite samples (Cat. No. KA1662; Abnova, Taipei City, Taiwan).

The simultaneous phagocytosis capacity and oxidative burst activity of peripheral monocytes and neutrophils was determined upon challenge with enteropathogenic bacteria (*Escherichia coli* 0118:H8) as described by Hulbert et al. (2011) with modifications. Briefly, 200 µL of whole blood with 40 µL 100 µ*M* dihydrorhodamine 123 (Sigma-Aldrich, St. Louis, MO), and 40 µL of propidium iodine labeled bacteria (10^9 cfu/mL) at 38.5°C for 10 min. After red blood cells were lysed with ice cold distilled deionized water, cells were resuspended in PBS solution. Subsequently, monocytes were marked with allophycocyanin-labeled anti-CD14 antibody (Cat. No. 301808; Biolegend, San Diego, CA) while neutrophils were stained with CH138A primary anti-bovine granulocyte monoclonal antibody (Cat. No. BOV2067, Washington State University, WA) and phycoerythrin-labeled secondary antibody (Cat. No. 1020-09S, Southern Biotech, AL). Lastly, the cells were re-suspended in PBS solution for flow cytometry analyses (LSR II; Becton Dickinson, San Jose, CA). Neutrophils and monocytes were

gated based on their side scatter properties in combination with the phycoerythrin and allophycocyanin signal, respectively. Compared with the negative controls, the neutrophils and monocytes from *E. coli*-stimulated samples with greater emissions of propidium iodine were considered positive for phagocytosis. Similarly, compared with negative controls the *E. coli*-stimulated samples with greater emissions of rhodamine 123 were considered positive for oxidative burst. Data are reported as percentages of CD14/CH138A positive cells with phagocytosis and oxidative burst capability. The detailed protocol of the assay is described in the supplemental file.

Liver tissue total and oxidized GSH were measured at -10, 7, 20, and 30 d relative to parturition using a commercial kit (Cat. No. NWH-GSH01; Northwest Life Science Specialties LLC, Vancouver, WA). Reduced GSH was calculated as reduced GSH = total GSH – oxidized GSH.

Statistical analysis

Data were analyzed using PROC MIXED of SAS (SAS Institute Inc., Cary, NC) according to the following model:

$$y_{ijklm} = \mu + b_i + M_j + C_k + MC_{jk} + T_l + TM_{jl} + TC_{kl} + TMC_{jkl} + A_{m:ijk} + \varepsilon_{ijklm}$$

where Y_{ijklm} is the dependent, continuous variable; μ is the overall mean; B_i is the random effect of the ith block; M_j is the fixed effect of MET (j = with or without); C_k is the fixed effect of CHOL (k = with or without);T_l is the fixed effect of time (day or week) of the experiment; $A_{m:ijk}$ is the random effect of the mth animal (cow) nested within block×MET×CHOL; ε_{ijklm} is the residual error. The covariate of parity (2nd vs. 3rd lactation and above), was maintained in the model for all variables when significant ($P < 0.05$). Blood metabolites 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. Least square means separation between time points was performed using the PDIFF statement. Statistical differences were declared significant at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

RESULTS

Biomarkers of Muscle Mass Catabolism and N Metabolism

Main effects of MET, CHOL, time, and interactions are presented in Table 3.1. As expected, creatinine concentrations in all treatments gradually decreased after calving (Suppl. Fig. 3.1). A lower creatinine concentration in plasma was detected due to the main effect of CHOL ($P = 0.02$, Suppl. Fig. 3.1B) but not MET ($P > 0.05$, Suppl. Fig. 3.1A). Unlike creatinine, overall concentration of urea in plasma did not differ in response to MET or CHOL, and was only affected by time ($P < 0.01$) because of an increase after calving.

Biomarkers of Liver Function

Main effects of MET, CHOL, time, and interactions for liver function biomarkers are summarized in Table 3.1. MET or CHOL supplementation had no effect on cholesterol concentrations or AST/AGT ratio ($P > 0.10$, Table 3.1). However, a tendency ($P = 0.07$) was detected in cows fed MET due to an overall greater increase in PON concentrations compared with cows without MET supplementation, especially after calving. Although concentration of PON in cows fed CHOL also increased ($P < 0.01$) over time after calving, CHOL did not result in greater PON compared with cows without CHOL supplementation ($P = 0.87$, Fig. 3.1D).

Biomarkers of Choline Metabolism

The main effect of MET, CHOL, and their interactions for blood and milk CHOL metabolism biomarkers are reported in Table 3.1. The main effects of MET and CHOL and their interactions were not significant ($P > 0.05$) for any of the CHOL metabolism biomarkers (Table 3.1). Regardless of treatment, plasma PC and free CHOL concentrations decreased ($P < 0.05$) around calving and reached a nadir at 4 d after parturition. Unlike PC, plasma free CHOL concentration remained low after 4 d and did not return to prepartum levels by 30 DIM (Suppl. Fig. 3.3), which could be mainly attributed to the constant increase in milk CHOL regardless of treatment ($P < 0.01$, Suppl. Fig. 3.3).

Biomarkers of Inflammation and APP

Among the APP determined in the present study, MET-supplemented cows had greater albumin concentration ($P = 0.04$) compared with cows without MET. In fact, plasma albumin in MET cows increased ($P < 0.05$, Fig. 3.2C) after calving, whereas albumin concentration in cows without MET supplementation remained unchanged compared with the prepartum period ($P > 0.05$). As expected, plasma haptoglobin concentration increased ($P < 0.01$, Fig. 3.2A and 3.2B) soon after calving. Although plasma haptoglobin reached a zenith on d 4 regardless of treatment, a tendency ($P = 0.08$) for lower haptoglobin was detected in response to MET supplementation but not CHOL ($P = 0.94$).

For pro-inflammatory cytokines, there was no main effect or interactions for CHOL. In contrast, a main effect with greater IL-6 in MET-supplemented cows was detected ($P = 0.03$, Fig. 3.3G). IL-1β concentration did not change ($P = 0.14$, Fig. 3.2E) in MET-supplemented cows.

Biomarkers of Oxidative Stress

Although no main effect or interactions of MET and CHOL were detected for plasma MPO, ROM, or FRAP, the concentration of ROM increased after calving and did not return to prepartum level by 30 DIM regardless of treatment (data not shown). In accordance with the temporal changes in ROM, liver total and reduced GSH concentrations decreased after calving and did not return to prepartum level by 30 DIM. However, unlike ROM, liver total ($P = 0.01$, Fig. 3.3A) and reduced GSH ($P = 0.01$, Fig. 3.3C) were greater in MET-supplemented cows, which was mainly attributed to greater concentration of total and reduced GSH on -10 d and 30 d relative to parturition.

Whole Blood Phagocytosis and Oxidative Burst

Upon challenge, a greater increase in neutrophil phagocytosis capability was detected in cows supplemented with MET ($P = 0.01$, Fig. 3.4A) but not CHOL ($P = 0.81$, Fig. 3.4B). In contrast, for blood monocytes, phagocytosis capability did not change in response to MET ($P = 0.28$, Suppl. Fig. 3.2A) or CHOL ($P = 0.15$, Suppl. Fig. 3.2B).

As expected, MET supplementation also resulted in greater blood neutrophil oxidative burst ($P = 0.03$, Fig. 3.4C). In contrast, blood neutrophil oxidative burst did not change in response to CHOL supplementation ($P = 0.54$, Fig. 3.4D). Although changes in monocyte oxidative burst capability were not detected for main effects of MET or CHOL ($P > 0.10$), a significant MET×CHOL interaction ($P < 0.01$, Table 3.2) were shown mainly due to increased monocyte oxidative burst capability in SMA and REA supplemented cows. No temporal changes were detected for blood monocyte ($P = 0.77$, Suppl. Fig 3.2) and neutrophil ($P = 0.45$, Fig. 3.4) oxidative burst activity.

DISCUSSION

Biomarkers of Muscle Mass and N Metabolism

During the periparturient period, the inability of cows to consume sufficient protein leads to negative MP balance and lower plasma AA, especially around parturition (Bell et al., 2000, Zhou et al., 2016da). Considering that the demands for fetal development in late-pregnancy and the onset of lactation greatly increase the tissue requirements for AA, muscle mobilization during this period can be expected. Creatinine is a suitable indicator of body muscle mass and the plasma level of creatinine has been used as an indirect marker for evaluation of body muscle catabolism both in the peripartal period (Kokkonen et al., 2005, Osorio et al., 2014b) and mid lactation (Pires et al., 2013). Thus, the lower plasma concentration of creatinine in CHOL cows indicated less body mass in these cows and therefore a higher degree of muscle mass mobilization. The mechanism for such response is unclear, however, it is likely to be associated with reduced insulin sensitivity in these cows because blood glucose:insulin ratio was lower in response to CHOL supplementation during the peripartal period (Zhou et al., 2016b).

Biomarkers of Liver Function and Choline Metabolism

It is well-known that cholesterol is required for synthesis of the hydrophobic core of lipoproteins synthesized in liver or the intestine (Cohen, 2008), and the concentrations of the various lipoprotein fractions are modified greatly around parturition (Bionaz et al., 2007). The suitability of cholesterol as a biomarker of liver function during the peripartal period is partly because the variation in concentrations depends to a large extent on the lipoprotein level in blood (Bionaz et al., 2007).

As lipotropic agents, MET and CHOL fed to ruminants may help clear lipid from the liver through stimulating VLDL formation and export (Waterman and Schultz, 1972). Previous

reports from both non-ruminant and ruminant studies have illustrated the importance of adequate lipotropic agents in the prevention of hepatic lipidosis (Cooke et al., 2007, Corbin and Zeisel, 2012).

As precursor of PC, which is essential for VLDL synthesis (Auboiron et al., 1995), CHOL supplementation also was expected to improve liver function at least in part by enhancing lipoprotein export from liver. In contrast with this notion, results from previous reports either observed no differences (Guretzky et al., 2006) or detected a lower concentration (Zahra et al., 2006) of blood cholesterol in response to CHOL supplementation. Thus, the lack of change in plasma cholesterol in CHOL-supplemented cows is not entirely surprising (Table 3.1). In agreement with these results, the fact that plasma PC concentration also was not affected by CHOL supplementation together with the higher liver TAG on 20 d in the CHOL-supplemented cows (Zhou et al., 2016b) indicated that the export of lipoproteins from liver was not increased.

Paraoxonase is synthesized in the liver after which it is released into the blood stream where it associates with high-density lipoprotein (HDL) and protects it from oxidative damage (Turk et al., 2004). Therefore, PON concentration has been used as one biomarker of liver function in peripartal cows (Bionaz et al., 2007). The general trend of decrease in PON around parturition with a nadir at 4 d postpartum (Figure 3.1C) was in agreement with previous work (Bionaz et al., 2007, Grossi et al., 2013, Osorio et al., 2014b). It was proposed previously that excessive deposition of triacylglycerol (**TAG**) in liver and its negative impact on hepatocytes was partly responsible for the decrease in PON after calving (Turk et al., 2004). However, the fact that liver TAG increased after calving (Zhou et al., 2016b) regardless of treatment does not seem to support a negative effect on PON. What is apparent from the present study and data on HDL profiles after calving (Bernabucci et al., 2004) is that PON plays a role in lipoprotein

metabolism besides potentially alleviating negative effects of oxidative stress (Aviram and Rosenblat, 2004). In that context, it is noteworthy that the overall correlation between PON and cholesterol was positive regardless of treatment (240 observations total; r = 0.50, $P < 0.01$).

Biomarkers of Inflammation and APP

As a negative APP, hepatic production of albumin is commonly decreased during inflammation, and serves as a useful biomarker to evaluate chronic inflammation (Bertoni et al., 2008, Ceciliani et al., 2012a). The substantially lower concentration of albumin in cows without MET supplementation was suggestive of a more profound inflammatory status during the peripartal period. In fact, albumin concentration in MET cows was already numerically greater at 4 compared with -10 d relative to parturition and continued to increase at 8 d postpartum. It is also noteworthy that cows without MET maintained a constant concentration of albumin throughout the study. Considering the detoxification function of albumin upon binding to NEFA as well as bilirubin, the greater concentration of albumin in MET-supplemented cows may have helped alleviate inflammation (Weinberg, 2006, van der Vusse, 2009) during the peripartal period. It is also noteworthy that in spite of an apparently greater hepatic synthesis of albumin in MET-supplemented cows, they also had greater overall milk protein percentage and milk yield (Zhou et al., 2016b). It has been suggested that a reduction in the synthesis of albumin within the liver would spare AA for gluconeogenesis and production of hepatic proteins, especially positive APP (Ceciliani et al., 2012a).

Production of the positive APP such as haptoglobin and ceruloplasmin are expected to increase during an inflammatory event. In transition cow research, haptoglobin has been of particular interest due to its quick increase during the acute-phase response and has, therefore, been used as diagnostic biomarker previously (Hirvonen et al., 1999, Sheldon et al., 2001). In

terms of the peripartal period, haptoglobin concentration in blood is commonly elevated as a consequence of common inflammatory conditions around calving (Bionaz et al., 2007), e.g. stimulation of parenchymal cells by fatty acid infiltration of the liver in transition cows (Eckersall, 2000, Katoh et al., 2002). The overall positive correlation (240 observations total; r = 0.14, $P = 0.03$) between haptoglobin and IL-1β was indicative of a pro-inflammatory state during the transition period. In accordance with the greater albumin in MET cows, the tendency for lower concentration of blood haptoglobin confirmed the less pronounced inflammatory status in these cows.

Although IL-6 is well-known for its pro-inflammatory properties, recent findings have revealed an anti-inflammatory property for this cytokine (Scheller et al., 2011). In fact, the anti-inflammatory activities of IL-6 are mediated by gp130 and IL-6R (classic signaling) whereas pro-inflammatory activities are mediated by the IL-6/soluble IL-R complex (trans-signaling) (Scheller et al., 2011). Therefore, depending on a given stimulus inducing a given signaling pathway, IL-6 should be considered as both a pro- and an anti-inflammatory cytokine. Because IL-6 does not upregulate major inflammatory mediators and directly inhibits TNFα and IL-1 expression and their signal transduction (Rehman et al., 1997), the current view is that IL-6 has primarily an anti-inflammatory effect (Pedersen et al., 2001, Lauder et al., 2013, Hunter and Jones, 2015). Therefore, considering the tendency for lower haptoglobin as well as the markedly greater albumin in MET-supplemented cows, it is reasonable to speculate that the higher IL-6 concentration in MET-supplemented cows reflected a less pronounced inflammatory status. It also is possible that the greater IL-6 concentration in MET-supplemented cows served to maintain a certain degree of peripheral tissue inflammation (Vailati Riboni et al., 2016).

It is noteworthy that complex indices such as liver activity index (LAI) and liver functionality index (LFI) have been generated to evaluate changes in health and productivity caused by inflammatory events (Bertoni and Trevisi, 2013a). Previous studies have shown greater plasma AA concentrations, milk yield, DMI as well as better health and fertility in high vs. low LFI or LAI cows (Bertoni et al., 2008, Bertoni and Trevisi, 2013a, Zhou et al., 2016d). Considering such indices were generated from plasma inflammation biomarkers (e.g. albumin, ceruloplasmin), it is not surprising that high LFI cows have on average 2.4 g/L greater plasma albumin as well as 0.08 g/L lower haptoglobin compared with low LFI cows postpartum (Zhou et al., 2016d). Similar differences in haptoglobin (0.12 g/L) was observed in the present study between cows with or without MET supplementation. Considering greater albumin was also observed in MET cows, although less in magnitude of difference (1.2 g/L), the favorable changes in health and productivity observed previously (Zhou et al., 2016b) are likely due in part to alterations in inflammation status suggested by inflammation biomarkers such as albumin and haptoglobin.

Biomarkers of Oxidative Stress

Oxidative stress, defined as the serious imbalance between oxidants and antioxidants, as a consequence of augmented ROM production due to the increased demand for nutrients and energy can exert pleiotropic actions in peripartal dairy cows (Sordillo and Aitken, 2009). In fact, ROM has been widely-adopted as a biomarker for oxidative stress in both non-ruminants (Fukui et al., 2011, Jansen et al., 2013) and ruminants (Bernabucci et al., 2002, 2005, Trevisi et al., 2009, Celi et al., 2010). Although ROM concentrations in the present study were not affected by MET or CHOL supplementation, the substantially higher ROM starting at 4 d postpartum was indicative of an increased risk for the onset of oxidative stress. In that context, it is noteworthy

that cows supplemented with MET had clear signs of a reduced inflammatory status. The reason for this is unclear but it could have been related with the differences between groups in the concentration of intracellular antioxidants such as GSH. In fact, GSH is the most abundant endogenous antioxidant due to its marked ability to scavenge ROM and free radicals. Hence, concentration of GSH has been used as biomarker in various oxidative stress-related diseases (Romeu et al., 2010, Vetrani et al., 2013, Saharan and Mandal, 2014). The overall decrease in total and reduced GSH concentration after parturition could have been due to the increased production of ROM and free radicals.

Previous work with dairy cows has demonstrated a positive effect of MET supplementation on intrahepatic GSH concentration during the peripartal period (Osorio et al., 2014b). Such effect may be directly associated with MET supplementation considering that MET can be incorporated upstream in the *de novo* synthesis pathway for GSH (Halsted, 2013). Studies with non-ruminants also have observed reduced concentrations of hepatic GSH in cases of protein-energy malnutrition, with GSH being replenished when sulfur AA or methyl donors are supplemented (Cho et al., 1984, Bauman et al., 1988, Goss et al., 1994). In fact, both *in vitro* (Hartman et al., 2002) and *in vivo* (Tabachnick and Tarver, 1955) studies using radioactive-labelled methionine have demonstrated hepatic incorporation of [^{35}S] into GSH. Therefore, the higher GSH concentration in MET-supplemented cows was in agreement with these reports and confirm a direct association between MET supplementation and hepatic GSH concentration.

Although CHOL does not contain sulfur, MET can be generated in tissues like the liver from CHOL when homocysteine accepts a methyl group from CHOL through betaine (Wong and Thompson, 1972a, Li and Vance, 2008b). Therefore, an increase in hepatic GSH also was expected from CHOL supplementation but it did not occur (Fig. 3.3). Because cysteine is the

limiting substrate for GSH synthesis and receives sulfur from MET (Franklin et al., 2009), it is reasonable to speculate that lack of sulfur was a key limiting factor for GSH synthesis from CHOL supplementation. Alternatively, it also is possible that MET synthesis from CHOL was limited during the peripartal period, either as a result of insufficient expression or activity of key enzymes (e.g., betaine homocysteine methyltransferase), or because CHOL was predominantly used for synthesis of other compounds such as PC or acetylcholine. The specific mechanisms for this response merit further study.

Blood Neutrophil and Monocyte Killing Capacity

Compromised neutrophil and monocyte phagocytic capacity and oxidative burst activity in response to pathogen challenge are direct indicators of an impaired immune response, resulting in increased risk of bacterial infections to the host (Unanue, 1976). Data available to date seem to indicate that peripartal dairy cows have a dysfunctional immune system around calving (Loor et al., 2013b). Therefore, the greater neutrophil phagocytosis and oxidative burst in blood from MET-supplemented cows indicated a better immune system status. However, the precise mechanisms for the "priming" effect are unknown. Human studies have reported that greater Ca^{2+} flux into PMNL increases markedly the oxidative burst intensity and the initiation of the oxidative burst (Bei et al., 1998). It is noteworthy that IL-6 increases intracellular stores of Ca^{2+}, which is the known basis for PMNL activation and degranulation of oxidative burst oxidase-containing granules (Sitaraman et al., 2001). Therefore, considering the sustained greater concentration of IL-6 together with the overall lower disease incidence in MET-supplemented cows (Zhou et al., 2016b), it can be speculated that MET supplementation enhanced phagocytosis and oxidative burst in PMNL at least in part by increasing intracellular Ca^{2+} stores which "sensitized" or "primed" these cells for pathogen challenge.

Although the intensity of the oxidative burst in monocytes stimulated by *E. coli* has been suggested to be lower than in PMNL (Yan et al., 2012), monocytes also have the ability to phagocytose and carry out oxidative burst (Keogh et al., 2011). The fact that CHOL supplementation lead to greater monocyte oxidative burst without elevating plasma Ca^{2+} suggest existence of alternative mechanisms. Other mechanisms related directly to metabolism of MET and CHOL or their metabolites by innate immune cells also could play a role. For instance, the respiratory burst in monocytes is accompanied by increased uptake of GSH (Seres et al., 2000). Studies with GSH reductase-deficient mice indicated they had an impaired oxidative burst and produced less ROM due to GSH depletion (Yan et al., 2012). In agreement with this, reduced neutrophil oxidative burst in humans was detected in response to chronic oxidative stress (Amer and Fibach, 2005). As precursors of GSH upstream in methionine cycle via transsulfuration pathway, it could be possible that supplemental MET and CHOL contributes to increased phagocytosis and oxidative burst upon challenge through increasing GSH availability to neutrophils and monocytes.

CONCLUSIONS

Overall, the changes observed in plasma PON, haptoglobin, and higher albumin revealed favorable alterations of liver function as well as inflammation status during lactation in MET-supplemented cows. MET supplementation resulted in better immune response in terms of greater phagocytosis and oxidative burst capabilities upon pathogen challenge. Biomarker analyses in blood, milk, and liver indicated that the effect of feeding MET on postpartal cow performance is likely due, in part, to a better immunometabolic status. Although some previous studies have demonstrated beneficial effects of CHOL supplementation on performance, the contrasting responses detected between MET and CHOL supplementation on immunometabolic

biomarkers in this study could be due, for example, to the fact that CHOL supplementation was insufficient for cows during the transition period. The requirements of CHOL and methionine during the transition period, along with potential interactions at a mechanistic level, merit further study.

TABLES AND FIGURES

Table 3.1. Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected MET (Smartamine M, Adisseo NA) or rumen-protected CHOL (ReaShure; Balchem Inc.) on blood and liver biomarkers.

	MET			CHOL			*P*-value					
Parameter[1]	Without	With	SEM[2]	Without	With	SEM	MET[3]	CHOL[4]	Parity	Time	M×T[5]	C×T[6]
Muscle body mass												
Creatinine (µmol/L)	90.80	92.29	1.58	94.17	88.92	1.58	0.51	0.02	-	<0.01	0.33	0.63
Urea (mmol/L)	5.16	5.14	0.13	5.16	5.13	0.13	0.91	0.88	-	0.01	0.90	0.52
Liver function markers												
Bilirubin (µmol/L)	4.38	4.22	0.49	4.44	4.16	0.49	0.82	0.69	-	<0.01	0.67	0.98
AST/AGT (U/L)	100.11	100.91	2.85	99.23	101.79	2.85	0.84	0.53	-	<0.01	0.54	0.77
Cholesterol (mmol/L)	3.31	3.62	0.15	3.41	3.53	0.16	0.11	0.55	-	<0.01	0.17	0.82
GGT (U/L)	24.58	24.03	1.44	23.41	25.20	1.44	0.79	0.38	-	<0.01	0.75	0.41
Paraoxonase (U/ml)	84.54	93.09	3.20	88.45	89.18	3.20	0.07	0.87	-	<0.01	0.17	0.74
Inflammation and APP												
Albumin (g/L)	35.53	36.55	0.35	36.21	35.87	0.35	0.04	0.50	-	0.37	0.15	0.68
Ceruloplasmin (µmol/L)	2.84	2.73	0.10	2.74	2.82	0.10	0.45	0.56	-	<0.01	0.18	0.68
Haptoglobin (g/L)	0.47	0.35	0.05	0.41	0.41	0.05	0.08	0.94	-	<0.01	0.90	0.70
Myeloperoxidase (U/L)	415.40	432.02	14.60	423.50	423.92	14.60	0.42	0.98	-	<0.01	0.96	0.49
IL-1β (pg/mL)	7.12	4.98	1.04	6.25	5.86	1.02	0.14	0.79	<0.01	<0.01	0.88	0.37
IL-6 (pg/mL)	835.16	1086.51	62.2	963.74	957.93	82.08	0.03	0.96	-	<0.01	0.87	0.55
Oxidative stress												
ROM (mg H_2O_2/100mL)	13.71	13.42	0.39	13.46	13.66	0.39	0.60	0.72	-	<0.01	0.44	0.84
FRAP (µmol/L)	135.84	135.54	3.66	135.02	136.36	3.66	0.95	0.80	-	0.03	0.28	0.31
Glutathione (µmol/g protein)	23.32	62.83	10.65	46.55	39.61	10.65	0.01	0.64	-	<0.01	0.22	0.58
Reduced glutathione (µmol/g protein)	22.81	62.10	10.59	45.87	39.04	10.59	0.01	0.65	-	<0.01	0.21	0.58
Choline metabolism												
Plasma choline (mg/dL)	38.04	37.38	1.16	37.25	38.16	1.16	0.69	0.58	-	<0.01	0.69	0.82
Plasma phosphatidylcholine (mg/mL)	111.56	115.96	3.77	112.79	114.73	3.77	0.41	0.72	<0.01	<0.01	0.60	0.65
Milk choline (mg/L)	26.78	27.13	1.70	27.43	26.47	1.70	0.88	0.68	-	<0.01	0.41	0.21

[1]MET × CHOL interaction was not significant for any of the parameters and included in the supplemental file.
[2]Greatest SEM.
[3]Overall effect of MET supplementation.
[4]Overall effect of CHOL supplementation.
[5]Interaction of MET × time.
[6]Interaction of CHOL × time.

Table 3.2. Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected MET (Smartamine M, Adisseo NA) or rumen-protected CHOL (ReaShure; Balchem Inc.) on blood neutrophil and monocyte phagocytosis and oxidative burst.

	MET		CHOL			Treatments[2]					*P*-value						
Parameter (%)	Without	With	Without	With	SEM[1]	CON	SMA	REA	MIX	SEM	MET[3]	CHOL[4]	M×C[5]	Time	M×T[6]	C×T[7]	M×C×T
Phago_mono[8]	43.03	45.28	42.62	45.69	1.59	40.01	45.22	46.04	45.34	2.30	0.28	0.15	0.16	0.08	0.27	0.48	0.03
Phago_neutro	54.69	61.05	57.60	58.14	1.67	53.14	62.08	56.23	60.05	2.43	0.01	0.81	0.25	0.10	0.73	0.80	0.42
Oxi_mono	21.35	23.99	21.96	23.38	1.37	17.72[b]	26.19[a]	24.99[a]	21.78[ab]	2.00	0.15	0.43	<0.01	0.77	0.12	0.90	0.41
Oxi_neutro	49.28	57.27	52.20	54.34	2.63	45.05	59.36	53.51	55.18	3.83	0.03	0.54	0.07	0.45	0.57	0.19	0.76

[a,b] Mean values with different superscripts differ ($P < 0.05$).
[1]Greatest SEM.
[2]CON = control; SMA = Smartamine M (0.08% of DMI); REA = ReaShure (60 g/d); MIX = SMA+REA.
[3]Overall effect of MET supplementation.
[4]Overall effect of CHOL supplementation.
[5]Interaction of MET × CHOL.
[6]Interaction of MET × time.
[7]Interaction of CHOL × time.
[8]Phago_mono = monocytes phagocytosis; Phago_neutro = neutrophils phagocytosis; Oxi_mono = monocytes oxidative burst; Oxi_neutro = neutrophils oxidative burst

Fig. 3.1

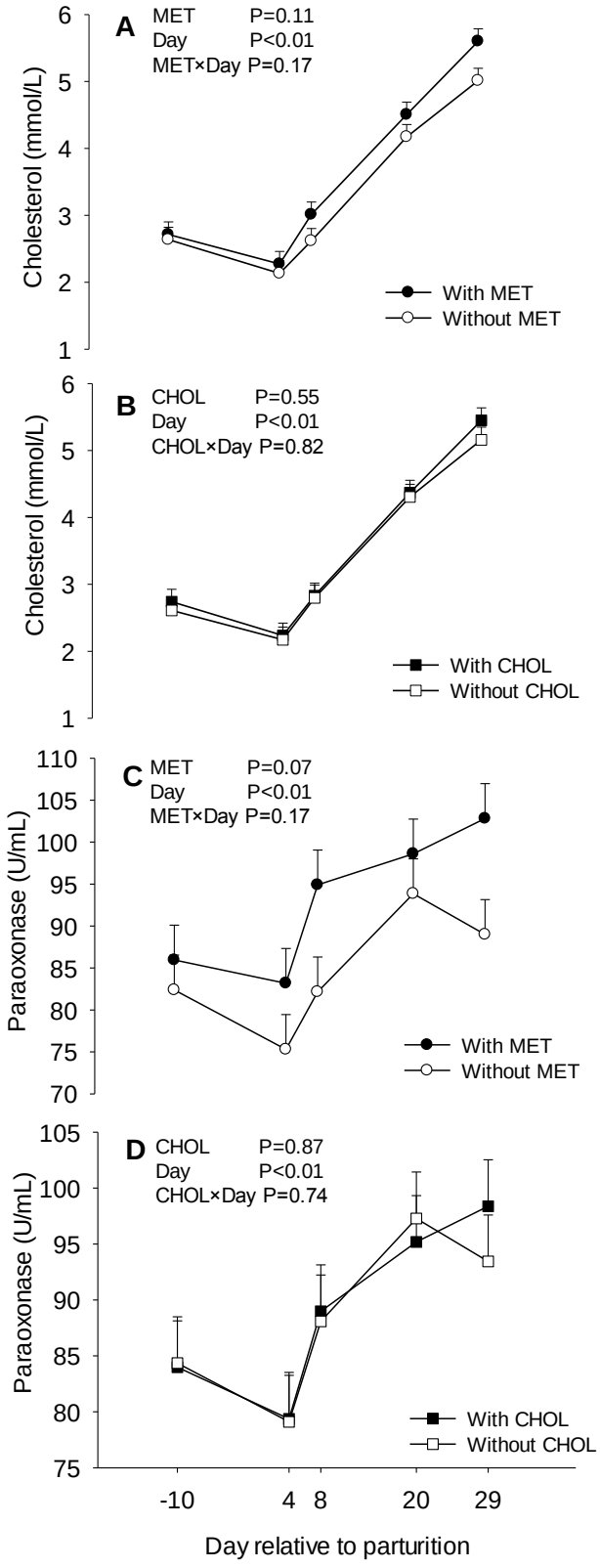
A
MET P=0.11
Day P<0.01
MET×Day P=0.17
Cholesterol (mmol/L)
With MET
Without MET
B
CHOL P=0.55
Day P<0.01
CHOL×Day P=0.82
Cholesterol (mmol/L)
With CHOL
Without CHOL
C
MET P=0.07
Day P<0.01
MET×Day P=0.17
Paraoxonase (U/mL)
With MET
Without MET
D
CHOL P=0.87
Day P<0.01
CHOL×Day P=0.74
Paraoxonase (U/mL)
With CHOL
Without CHOL
-10
4
8
20
29
Day relative to parturition

Fig. 3.2

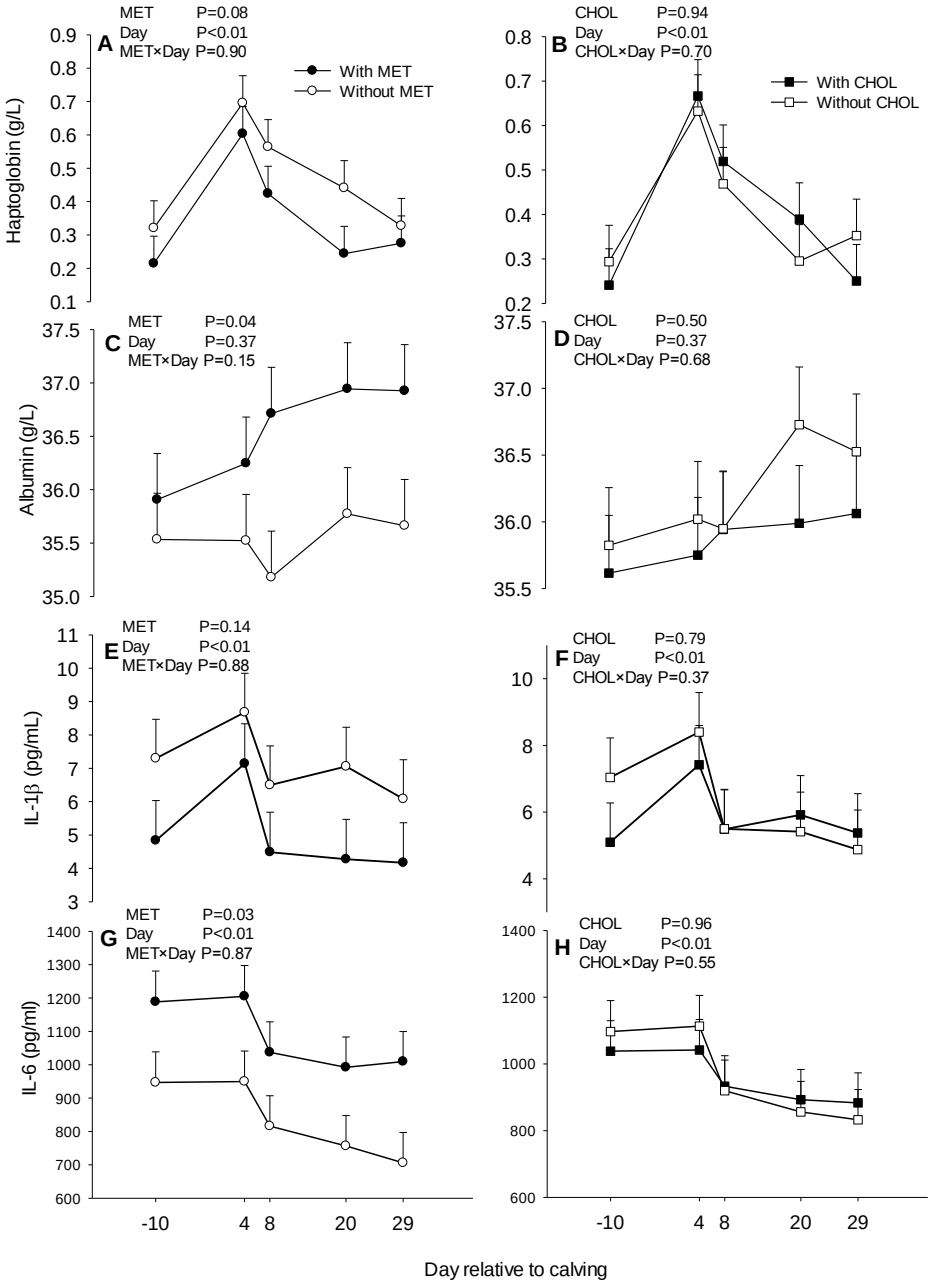
A
MET P=0.08
Day P<0.01
MET×Day P=0.90
With MET
Without MET
Haptoglobin (g/L)
B
CHOL P=0.94
Day P<0.01
CHOL×Day P=0.70
With CHOL
Without CHOL
C
MET P=0.04
Day P=0.37
MET×Day P=0.15
Albumin (g/L)
D
CHOL P=0.50
Day P=0.37
CHOL×Day P=0.68
E
MET P=0.14
Day P<0.01
MET×Day P=0.88
IL-1β (pg/mL)
F
CHOL P=0.79
Day P<0.01
CHOL×Day P=0.37
G
MET P=0.03
Day P<0.01
MET×Day P=0.87
IL-6 (pg/ml)
H
CHOL P=0.96
Day P<0.01
CHOL×Day P=0.55
-10
4
8
20
29
Day relative to calving

Fig. 3.3

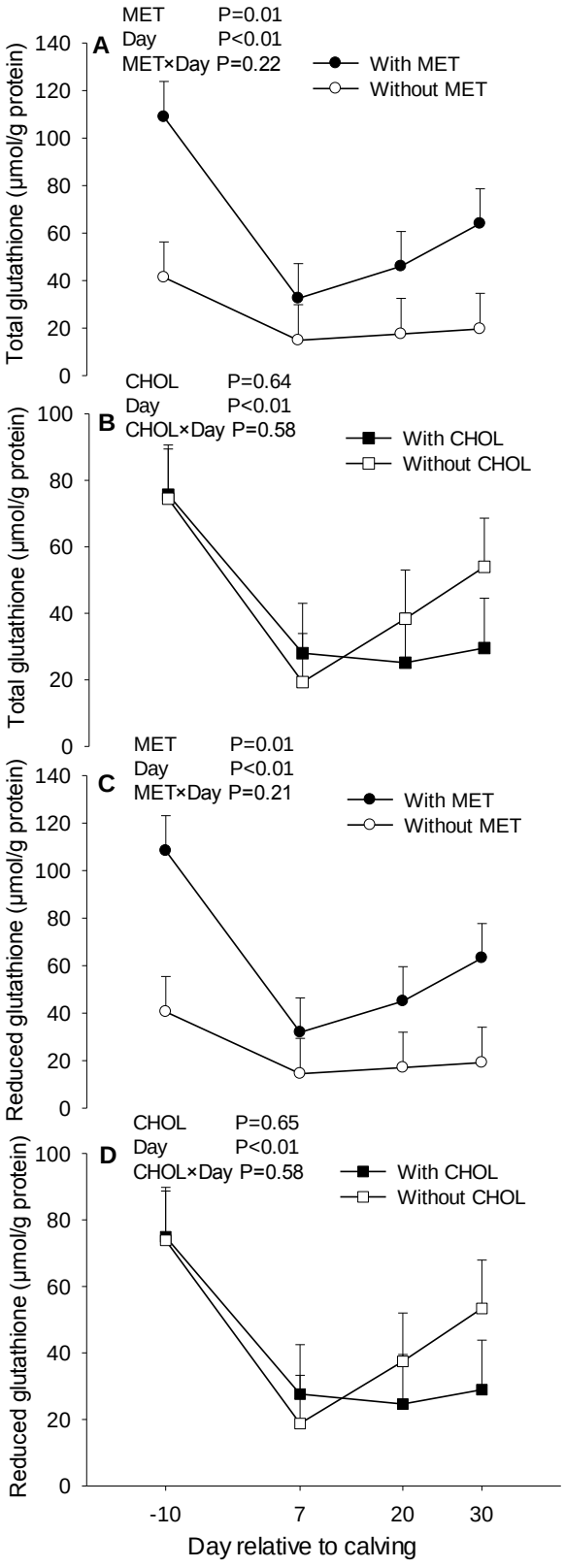
A
MET P=0.01
Day P<0.01
MET×Day P=0.22
With MET
Without MET
Total glutathione (µmol/g protein)
B
CHOL P=0.64
Day P<0.01
CHOL×Day P=0.58
With CHOL
Without CHOL
Total glutathione (µmol/g protein)
C
MET P=0.01
Day P<0.01
MET×Day P=0.21
With MET
Without MET
Reduced glutathione (µmol/g protein)
D
CHOL P=0.65
Day P<0.01
CHOL×Day P=0.58
With CHOL
Without CHOL
Reduced glutathione (µmol/g protein)
0
20
40
60
80
100
120
140
-10
7
20
30
Day relative to calving

Fig. 3.4

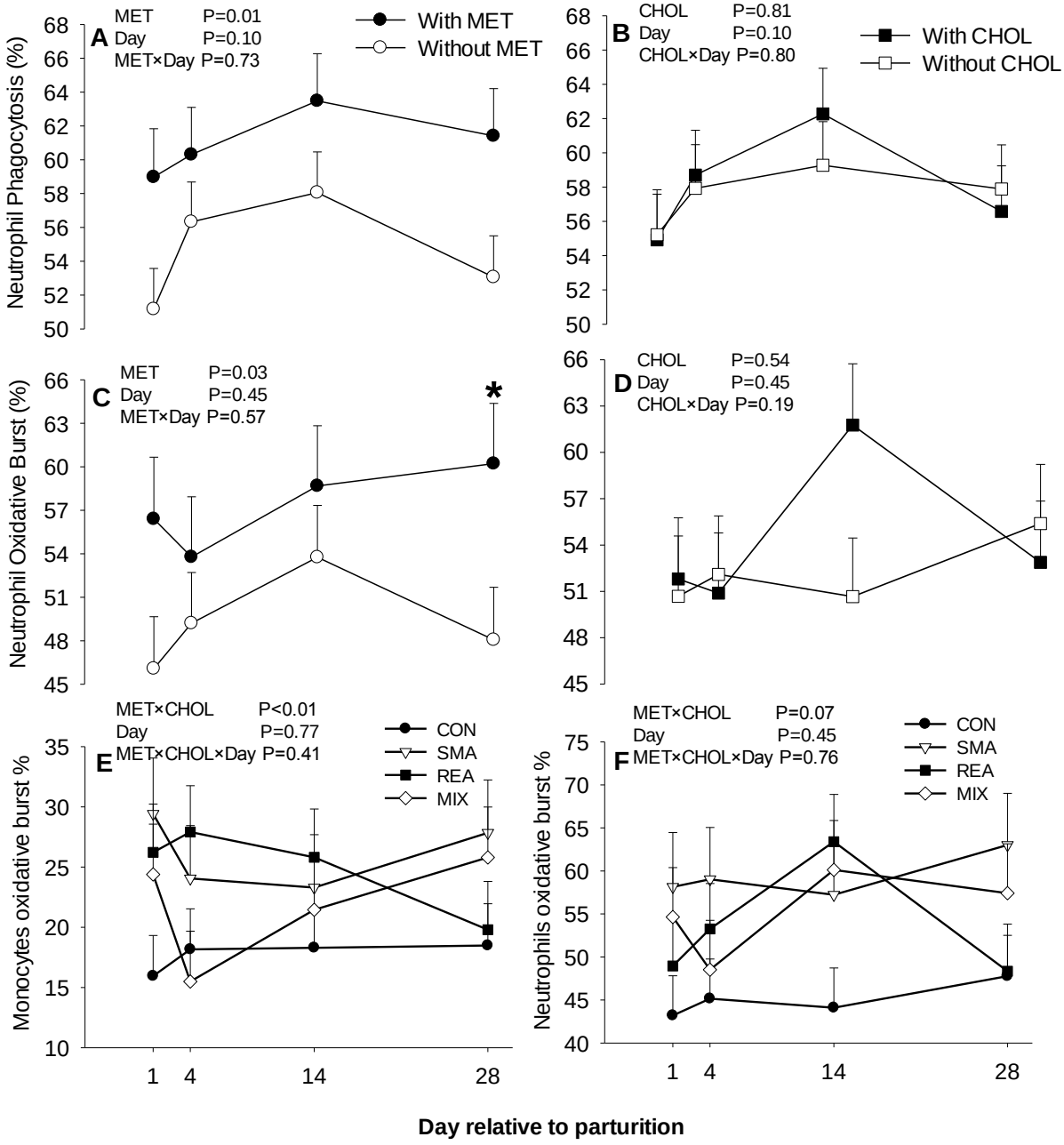
A
MET P=0.01
Day P=0.10
MET×Day P=0.73
With MET
Without MET
Neutrophil Phagocytosis (%)
B
CHOL P=0.81
Day P=0.10
CHOL×Day P=0.80
With CHOL
Without CHOL
C
MET P=0.03
Day P=0.45
MET×Day P=0.57
*
Neutrophil Oxidative Burst (%)
D
CHOL P=0.54
Day P=0.45
CHOL×Day P=0.19
E
MET×CHOL P<0.01
Day P=0.77
MET×CHOL×Day P=0.41
CON
SMA
REA
MIX
Monocytes oxidative burst %
F
MET×CHOL P=0.07
Day P=0.45
MET×CHOL×Day P=0.76
Neutrophils oxidative burst %
1 4 14 28
Day relative to parturition

Figure 3.1. Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected methionine (MET; Smartamine M, Adisseo NA) or rumen-protected choline (CHOL; ReaShure, Balchem Inc.) on blood cholesterol (A, B) and paraoxonase (C, D). Values are means, with standard errors represented by vertical bars. Only MET and CHOL main effect were shown since MET × CHOL interaction was not significant for any of the parameters.

Figure 3.2. Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected methionine (MET; Smartamine M, Adisseo NA) or rumen-protected choline (CHOL, ReaShure, Balchem Inc.) on blood haptoglobin (A, B), albumin (C, D), IL-1β (E, F), and IL-6 (G, H). Values are means, with standard errors represented by vertical bars. Only MET and CHOL main effect were shown since MET × CHOL interaction was not significant for any of the parameters.

Figure 3.3. Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected methionine (MET; Smartamine M, Adisseo NA) or rumen-protected choline (CHOL, ReaShure, Balchem Inc.) on liver total glutathione (A, B) and reduced glutathione (C, D). Values are means, with standard errors represented by vertical bars. Only MET and CHOL main effect were shown since MET × CHOL interaction was not significant for any of the parameters.

Figure 3.4. Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected methionine (MET; Smartamine M, Adisseo NA) or rumen-protected choline (CHOL; ReaShure, Balchem Inc.) on phagocytosis (A, B) and oxidative burst (C, D, E, F). CON = control; SMA = Smartamine M (0.08% of DMI); REA = ReaShure (60 g/d); MIX = SMA+REA; Values are means, with standard errors represented by vertical bars.

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CHAPTER 4

HEPATIC ACTIVITY AND TRANSCRIPTION OF BETAINE-HOMOCYSTEINE METHYLTRANSFERASE, METHIONINE SYNTHASE, AND CYSTATHIONINE SYNTHASE IN PERIPARTURIENT DAIRY COWS ARE ALTERED TO DIFFERENT EXTENTS BY SUPPLY OF METHIONINE AND CHOLINE

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INTRODUCTION

The increased nutrient demands around parturition to sustain fetal growth and lactation coupled with the gradual decrease in voluntary feed intake as parturition approaches induce a tremendous metabolic stress on dairy cows. Consequently, the ensuing negative nutrient balance after parturition contributes greatly to disease incidence and sub-optimal production efficiency (Goff and Horst, 1997, Zhou et al., 2016e), e.g. decreased supply of amino acids (AA)[6] reaching the small intestine due to reduced feed intake. The fact that supplementation of rumen-protected methionine (MET) was able to stimulate milk production despite a marked negative metabolizable protein balance (i.e. microbial and rumen-undegradable protein reaching the small intestine) underscored the critical role of post-ruminal essential AA availability (Zhou et al., Osorio et al., 2013). Although the AA profile of microbial protein is excellent for supporting lactation (Schwab et al.), AA profiles of rumen undegradable protein may be less than ideal (Brake et al., 2013). Hence, improvements in health and production performance might be achieved either by dietary supplementation of rumen-protected MET (Overton et al., 1996, Piepenbrink et al., 1996, Osorio et al., 2013, Osorio et al., 2014c) or other essential nutrients that could potentially spare methionine (e.g. rumen-protected choline (CHOL)) (Piepenbrink and Overton, 2003, Pinotti et al., 2003, Elek et al., 2008, Zom et al., 2011).

Due to extensive microbial degradation in the rumen, post-ruminal availability of MET and CHOL is limited (Sharma and Erdman, 1989, Girard and Matte, 2005). Inadequate MET and CHOL may be an important challenge for "transition" dairy cows, i.e. cows navigating the last 3-4 weeks prior to parturition through the first 3-4 weeks postpartum. Increasing the delivery of MET to the liver via supplementation is particularly important for transition cows not only because of its key role in milk protein synthesis but also for intra-hepatic VLDL synthesis, production of intracellular antioxidants (glutathione and taurine) (Atmaca, 2004), and provision of methyl groups to various compounds including choline (Finkelstein, 1990). Similarly, CHOL containing three methyl groups also acts an important source for the biosynthesis of methylated compounds. More importantly, CHOL is a precursor for VLDL assembly in the liver and, thus, has a crucial role in the export of triacylglycerol that helps prevent fatty liver (Pinotti et al., 2002). Therefore, both MET and CHOL are indispensable for the dairy cow's adaptation to metabolic challenges during the transition period.

It is noteworthy that the metabolism of MET and CHOL is closely interrelated (**Figure 4.1**). For instance, MET can be regenerated when homocysteine receives a methyl group from CHOL through betaine (Wong and Thompson, 1972b, Li and Vance, 2008a). Alternatively, MET donates its methyl group for *de novo* synthesis of CHOL through S-adenosyl methionine (SAM) (Vance et al., 1997). Because deficiency in one or more of the methyl group donors results in compensatory changes in the other (Wells and Remy, 1961, Selhub et al., 1991), supplementing MET can potentially spare a portion of choline needed and vice versa. However, as the quantity of MET utilized to generate the required CHOL (and vice versa) during the transition period has yet to be determined, it has been debatable whether supplementing MET or CHOL alone will be enough to satisfy the transition cow's requirement for both. More

importantly, the proportion of flux from MET to CHOL and vice versa utilized to alleviate metabolic stress in transition dairy cow remains unknown.

Considering that MET and CHOL are interrelated *in vivo*, it can be expected that enhancing post-ruminal delivery of MET and CHOL via rumen-protected supplementation should be able to exert similar beneficial effect on cow performance, health, and stress. However, results from our recent study indicated better production performance, immune function, and inflammatory status in cows supplemented with MET than CHOL (Zhou et al., Zhou et al.). Because the limitations in substrate availability as well as enzyme abundance and activity could potentially influence the rate by which methyl groups from CHOL can be used for the re-methylation of homocysteine to regenerate CHOL and vice versa, the hypothesis for the present study was that the increase, if any, in flux from CHOL to MET during the transition period through rumen-protected CHOL supplementation is diminished due to limitations in substrate availability and enzymes involved in MET and CHOL metabolism. Therefore, specific objectives were to characterize mRNA expression and activity of key enzymes involved in MET and CHOL metabolism to identify potential mechanism for the production and immuno-metabolic status differences observed previously (Zhou et al., Zhou et al.).

METHODS

Experimental design and treatments

All procedures for this study (protocol no. 13023) were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Illinois. Details of the experimental design have been reported previously (Zhou et al.). Briefly, the experiment was conducted as a randomized complete block 2×2 factorial design. The main effects include MET

(cows either supplemented with (+MET) or without (-MET) Smartamine M, Adisseo NA) and CHOL (cows either supplemented with (+CHOL) or without (-CHOL) ReaShure, Balchem Inc). Cows within each block were balanced for parity, previous lactation milk yield, and BCS before MET and/or CHOL treatments were applied starting at -21 d before expected parturition. A total of 40 cows were used. Treatments (MET×CHOL) were control (CON, n = 10), with no MET or CHOL supplementation; Smartamine (SMA, n = 10), with only MET supplementation at a rate of 0.08% of DM; Reashure (REA, n = 10), with only CHOL supplementation at 60 $g \cdot d^{-1}$; or Smartamine and Reashure (MIX, n = 10), with both MET and CHOL supplementation. Dosage of MET was based on a previous study (Osorio et al., 2013), whereas CHOL was supplemented following the manufacturer's recommendations. All cows received the same controlled-energy diet (far-off diet, 1.40 $Mcal \cdot kg^{-1}$ of dry matter) from -50 to -22 d before expected parturition, a moderate-energy diet (close-up diet, 1.52 $Mcal \cdot kg^{-1}$ of dry matter) from -21 d to expected parturition, and a higher-energy diet (lactation diet, 1.71 $Mcal \cdot kg^{-1}$ of dry matter) from parturition through 30 DIM. Diet composition is reported in **Supplemental Table 4.1**. The MET and CHOL were supplemented to each cow by placing on top of the mixed diet from -21 ± 2 to 30 DIM once daily at the AM feeding using approximately 50 g of ground corn as carrier for all treatments. Supplementation of SMA (0.08% DM of TMR offered) was calculated daily for each cow. Smartamine M was supplied as small beads containing a minimum of 75% DL-Met, physically protected by a pH sensitive coating, which is considered to have a Met bioavailability of 80% (Zhou et al.); therefore, per 10 g of SMA, the cows received 6 g of MET available for absorption. The REA supplement is reported to contain 28.8% choline chloride and is protected by microencapsulation. The product is considered to have CHOL bioavailability of 72% (Zhou et al.); therefore, per 60 g of REA, the cows received 12.4 g of choline chloride available for

absorption. To our knowledge, neither SMA nor REA have specific characteristics that may affect palatability of diets.

Animal management

Dry cows were housed in a ventilated enclosed barn during the dry period and fed individually once daily at 0630 h using an individual gate system (American Calan Inc., Northwood, NH). After parturition, cows were housed in a tie-stall barn and were fed a common lactation diet once daily. Feed offered was adjusted daily to achieve ~10% refusals. Nutrient composition and evaluation of the diets are reported in **Supplemental Table 4.2**.

Liver biopsy

Liver was sampled via puncture biopsy (Zhou et al.) from cows under local anesthesia at approximately 0800 h on d -10, 7, 20, and 30 d relative to parturition before they had access to feed and MET and/or CHOL supplements (fed after the morning milking). Liver was frozen immediately in liquid nitrogen and stored until analysis.

Hepatic MET and CHOL metabolites

Liver tissue L-methionine was measured at -10, 7, 20, and 30 d relative to parturition using a commercial kit (Cat. No. TRF-1-1-1006B; Mediomics, LLC; St. Louis, MO); hepatic phosphatidylcholine (PC) concentrations were determined using a commercial kit (Cat. No. K576-100; BioVision Inc., Mountain View, CA).

qRT-PCR

The qPCR was performed as describe previously (Osorio et al., 2014a). qPCR performance, relative mRNA abundance, and primer information are included in the Online Supporting Material.

Enzyme activity assays

BHMT activity was measured as described by Garrow (Garrow, 1996). MTR activity was determined based on Banerjee et al. (Banerjee et al., 1997). CBS activity was determined based on Lambert et al. (Lambert et al., 2002), except that the separation of ^{14}C-cystathionine from ^{14}C-Ser was done according to Taoka et al. (Taoka et al., 1998). Enzyme assay details are included in Online Supporting Material.

Statistical analysis

Data were analyzed using PROC MIXED of SAS (SAS Institute Inc., Cary, NC) appropriate for a 2 × 2 factorial design as described previously (Zhou et al.). Briefly, the statistical model included the main effects of MET, CHOL, day, and their interactions. The covariate of parity (2nd vs. 3rd lactation and above), was maintained in the model for all variables when significant ($P < 0.05$). Gene expression and enzyme activities were analyzed at various time points that were not equally spaced. Therefore, the first order ante-dependence covariance structure ANTE (1) were used for repeated measures. Least square means separation between time points was performed using the PDIFF statement. Data were reported as means ± pooled SEM. Statistical differences were declared significant at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

RESULTS

CHOL oxidation to Betaine

Main effects of MET and CHOL for genes involved in CHOL oxidation are presented in **Table 4.1**. Means for MET×CHOL are presented in **Supplemental Table 4.1**. Regardless of treatments, *BADH* and *CHDH* expression was lower ($P = 0.01$) postpartum (7 d) compared with prepartum. CHOL supplementation lead to an overall tendency towards greater ($P = 0.10$) expression of *CHDH*, which agreed with the greater (C×T $P = 0.01$) transcript expression at -10 d **(Supplemental Figure 4.1D)**. Overall *BADH* expression also was greater ($P = 0.01$) in CHOL cows compared with cows without CHOL supplementation. However, both the prepartum (-10 d, C×T $P < 0.01$) and postpartum (7 d, C×T $P < 0.01$) *BADH* expression was greater in response to CHOL supplementation **(Supplemental Figure 4.1B).** Similarly, an overall greater expression of *BADH* ($P = 0.03$) and a tendency for greater *CHDH* ($P = 0.07$) were detected in MET-supplemented cows.

Re-methylation of homocysteine to MET

Main effects of MET, CHOL, time, and interactions for mRNA abundance of enzymes involved homocysteine re-methylation to MET are presented in **Table 4.1** and **Table 4.2**, respectively. Means for MET×CHOL are presented in **Supplemental Table 4.1**. Compared with the prepartum level, overall *BHMT* expression and enzyme activity increased 0.7- and 1.7-fold respectively soon after parturition (7d, **Fig. 4.2**). Although there was a decrease in *MTR* expression ($P < 0.01$) after parturition the MTR activity was greater ($P < 0.01$) postpartum regardless of treatment. There was no overall effect ($P > 0.10$) of MET or CHOL for *BHMT* expression or BHMT enzyme activity. However, a tendency ($P = 0.06$) for greater *DMGDH* expression was detected in response to CHOL supplementation. Although no MET or CHOL main effects were detected ($P > 0.10$) for *MTR*, MTR activity was greater ($P = 0.04$) overall in response to MET supplementation. In contrast, CHOL supplementation led to lower overall ($P =$

0.05) MTR activity, mainly due to lower MTR activity at -10 d (C×T $P = 0.04$, **Figure 4.3D**). Although *SAHH* expression was lower ($P < 0.01$) postpartum regardless of treatment, MET cows had overall greater *SAHH* as a result of greater expression at -10 d and 7 d (M×T $P < 0.05$). Despite the fact that *SAHH* expression was also greater in CHOL cows on -10 d and 7 d (C×T $P < 0.05$), there was no significant ($P = 0.32$) CHOL main effect (**Supplemental Figure 4.3F)**.

PC synthesis from MET

Main effects of MET, CHOL, time, and interactions for genes involved in PC synthesis from MET are presented in **Table 4.1**. Means for MET×CHOL are presented in **Supplemental Table 4.1**. Expression of *MAT1A* and *MAT2A* both increased ($P < 0.05$) after parturition. Unlike the abrupt increase in *MAT1A* and *MAT2A* expression soon after parturition, *PEMT* expression increased gradually, reaching statistical significance ($P < 0.05$) at 20 d postpartum. Although *MAT2A* expression was not affected ($P > 0.10$) by MET or CHOL, greater ($P = 0.02$) expression and a tendency ($P = 0.06$) for greater *MAT1A* expression were observed in MET and CHOL cows, respectively. However, greater ($P = 0.05$) expression of *PEMT* was only observed in response to MET supplementation mainly due to greater (M×T $P < 0.05$) expression of *PEMT* at -10 d and 7 d in MET cows (**Supplemental Figure 4.3C)**.

CDP-choline pathway

Main effects of MET, CHOL, time, and interactions for genes in CDP-choline pathway are presented in **Table 4.1**. Means for MET×CHOL are presented in **Supplemental Table 4.1**. Overall expression of *CHKA* was greater ($P = 0.05$) in CHOL compared with MET cows. Although *CHKB* expression increased soon after parturition ($P = 0.03$), there was no overall effect of MET and CHOL ($P > 0.10$). Both *PCYT1A* and *PCYT1B* expression was greater ($P <$

0.05) in response to CHOL supplementation, mainly due to greater *PCYT1A* and *PCYT1B* expression on -10 d and 7 d in CHOL cows (C×T $P < 0.05$, **Supplemental Figure 4.2D & Figure 4.2F**). Similarly, a tendency ($P = 0.06$) for greater *PCYT1B* expression and greater *PCYT1A* expression were observed in MET cows. However, *CEPT1* expression ($P > 0.10$) was not affected by MET or CHOL supplementation.

Transsulfuration pathway

Main effects of MET, CHOL, time, and interactions for genes involved in the transsulfuration pathway are presented in **Table 4.1**. Means for MET×CHOL are presented in **Supplemental Table 4.1**. Both *CBS* expression and enzyme activity increased ($P < 0.05$) after parturition (**Figure 4.4**). However, CBS enzyme activity was not affected ($P > 0.10$) by MET or CHOL supplementation (**Table 4.2**). At the transcription level, *CBS* expression was overall greater in MET but not CHOL cows. Despite the increase ($P < 0.05$) in *CDO1*, *CSAD*, and *GSR* expression soon after parturition, there was no overall effect ($P > 0.10$) of MET or CHOL. Similarly, although a decrease in expression of *GCLC* and *GSS* was detected postpartum, their expression was not affected ($P > 0.10$) by MET or CHOL supplementation.

Hepatic L-MET and PC concentrations

Main effects of MET, CHOL, time, and interactions for L-MET and PC are presented in **Table 4.2**. Means for MET×CHOL are presented in **Supplemental Table 4.2**. As expected, there was a decrease ($P < 0.01$) in hepatic L-MET concentration postpartum, with a nadir at 7 d. Regardless, MET and CHOL supplementation did not affect ($P > 0.10$) hepatic L-MET concentration. In contrast, hepatic PC concentration was constant ($P = 0.91$) during the transition period. There

was an overall lower hepatic PC concentration in response to MET (P = 0.05) and CHOL (P = 0.03).

DISCUSSION

Synthesis of PC from MET and CHOL

As a nutritionally-important methyl donor, rumen-protected MET supplementation during the transition period is expected to promote overall production and health of dairy cows not only by serving as a limiting AA for milk synthesis, but also by promoting synthesis of important methylated compounds including PC. Apart from the well-characterized demand for PC during VLDL synthesis as a way to prevent fatty liver, transition dairy cows also are expected to have high PC requirements owing to the high PC output in milk (Pinotti et al., 2002). In fact, levels of CHOL secretion into milk are maintained even at the cost of depleting liver reserves of choline and its metabolites (Pinotti et al., 2002). Therefore, to achieve the normal high rates of milk synthesis after parturition without compromising liver VLDL export, MET supplementation is expected to increase synthesis of PC in transition dairy cows.

In vivo synthesis of PC from MET can be achieved by sequential methylation of PE, acquiring three methyl groups from the global methyl donor SAM. *MAT1A* mediates the first step in the hepatic synthesis of PC from MET to generate SAM (Kotb et al., 1997, Martinov et al., 2010a). Subsequently, *PEMT* controls the committed step in PC synthesis by sequentially transferring three methyl groups from SAM to PE (Zeisel, 1992). The observed increase in *MAT1A* and *PEMT* in MET cows support the thesis that MET supplementation led to greater availability of SAM to synthesize PC.

In vivo synthesis of PC from CHOL can be achieved through the CDP-choline pathway. Choline kinases catalyzes the first committed step in PC synthesis from CHOL (Fagone and Jackowski, 2013). Due to the micromolar affinity of choline kinase for CHOL, the CHOL taken up into cells is readily trapped as phosphocholine (Fagone and Jackowski, 2013). Therefore, the greater *CHKA* in CHOL cows indicates an enriched phosphocholine pool during the transition period. Various studies have detected intracellular accumulation of phosphocholine due to limited intracellular amount of PCYT enzymes which convert phosphocholine to CDP-choline (Clement and Kent, 1999, Sugimoto et al., 2008). In fact, it is the rate-limiting step in PC synthesis and thereby determines the biosynthetic flux from CHOL to PC (Weinhold and Feldman, 1992). The observed increase in *PCYT1A* and *PCYT1B* in CHOL cows support the thesis that CHOL supplementation led to greater availability of CDP-choline to synthesize PC. However, expression of *CEPT1*, which catalyzes the final step of PC synthesis via the Kennedy pathway, was not affected by CHOL supplementation. Whether such lack of change at the transcription level leads to accumulation of CDP-choline in hepatocytes of CHOL cows during the transition period remains unknown, but it is possible that biosynthetic flux from CHOL to PC can increase without elevating *CEPT1* mRNA expression. For instance, greater CEPT1 activity could also lead to increased flux from CHOL to PC.

Synthesis of MET from CHOL

The essential role of MET as liming AA for milk protein synthesis during the transition period has been well-established (Schwab et al., 1992, Pisulewski et al., 1996). The fact that circulating MET concentration decreased markedly through parturition and was not restored to prepartum levels until 28 d postpartum underscored the deficiency of MET during this period (Zhou et al., 2016e). Previous studies have observed benefits of supplementing MET in terms of

production, health, and stress status (Overton et al., 1996, Osorio et al., 2013, Osorio et al., 2014c). Considering that MET can be re-synthesized in vivo from CHOL-derived methyl groups and homocysteine, similar benefits during the transition period would be expected if a comparable amount of MET is generated from CHOL.

CHOL could promote MET synthesis *in vivo* by supplying methyl groups for homocysteine re-methylation. Although re-methylation of homocysteine to MET can be achieved either through BHMT in the MET cycle or MTR from the THF cycle, betaine and methyl-THF, rather than CHOL, are the direct methyl donors for these reactions. To generate methyl groups for MET synthesis, there is an irreversible two-step oxidation process from CHOL to betaine. CHDH mediates the first and committed step in which choline is first oxidized to betaine aldehyde. Subsequently, betaine aldehyde is further oxidized to betaine by BADH (Slow and Garrow, 2006).

The tendency towards greater *CHDH* expression together with greater *BADH* expression in CHOL cows indicated there might have been greater betaine generated in response to CHOL supplementation. However, no differences in mRNA expression or activity of BHMT were observed in CHOL cows. Therefore, despite potentially increased betaine synthesis, MET synthesis might not have been increased in CHOL cows. In fact, the lack of change in *MTR* expression and the lower MTR activity further support the idea of decreased MET synthesis in those cows. It is noteworthy that CHOL relies on homocysteine to provide the sulfur required for MET synthesis. Because of its key role in the re-methylation and transsulfuration pathway, homocysteine could have been a limiting substrate for MET synthesis, especially considering the greater demands after parturition for intracellular sulfur-containing antioxidants such as GSH and taurine.

Despite the fact that transcription and activity data both indicate that CHOL supplementation did not seem to have promoted MET synthesis during the transition period, it is noteworthy that flux from CHOL to MET could still have increased in CHOL cows. For instance, it is possible that hepatic MTR and BHMT activity are not limiting but rather the availability of homocysteine and their respective methyl donors control the flux of homocysteine to MET. Hence, no change in expression or activity in these enzymes would be necessary to support the increase in flux. In fact, the greater *MAT1A* in CHOL cows could be an indication of increased MET synthesis from CHOL because high hepatic MET concentrations lead to increased hepatic SAM concentrations (Corrales et al., 2002). Similarly, the tendency towards greater *DMGDH* in CHOL cows also could be due to increased dimethylglycine generated from MET synthesis. Therefore, a better question would be whether the increase in flux from CHOL to MET, if any, could provide sufficient MET to achieve comparable benefits as MET supplementation. Previous results in these same cows revealed that CHOL supplementation failed to induce equal benefits as MET in terms of production performance, health, and stress status (Zhou et al., Zhou et al.). Therefore, it seems that the difference observed between MET and CHOL supplementation during the transition period was due, at least in part, to insufficient synthesis of MET from CHOL.

Synthesis of taurine and glutathione through the transsulfuration pathway

Around parturition, production of reactive oxygen metabolites (ROM) is greatly enhanced (Reynolds et al., 2003). Although cellular antioxidants are capable of neutralizing ROM, depletion of such antioxidants leads to ROM accumulation giving rise to substantial tissue damage and inflammation (Sordillo and Aitken, 2009). Both glutathione (GSH) and taurine are potent sulfur-containing antioxidants that can be synthesized through the transsulfuration

pathway. The committed step in the transsulfuration pathway is regulated by CBS, which converts homocysteine to cystathionine. Regardless of treatment, the overall increase in both *CBS* expression and enzyme activity soon after calving was highly suggestive of a greater flux in the transsulfuration pathway postpartum.

Biomarker analysis from these same cows revealed a reduced oxidative stress status in MET cows (Zhou et al.). In agreement with this, the greater *SAHH* in MET cows indicated the potential for greater homocysteine being generated in response to MET supplementation. Additionally, the lower MTR activity as well as greater *CBS* expression observed in MET cows suggested an increase in flux through the transsulfuration pathway instead of re-methylation to MET during the transition period. Although expression of genes in the steps leading to GSH and taurine synthesis was not affected by MET or CHOL, the fact that hepatic total and reduced GSH concentrations (Zhou et al.) as well as plasma taurine (Zhou et al., 2016f) were greater in response to MET supplementation indicated that synthesis of GSH and taurine might not be regulated at the transcriptional level.

Limitations

The dynamic nature of one carbon metabolism makes it less ideal to use substrate concentrations as indicators of flux. For instance, one would expect hepatic MET concentration to increase in response to MET supplementation. However, increased flux via the transulfuration pathway for antioxidant synthesis could be responsible for the similar hepatic MET concentrations observed among treatments. Similarly, the decrease in hepatic PC concentration in response to MET or CHOL supplementation could have been the result of increased liver export.

Although enzyme activity can serve as an indicator of alterations in flux in response to treatments, it is important to note that changes in flux may exist without inducing transcriptional or activity level alterations (Fell, 2005). In fact, as a saturated concentration of substrate was used during incubations in the present study, it is reasonable to assume that changes in enzyme activities observed reflect corresponding changes in enzyme protein content plus potential post-translational changes such as phosphorylation, acetylation, and methylation. Therefore, a stable isotope flux study is required to confirm our speculation about alterations in flux.

CONCLUSIONS

Our findings provide evidence that MET and CHOL supplementation during the periparturient period can affect hepatic mRNA expression and enzyme activity of enzymes controlling flux between MET and CHOL as well as the transsulfuration pathway. The greater expression of *PEMT* and *CBS* indicates an increase in synthesis of PC and antioxidants in MET cows. In CHOL cows, the lack of change in *BHMT* and *MTR*, together with unchanged BHMT and a lower MS enzyme activity indicated that CHOL supplementation did not increase MET synthesis as postulated. In summary, the overall better health and production performance reported previously in MET cows was due, at least in part, to increased PC and antioxidant synthesis. Failure to generate a comparable amount of MET from CHOL could be one of the reasons preventing CHOL-fed cows from achieving comparable performance and health benefits during the transition period.

TABLES AND FIGURES

Table 4.1. Hepatic gene expression in periparturient cows that were unsupplemented or were supplemented with rumen-protected MET and CHOL for 51 d[1].

	MET		CHOL			Day				*P*-value[2]				
Gene	-MET	+MET	-CHOL	+CHOL	SEM	-10	7	20	30	MET[3]	CHOL[4]	Time	M×T[5]	C×T[6]
MET and CHOL metabolism														
BADH	0.95	1.05	0.93	1.07	0.05	1.06^{a}	0.98^{b}	0.97^{b}	0.99^{b}	0.03	0.01	0.01	0.17	0.05
BHMT	0.91	0.95	0.90	0.96	0.07	0.76^{c}	1.31^{a}	0.91^{b}	0.82^{bc}	0.58	0.41	<0.01	0.73	0.80
BHMT2	0.20	0.39	0.28	0.27	0.13	0.19^{b}	0.32^{a}	0.31^{a}	0.31^{a}	0.12	0.89	<0.01	0.02	0.12
CHDH[*]	0.32	0.43	0.33	0.42	0.06	0.45^{a}	0.33^{b}	0.37^{a}	0.36^{a}	0.07	0.10	<0.01	0.03	0.01
DMGDH	1.06	0.95	0.90	1.11	0.11	0.73^{c}	1.24^{a}	1.09^{b}	1.03^{bc}	0.33	0.06	<0.01	0.36	0.01
MAT1A[*]	0.27	0.38	0.28	0.37	0.05	0.26^{b}	0.32^{a}	0.33^{a}	0.37^{a}	0.02	0.06	<0.01	<0.01	<0.01
MAT2A	1.09	1.01	1.07	1.03	0.06	0.84^{c}	1.19^{a}	1.20^{a}	1.01^{b}	0.24	0.44	<0.01	0.49	0.34
MTHFR	1.15	1.09	1.13	1.11	0.05	1.26^{a}	1.16^{b}	1.04^{c}	1.04^{c}	0.29	0.79	<0.01	0.50	0.12
MTR	1.00	0.98	1.02	0.96	0.05	1.09^{a}	0.94^{b}	0.99^{b}	0.95^{b}	0.61	0.23	<0.01	0.05	0.54
PEMT[*]	0.58	0.74	0.65	0.66	0.08	0.59^{c}	0.63^{bc}	0.68^{ab}	0.73^{a}	0.05	0.90	<0.01	0.01	0.18
SAHH[*]	0.79	0.93	0.84	0.88	0.05	0.92^{a}	0.80^{c}	0.84^{bc}	0.88^{ab}	0.01	0.32	<0.01	<0.01	<0.01
SARDH	1.16	1.19	1.11	1.24	0.12	1.20^{ab}	0.96^{c}	1.21^{ab}	1.35^{a}	0.80	0.26	<0.01	0.48	0.46
Transsulfuration pathway														
CBS[*]	1.02	1.16	1.06	1.12	0.07	0.97^{b}	1.10^{a}	1.18^{a}	1.11^{a}	0.04	0.36	<0.01	0.07	0.11
CDO1	1.04	1.06	1.03	1.07	0.07	1.02	1.09	1.06	1.03	0.86	0.53	0.23	0.06	0.48
CSAD	0.91	0.81	0.95	0.78	0.11	0.64^{b}	0.95^{a}	0.97^{a}	0.92^{a}	0.34	0.12	0.01	0.40	0.87
GCLC	0.97	0.94	0.96	0.95	0.06	1.27^{a}	0.82^{b}	0.87^{b}	0.91^{b}	0.51	0.97	<0.01	0.85	0.34
GSR	0.99	0.99	1.00	0.98	0.04	0.88^{c}	1.09^{a}	1.01^{b}	0.97^{b}	0.99	0.62	<0.01	0.95	0.38
GSS[*]	1.12	1.15	1.11	1.16	0.04	1.19^{a}	1.07^{b}	1.15^{a}	1.14^{a}	0.40	0.22	<0.01	0.19	0.32
CDP-choline pathway														
CEPT1	1.11	1.01	1.14	0.99	0.11	1.23	1.05	1.08	0.91	0.41	0.17	0.23	0.37	0.36
CHKA[*]	1.13	1.15	1.06	1.22	0.08	1.13	1.14	1.15	1.14	0.88	0.05	0.98	0.48	0.29
CHKB	0.96	1.02	0.97	1.00	0.03	0.94^{b}	1.02^{a}	1.02^{a}	0.98^{ab}	0.11	0.31	0.03	0.69	0.13
PCYT1A	1.17	1.33	1.19	1.30	0.04	1.18^{c}	1.31^{a}	1.28^{ab}	1.22^{bc}	<0.01	0.01	0.02	0.36	<0.01
PCYT1B[*]	1.24	1.39	1.24	1.39	0.08	1.35	1.27	1.32	1.33	0.06	0.05	0.56	0.66	0.01

[*]Significant ($P < 0.05$) parity effect observed.[1]Values are means and pooled SEM, n = 20/group. Labeled means in a row without a common letter differ, $P < 0.05$. [2]MET × CHOL interaction was included in Supplemental Table 4.1. [3]Overall effect of MET supplementation. [4]Overall effect of CHOL supplementation. [5]Interaction of MET × time. [6]Interaction of CHOL × time. *BADH*, Betaine

Table 4.1 (Continued)

aldehyde dehydrogenase; *BHMT*, Betaine homocysteine methyltransferase; *BHMT2*, Betaine homocysteine methyltransferase 2; *CBS*, Cystathionine β-synthase; *CDO*, cysteine dioxygenase; *CEPT1*, Choline/ethanolamine phosphotransferase 1; *CHDH*, Choline dehydrogenase; *CHKA*, choline kinase A; *CHKB*, choline kinase B; *CSAD*, Cysteine sulfinic acid decarboxylase; *DMGDH*, Dimethylglycine dehydrogenase; *GCLC*, Glutamate-cysteine ligase, catalytic subunit; *GSR*, Glutathione reductase; *GSS*, Glutathione synthase; *MAT1A*, Methionine adenosyltransferase 1A; *MAT2A*, Methionine adenosyltransferase 2A; *MTHFR*, Methylenetetrahydrofolate reductase; *MTR*, 5-methyltetrahydrofolate-homocysteine methyltransferase; *PCYT1A*, Phosphate cytidylyltransferase 1A; *PCYT1B*, Phosphate cytidylyltransferase 1B; *PEMT*, Phosphatidylethanolamine methyltransferase; *SAHH*, S-adenosylhomocysteine hydrolase; *SARDH*, Sarcosine dehydrogenase.

Table 4.2. Hepatic one carbon metabolism enzyme activity and metabolites in periparturient cows that were unsupplemented or were supplemented with rumen-protected MET and CHOL for 51 d[1].

	MET		CHOL			Day				*P*-value[2]				
Parameters	-MET	+MET	-CHOL	+CHOL	SEM	-10	7	20	30	MET[3]	CHOL[4]	Time	M×T[4]	C×T[5]
Enzyme activity (nmol product·h^{-1}·mg $protein^{-1}$)														
BHMT	22.8	22.8	22.7	23.0	1.9	12.3^{d}	29.1^{a}	27.0^{ab}	23.0^{c}	0.99	0.88	<0.01	<0.01	0.99
MTR	29.7	23.4	29.6	23.5	2.9	22.8^{b}	28.7^{a}	26.5^{ab}	28.2^{a}	0.04	0.05	0.04	0.68	0.04
CBS	48.1	49.6	49.4	48.3	2.5	39.1^{d}	47.1^{a}	59.3^{c}	50.1^{ab}	0.57	0.67	<0.01	0.79	0.84
Metabolites														
L-methionine (μmol·g^{-1} protein)	52.4	53.4	55.3	50.5	4.0	60.0^{a}	47.9^{b}	52.0^{b}	51.8^{b}	0.81	0.25	< 0.01	0.87	0.27
Phosphatidylcholine*(mg·g^{-1})	10.2	9.4	10.2	9.3	0.4	9.8	9.8	9.6	9.8	0.05	0.03	0.91	0.76	0.88

*Significant ($P < 0.05$) parity effect observed. [1]Values are means and pooled SEM, n = 20/group. Labeled means in a row without a common letter differ, $P < 0.05$. [2]MET × CHOL interaction is included in Supplemental Table 4.2. [3]Main effect of MET supplementation (SMA+MIX compared with CON+REA). [4]Main effect of CHOL supplementation (REA+MIX compared with CON+SMA). [5]Interaction of MET × time. [6]Interaction of CHOL × time. BHMT, Betaine homocysteine methyltransferase; CBS, Cystathionine β-synthase; MTR, 5-methyltetrahydrofolate-homocysteine methyltransferase. Labeled means in a row without a common letter differ, $P < 0.05$.

Fig. 4.1

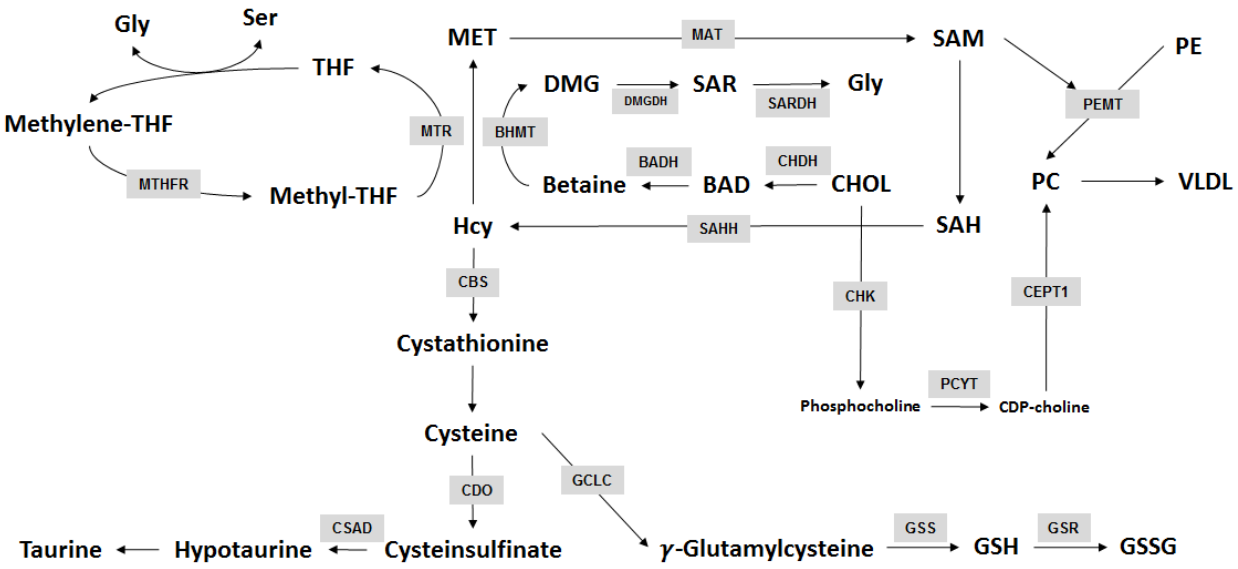

Gly
Ser
THF
Methylene-THF
MTHFR
Methyl-THF
MTR
BHMT
MET
MAT
SAM
PE
PEMT
DMG
DMGDH
SAR
SARDH
Gly
Betaine
BADH
BAD
CHDH
CHOL
PC
VLDL
Hcy
SAHH
SAH
CBS
CHK
CEPT1
Cystathionine
Phosphocholine
PCYT
CDP-choline
Cysteine
GCLC
CDO
CSAD
Taurine
Hypotaurine
Cysteinsulfinate
γ-Glutamylcysteine
GSS
GSH
GSR
GSSG

Fig. 4.2

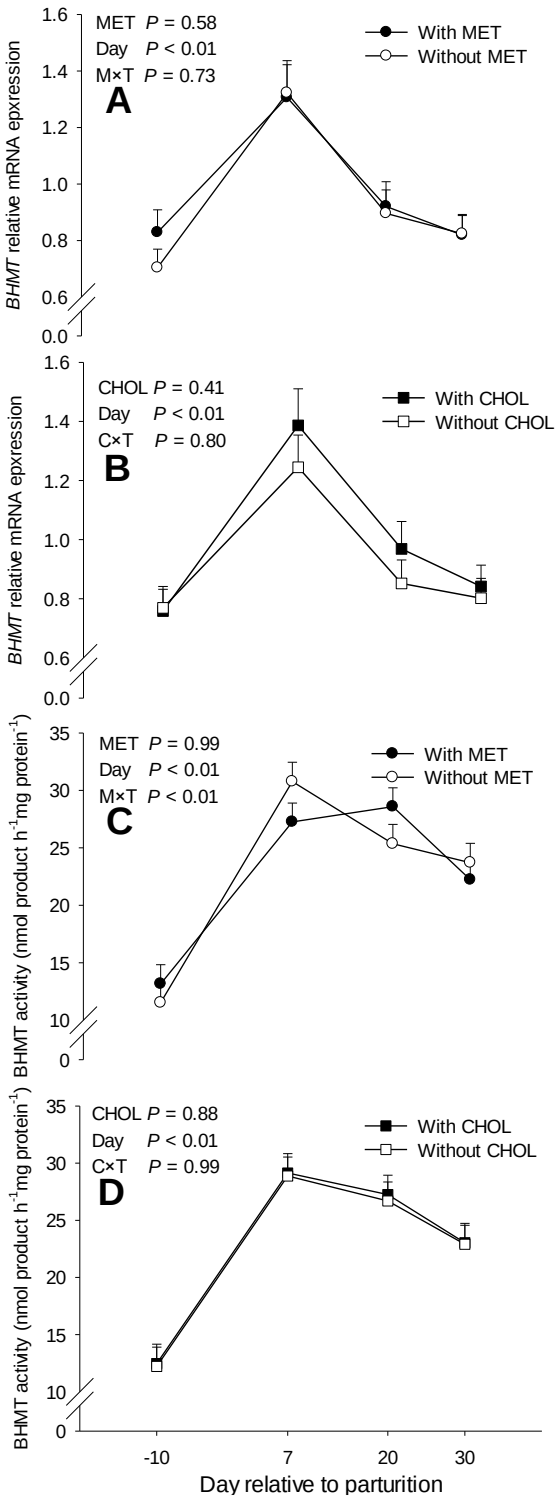

MET P = 0.58
Day P < 0.01
M×T P = 0.73
A
With MET
Without MET
BHMT relative mRNA epxression
CHOL P = 0.41
Day P < 0.01
C×T P = 0.80
B
With CHOL
Without CHOL
BHMT relative mRNA epxression
MET P = 0.99
Day P < 0.01
M×T P < 0.01
C
With MET
Without MET
BHMT activity (nmol product h-1mg protein-1)
CHOL P = 0.88
Day P < 0.01
C×T P = 0.99
D
With CHOL
Without CHOL
BHMT activity (nmol product h-1mg protein-1)
-10
7
20
30
Day relative to parturition

Fig. 4.3

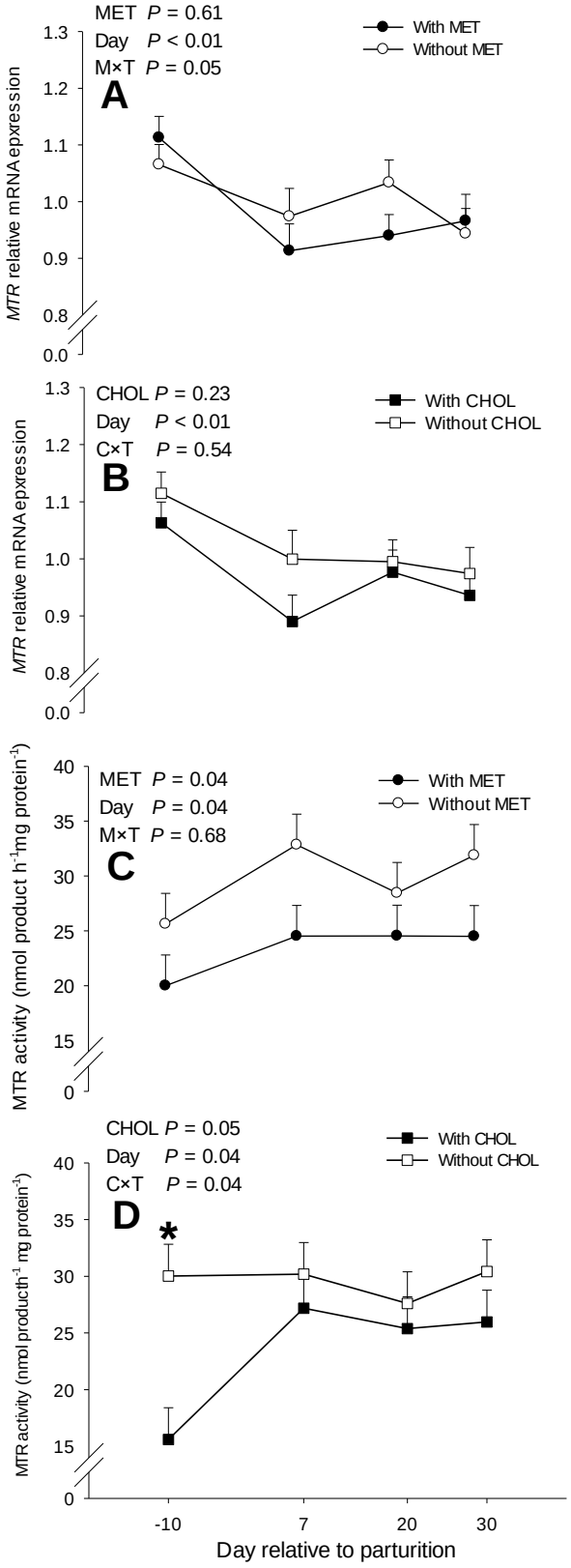

MET P = 0.61
Day P < 0.01
M×T P = 0.05
With MET
Without MET
A
MTR relative mRNA epxression
1.3
1.2
1.1
1.0
0.9
0.8
0.0
CHOL P = 0.23
Day P < 0.01
C×T P = 0.54
With CHOL
Without CHOL
B
MTR relative mRNA epxression
MET P = 0.04
Day P = 0.04
M×T P = 0.68
With MET
Without MET
C
MTR activity (nmol product h-1mg protein-1)
40
35
30
25
20
15
0
CHOL P = 0.05
Day P = 0.04
C×T P = 0.04
With CHOL
Without CHOL
D
*
MTR activity (nmol producth-1 mg protein-1)
-10
7
20
30
Day relative to parturition

Fig. 4.4

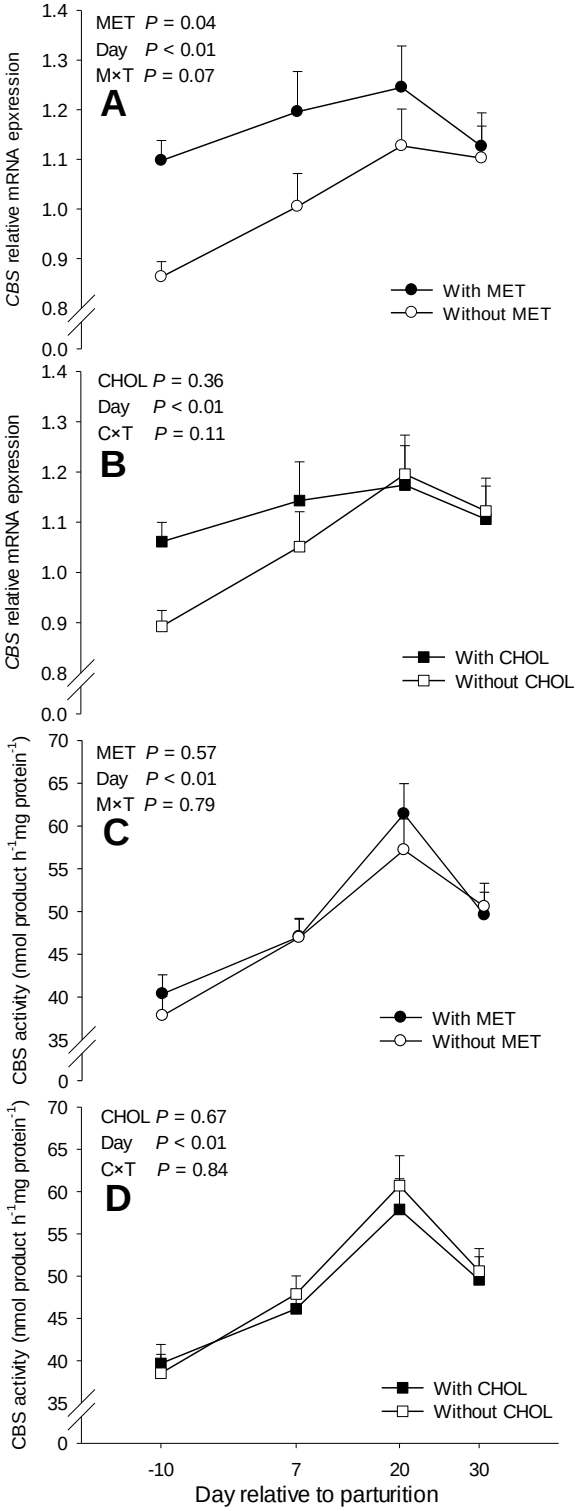

MET P = 0.04
Day P < 0.01
M×T P = 0.07
A
CBS relative mRNA epxression
With MET
Without MET
CHOL P = 0.36
Day P < 0.01
C×T P = 0.11
B
CBS relative mRNA epxression
With CHOL
Without CHOL
MET P = 0.57
Day P < 0.01
M×T P = 0.79
C
CBS activity (nmol product h-1mg protein-1)
With MET
Without MET
CHOL P = 0.67
Day P < 0.01
C×T P = 0.84
D
CBS activity (nmol product h-1mg protein-1)
With CHOL
Without CHOL
-10
7
20
30
Day relative to parturition

Figure 4.1. Methionine and choline metabolism. BAD, Betaine aldehyde; BADH, Betaine aldehyde dehydrogenase; BHMT, Betaine homocysteine methyltransferase; CBS, Cystathionine β-synthase; CDO, cysteine dioxygenase; CEPT1, Choline/ethanolamine phosphotransferase 1; CHDH, Choline dehydrogenase; CHK, choline kinase; CHOL, Choline; CSAD, Cysteine sulfinic acid decarboxylase; DMG, Dimethylglycine; DMGDH, Dimethylglycine dehydrogenase; GCLC, Glutamate-cysteine ligase, catalytic subunit; GSH, Glutathione; GSR, Glutathione reductase; GSS, Glutathione synthase; GSSG, Glutathione disulfide; Hcy, Homocysteine; MAT, Methionine adenosyltransferase; MET, Methionine; MTHFR, Methylenetetrahydrofolate reductase; MTR, 5-methyltetrahydrofolate-homocysteine methyltransferase; PC, Phosphatidylcholine; PCYT, Phosphate cytidylyltransferase; PE, Phosphatidylethanolamine; PEMT, Phosphatidylethanolamine methyltransferase; SAH, S-adenosylhomocysteine; SAHH, S-adenosylhomocysteine hydrolase; SAM, S-adenosylmethionine; SAR, Sarcosine; SARDH, Sarcosine dehydrogenase; THF, Tetrahydrofolate.

Figure 4.2. Hepatic mRNA expression and activity of BHMT in periparturient cows that were unsupplemented or were supplemented with rumen-protected MET or CHOL for 51 d. Hepatic *BHMT* mRNA expression (A, B) and hepatic BHMT activity (C, D) are presented as means and SEM (n = 20). BHMT, Betaine homocysteine methyltransferase.

Figure 4.3. Hepatic mRNA expression and activity of MTR in periparturient cows that were unsupplemented or were supplemented with rumen-protected MET or CHOL for 51 d. Hepatic *MTR* mRNA expression (A, B) and hepatic MTR activity (C, D) are presented as means and SEM, (n = 20). *Means at that time differ, $P < 0.05$. MTR, 5-methyltetrahydrofolate-homocysteine methyltransferase.

Figure 4.4. Hepatic mRNA expression and activity of CBS in periparturient cows that were unsupplemented or were supplemented with rumen-protected MET or CHOL for 51 d. Hepatic *CBS* mRNA expression (A, B), and hepatic CBS activity (C, D) are presented as means and SEMs, (n = 20). CBS, Cystathionine β-synthase.

Supplemental Table 4.1. Ingredient composition of diets fed to periparturient cows that were unsupplemented or were supplemented with Smartamine M or Reashure.

		Close-up				Lactation			
Ingredients (g·kg^{-1} DM)	Far off	CON[1]	SMA	REA	MIX	CON	SMA	REA	MIX
Alfalfa silage	120	83.4	83.4	83.4	83.4	50.7	50.7	50.7	50.7
Alfalfa hay	-	42.9	42.9	42.9	42.9	29.8	29.8	29.8	29.8
Corn silage	330	364	364	364	364	334	334	334	334
Wheat straw	360	156	156	156	156	29.8	29.8	29.8	29.8
Cottonseed	-	-	-	-	-	35.8	35.8	35.8	35.8
Wet brewers grains	-	42.9	42.9	42.9	42.9	90.9	90.9	90.9	90.9
Ground shelled corn	40	129	129	129	129	239	239	239	239
Soy hulls	20	42.9	42.9	42.9	42.9	41.8	41.8	41.8	41.8
Soybean meal, 48% CP	79.2	25.7	25.7	25.7	25.7	23.9	23.9	23.9	23.9
Expeller soybean meal[2]	-	25.7	25.7	25.7	25.7	59.7	59.7	59.7	59.7
Soychlor[3]	1.5	38.6	38.6	38.6	38.6	-	-	-	-
Blood meal, 85% CP	10	-	-	-	-	-	-	-	-
ProVAAl AADvantage[4]	-	8.6	8.6	8.6	8.6	15.0	15.0	15.0	15.0
Urea	4.5	3.0	3.0	3.0	3.0	1.8	1.8	1.8	1.8
Rumen-inert fat[5]	-	-	-	-	-	10.2	10.2	10.2	10.2
Limestone	13	12.9	12.9	12.9	12.9	13.1	13.1	13.1	13.1
Salt	3.2	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Dicalcium phosphate	1.2	1.8	1.8	1.8	1.8	3.0	3.0	3.0	3.0
Magnesium oxide	2.1	0.8	0.8	0.8	0.8	1.2	1.2	1.2	1.2
Magnesium sulfate	9.1	9.9	9.9	9.9	9.9	-	-	-	-
Sodium bicarbonate	-	-	-	-	-	7.9	7.9	7.9	7.9
Potassium carbonate	-	-	-	-	-	3.0	3.0	3.0	3.0
Calcium sulfate	-	-	-	-	-	1.2	1.2	1.2	1.2
Mineral vitamin mix[6]	2	1.7	1.7	1.7	1.7	1.8	1.8	1.8	1.8
Vitamin A[7]	0.15	-	-	-	-	-	-	-	-
Vitamin D[8]	0.25	-	-	-	-	-	-	-	-
Vitamin E[9]	3.8	3.9	3.9	3.9	3.9	-	-	-	-
Biotin	-	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
SMA[10]	-	-	0.8	-	0.8	-	0.8	-	0.8
REA[11]	-	-	-	60 g	60 g	-	-	60 g	60 g

[1]CON = control; SMA = rumen-protected methionine; REA = rumen-protected choline; MIX = SMA+REA; MET = SMA+MIX; CHOL = REA+MIX. [2]SoyPLUS (West Central Soy, Ralston, IA). [3]By West Central Soy. [4]Blood meal-based product (Perdue AgriBusiness,Salisbury, MD). [5]Energy Booster 100 (Milk Specialties Global, Eden Prairie, MN). [6]Composition in g·kg^{-1} DM: Mg 50, S 100, K 75, Fe 20, Zn 30, Mn 30, Cu 5, I 0.25, Co 0.04, Se 0.15. Vitamins in mg·kg^{-1} DM: retinol 660, cholecaciferol 16.5, alpha-tocopherol 5159. [7]Contained 9 g·kg^{-1} retinol. [8]Contained 0.125 g·kg^{-1} cholecaciferol. [9]Contained 29.5g·kg^{-1} alpha-tocopherol. [10]Rumen protected methionine (Smartamine, Adisseo NA) was supplemented on top of the diet given to

Supplemental Table 4.1 (Continued)
the animals.[11]Rumen protected choline (Reashure, Balchem Inc. NH) was supplemented to cows at 60 g·cow^{-1}d $^{-1}$ to each cow.

Supplemental Table 4.2. Nutrient composition and evaluation (NRC, 2001) of prepartal and postpartal diets fed to periparturient cows that were unsupplemented or were supplemented with Smartamine M or Reashure for 51 d [1].

		Close-up[2]				Lactation			
Chemical component	Far off	CON	SMA	REA	MIX	CON	SMA	REA	MIX
CP (g·kg^{-1} DM)	143	146	146	144	144	172	173	172	173
RDP (g·kg^{-1} DM)	102	93	91	91	91	97	96	96	96
RUP (g·kg^{-1} DM)	41	53	55	53	54	75	77	75	76
NDF (g·kg^{-1} DM)	511	420	419	417	417	340	339	337	336
ADF (g·kg^{-1} DM)	354	283	283	282	281	214	214	213	212
NFC (g·kg^{-1} DM)	250	322	322	322	322	378	378	377	377
EE (g·kg^{-1} DM)	22	33	33	36	36	57	57	59	59
RDP supplied (g·d^{-1})	1248	1228	1377	1218	1340	1773	1911	1780	1918
RDP balance (g·d^{-1})	138	-50	-70	-61	-75	-130	-142	-138	-145
RUP supplied (g·d^{-1})	507	677	826	703	793	1369	1518	1390	1519
RUP required (g·d^{-1})	92	177	163	214	172	1922	2283	1813	2174
RUP balance (g·d^{-1})	415	500	663	489	621	-553	-765	-423	-655
MP supplied (g·d^{-1})	1058	1251	1494	1297	1446	2222	2435	2245	2440
MP balance (g·d^{-1})	324	406	541	397	506	-473	-656	-362	-562
Lys:Met	3.89:1	3.62:1	2.81:1	3.62:1	2.78:1	3.54:1	2.70:1	3.53:1	2.70:1
Lys (% of MP)	7.24	6.74	6.62	6.73	6.65	6.30	6.24	6.29	6.24
MP-Lys (g)	77	104	122	108	119	170	184	171	184
Met (% of MP)	1.86	1.86	2.36	1.86	2.39	1.78	2.31	1.78	2.31
MP-Met (g)	20	29	42	30	41	48	65	49	65

[1]The NRC (2001) evaluation of diets was based on final averaged pre and postpartum DMI, production data, and feed analysis. [2]CON = control; SMA = rumen protected methionine (Smartamine, Adissseo NA; 0.08% of DMI); REA = rumen protected choline (Reashure, Balchem Inc.NH; 60 g·d^{-1}); MIX = SMA+REA. ADF, acid detergent fiber; CP, crude protein; EE, ether extract; Lys, lysine; Met, methionine; NDF, neutral detergent fiber; NFC, Non-fat carbohydrate; MP, metabolizable protein; RDP, rumen degradable protein; RUP, rumen undegradable protein.

Supplemental Table 4.3. Hepatic gene expression in transition cows that were unsupplemented or were supplemented with Smartamine M and ReaShure for 51 d.

	Treatments					*P*-value	
Parameter	CON[1]	SMA	REA	MIX	SEM	M×C[2]	M×C×T[3]
MET and CHOL metabolism							
BADH	0.90	0.97	1.00	1.14	0.05	0.58	0.40
BHMT	0.85	0.95	0.97	0.95	0.07	0.47	0.02
BHMT2	0.13[b]	0.63[a]	0.30[b]	0.24[b]	0.16	0.04	0.48
CHDH[*]	0.24[b]	0.45[a]	0.43[a]	0.41[a]	0.06	0.03	<0.01
DMGDH	0.93	0.88	1.20	1.03	0.11	0.66	0.28
MAT1A[*]	0.19[b]	0.40[a]	0.38[a]	0.36[a]	0.05	0.01	0.17
MAT2A	1.13	1.01	1.04	1.01	0.06	0.45	0.16
MTHFR	1.13	1.13	1.17	1.06	0.05	0.31	0.79
MTR	1.00	1.04	1.00	0.93	0.05	0.24	0.51
PEMT[*]	0.50[b]	0.84[a]	0.66[b]	0.65[b]	0.08	0.04	0.36
SAHH[*]	0.71[b]	0.98[a]	0.89[a]	0.88[a]	0.05	<0.01	0.02
SARDH	1.09	1.12	1.22	1.25	0.12	0.98	0.33
Transsulfuration pathway							
CBS[*]	0.98	1.15	1.06	1.18	0.07	0.69	0.52
CDO1	1.03	1.02	1.06	1.09	0.07	0.75	0.13
CSAD	1.04	0.87	0.80	0.75	0.11	0.40	0.30
GCLC	0.94	0.97	1.01	0.91	0.06	0.85	0.68
GSR	1.01	0.98	0.96	0.99	0.04	0.95	0.13
GSS[*]	1.09	1.14	1.16	1.16	0.04	0.19	0.85
CDP-choline pathway							
CEPT1	1.14	1.14	1.07	0.90	0.11	0.42	0.47
CHKA[*]	1.07	1.06	1.20	1.24	0.08	0.75	0.70
CHKB	0.93	1.02	1.00	1.01	0.03	0.27	0.01
PCYT1A	1.01[c]	1.40[a]	1.35[ab]	1.26[b]	0.04	<0.01	0.21
PCYT1B[*]	1.01[c]	1.52[a]	1.52[a]	1.27[b]	0.08	<0.01	0.69

[*]Significant ($P < 0.05$) parity effect observed. [1]CON = control; SMA = Smartamine M (0.08% of DMI); REA = ReaShure (60 g·d^{-1}); MIX = SMA+REA. Values are means and pooled SEM, n = 10/group. [2]Interaction of MET × CHOL. [3]Interaction of MET × CHOL × Time. Labeled means in a row without a common letter differ, $P < 0.05$. *BADH*, Betaine aldehyde dehydrogenase; *BHMT*, Betaine homocysteine methyltransferase; *BHMT2*, Betaine homocysteine methyltransferase 2; *CBS*, Cystathionine β-synthase; *CDO*, cysteine dioxygenase; *CEPT1*, Choline/ethanolamine phosphotransferase 1; *CHDH*, Choline dehydrogenase; *CHKA*, choline kinase A; *CHKB*, choline kinase B; *CSAD*, Cysteine sulfinic acid decarboxylase; *DMGDH*, Dimethylglycine dehydrogenase; *GCLC*, Glutamate-cysteine ligase, catalytic subunit; *GSR*, Glutathione reductase; *GSS*, Glutathione synthase; *MAT1A*, Methionine adenosyltransferase 1A; *MAT2A*, Methionine adenosyltransferase 2A; *MTHFR*, Methylenetetrahydrofolate reductase; *MTR*, 5-methyltetrahydrofolate-homocysteine methyltransferase; *PCYT1A*, Phosphate cytidylyltransferase 1A; *PCYT1B*, Phosphate cytidylyltransferase 1B; *PEMT*,

Table 4.3 (Continued)

Phosphatidylethanolamine methyltransferase; *SAHH*, S-adenosylhomocysteine hydrolase; *SARDH*, Sarcosine dehydrogenase.

Supplemental Table 4.4. Hepatic one carbon metabolism enzyme activity and metabolites in transition cows unsupplemented or supplemented with Smartamine M and ReaShure.

	Treatments					*P*-value	
Parameter	CON[1]	SMA	REA	MIX	SEM	M×C[2]	M×C×T[3]
Enzyme activity (nmol product·h^{-1}·mg $protein^{-1}$)							
BHMT	21.3	24.0	24.4	21.6	1.9	0.16	0.73
MTR	32.3	26.8	27.1	19.9	2.9	0.76	0.63
CBS	50.1	48.8	46.2	50.4	2.5	0.29	0.12
Metabolites							
L-methionine (μmol·g^{-1} protein)	57.3	53.3	47.5	53.6	4.0	0.22	0.01
Phosphatidylcholine* (mg·g^{-1})	10.5	9.9	9.9	8.8	0.4	0.50	0.72

[1]CON = control; SMA = Smartamine M (0.08% of DMI); REA = ReaShure (60 g/d); MIX = SMA+REA. Values are means and pooled SEM, n = 10/group. [2]Interaction of MET × CHOL. [3]Interaction of MET × CHOL × Time. BHMT, Betaine homocysteine methyltransferase; CBS, Cystathionine β-synthase; MTR, 5-methyltetrahydrofolate-homocysteine methyltransferase.

Supplemental Table 4.5. qPCR performance and relative mRNA abundance of genes measured in periparturient cow liver.

Gene	Median Ct[1]	Median ΔCt[2]	Slope[3]	(R^2)[4]	Efficiency[5]	Relative mRNA abundance[6]
MET and CHOL metabolism						
BADH	22.957	2.175	-3.217	0.994	2.046	0.211
BHMT	20.303	-0.458	-3.359	0.995	1.985	1.369
BHMT2	25.524	4.835	-3.086	0.997	2.109	0.027
CBS	22.747	2.003	-3.400	0.999	1.968	0.258
CHDH	26.784	6.014	-3.223	0.972	2.043	0.014
DMGDH	17.986	-2.721	-3.315	0.981	2.003	6.620
MAT1A	21.475	0.681	-3.445	0.993	1.951	0.634
MAT2A	20.796	0.107	-3.132	0.994	2.086	0.924
MTHFR	22.488	1.74	-3.714	0.998	1.859	0.340
MTR	24.848	4.103	-3.171	0.997	2.067	0.051
PEMT	23.121	2.370	-3.286	0.969	2.015	0.190
SAHH	19.239	-1.524	-3.264	0.984	2.025	2.930
SARDH	19.942	-0.755	-3.096	0.997	2.104	1.753
Transsulfuration pathway						
CDO1	19.811	-0.961	-3.280	0.987	2.018	1.963
CSAD	23.701	3.011	-3.354	0.988	1.987	0.127
GCLC	22.902	2.132	-3.320	0.985	2.001	0.228
GSR	23.603	2.871	-3.454	0.976	1.948	0.148
GSS	24.964	4.236	-3.323	0.996	1.999	0.053
CDP-choline pathway						
CEPT1	20.860	0.151	-3.387	0.996	1.974	0.902
CHKA	20.953	0.232	-3.369	0.983	1.981	0.853
CHKB	20.364	-0.368	-3.457	0.993	1.947	1.278
PCYT1A	22.044	1.361	-3.844	0.991	1.820	0.443
PCYT1B	25.856	5.122	-2.821	0.996	2.262	0.015

[1]The median is calculated considering all time points and all cows. [2]The median of ΔCt is calculated as [Ct gene – geometrical mean of Ct internal controls] for each time point and each cow. [3]Slope of the standard curve. [4]R^2 stands for the coefficient of determination of the standard curve. [5]Efficiency is calculated as [$10^{(-1 / \text{Slope})}$]. [6]Relative mRNA abundance = 1/ Efficiency $^{\text{Median } \Delta Ct}$. *BADH*, Betaine aldehyde dehydrogenase; *BHMT*, Betaine homocysteine methyltransferase; *BHMT2*, Betaine homocysteine methyltransferase 2; *CBS*, Cystathionine β-synthase; *CDO*, cysteine dioxygenase; *CEPT1*, Choline/ethanolamine phosphotransferase 1; *CHDH*, Choline dehydrogenase; *CHKA*, choline kinase A; *CHKB*, choline kinase B; *CSAD*, Cysteine sulfinic acid decarboxylase; *DMGDH*, Dimethylglycine dehydrogenase; *GCLC*, Glutamate-cysteine ligase, catalytic subunit; *GSR*, Glutathione reductase; *GSS*, Glutathione synthase; *MAT1A*, Methionine adenosyltransferase 1A; *MAT2A*, Methionine adenosyltransferase 2A; *MTHFR*, Methylenetetrahydrofolate reductase; *MTR*, 5-methyltetrahydrofolate-homocysteine methyltransferase; *PCYT1A*, Phosphate cytidylyltransferase 1A; *PCYT1B*, Phosphate

Table 4.5 (Continued)

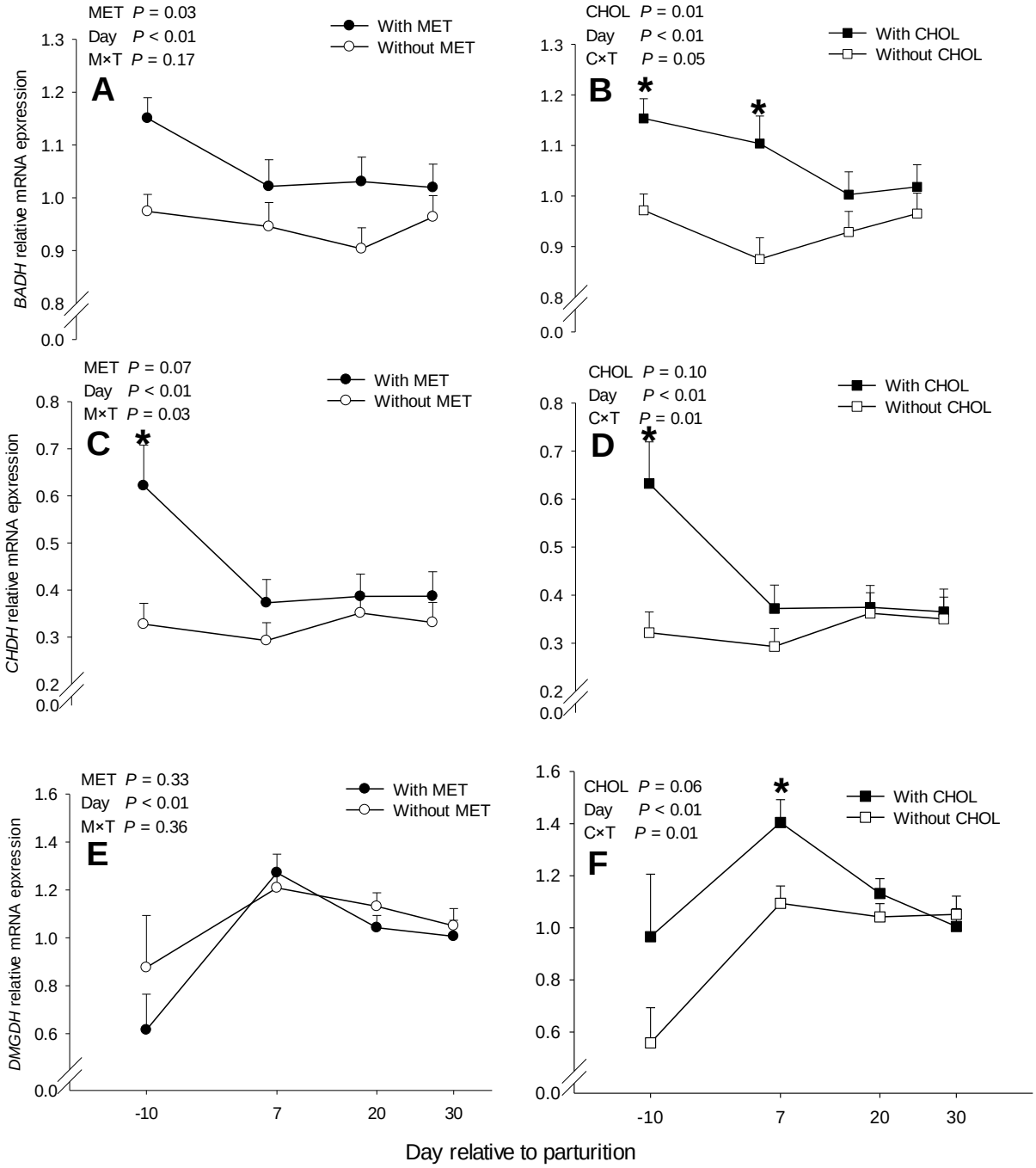
cytidylyltransferase 1B; *PEMT*, Phosphatidylethanolamine methyltransferase; *SAHH*, S-adenosylhomocysteine hydrolase; *SARDH*, Sarcosine dehydrogenase.

Supplemental Table 4.6. Accession number, gene symbol, and forward and reverse primer sequences of genes analyzed in periparturient cow liver.

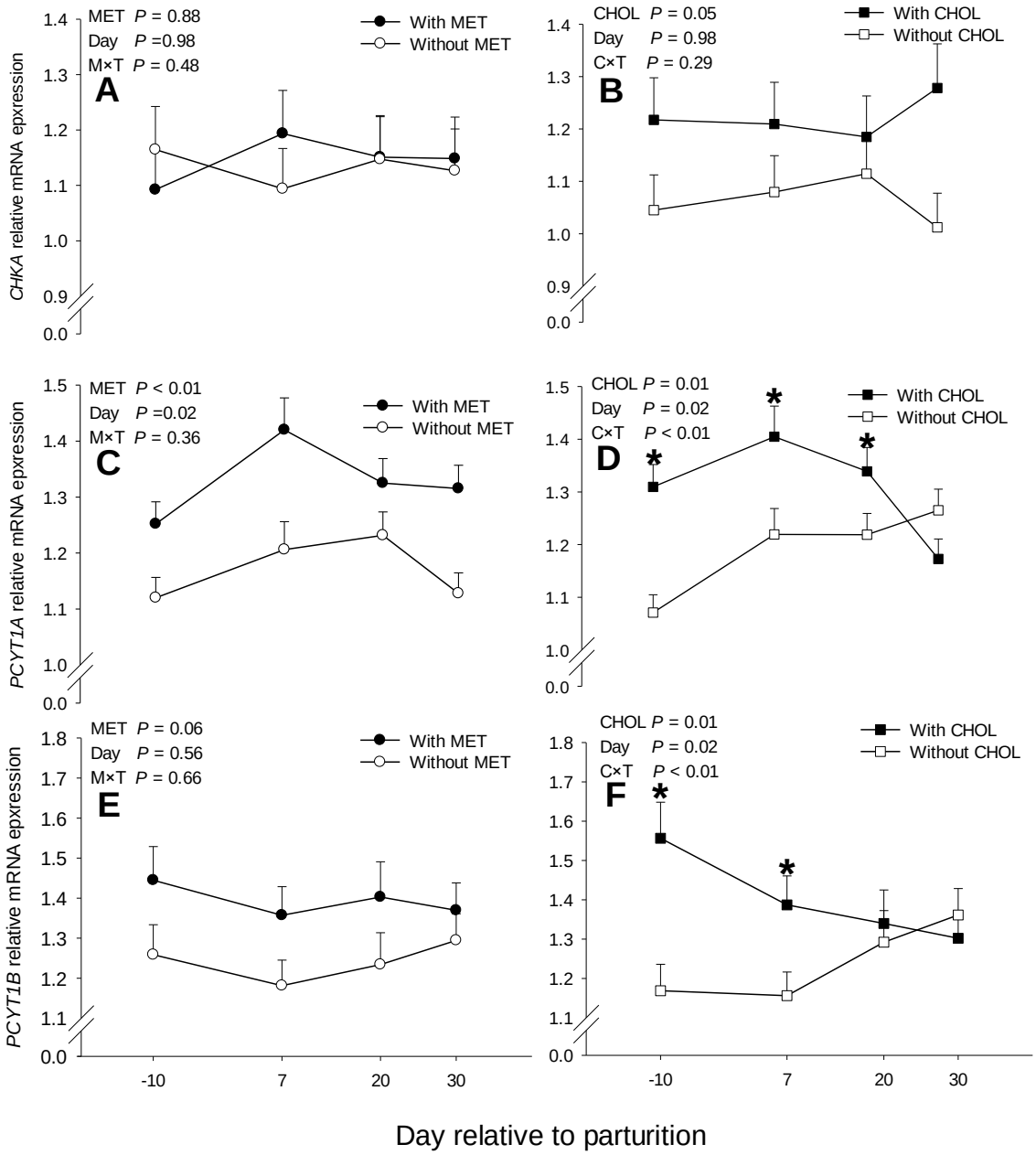
Gene	Accession number	Forward primer	Reverse Primer
BADH	NM_001045969	CAC CTA CTG TCC TGC TAA	ATC ACC AAT CTG TCT CAC TAC
BHMT	NM_001011679	GCT CTC CTC GTC CAT CCT CAT	CCG TTC TAG GAT GCC CTT CTT
BHMT2	XM_003586514	ACA CCA CAA GGA TGA AGT	CAG GCT CTC CAG ATT CTT
CBS	NM_001102000	GCC ACC ACC TCT GTC AAA TTC	GGA CAG AAA GCA GAG TGG TAA CTG
CDO1	NM_001034465	ATG GAA GCC TAT GAG AGC AA	TTC AGA AAG CAG TGG GAG TC
CEPT1	NM_001193130	GGA TAG CCC TGG TCT TCT CTT	ATT CGC CTC AAT ATG TTC AGA TTC TT
CHDH	NM_001205564	AAA CTG AGA AGT GCC AAC	ACG GAA GTC TTT AAT GTC A
CHKA	XM_002699402	GCA CAG GTT CCT CAG TTA	GCC ATC CAG CAG TAA GAT
CHKB	XM_010805827	CCA AGA GGA GCA GAG GAA	GTA GAA CTG GAA CCG AGA CT
CSAD	XM_005206242	GCC TCA ACA CCA GCC AGT A	GTT TCT TCA GCA CCT CCT CCT
DMGDH	NM_001205545	TCT GGT ATG ATG GCA AGG T	GCT TCT GGT TGG TTC TGT T
GCLC	NM_001083674	TAC GAT CAG TTG GCT ACC	CCG AGT TCT ATC ATC TAC AGA
GSR	NM_001114190	GAG AAC GCT GGC ATT GAG	AGC AGG CAG TCA ACA TCT
GSS	NM_001015630	CGA GTG ATC CAA TGC ATT TCA	ATG TCC CAC GTG CTT GTT CAT
MAT1A	NM_001046497	CAA GGG CTT TGA CTT TAA	CCG ACA TCC TCT TCA TTT
MAT2A	NM_001101131	AAT CTA TCA TCT ACA GCC AAG TG	CCA ACG AGC AGC ATA AGC
MTHFR	NM_001011685	TTC AAC TAT GCT ACG GAC TT	CCT TCA GGT GCT TCA GAT
MTR	NM_001030298	ATA CCG CCA ATG CCA AGG	ATG AGA CAC GCT GAT GAC AA
PCYT1A	XM_005201384	GAT GAG GTG GTG AGG AAT	CGA GTG ATG ATG TCT GAT G
PCYT1B	NM_001193051	ACT GTC GCT ATG TGG ATG AA	GCC TTC TGT TCT CTG TGT TG
PEMT	NM_182989	AAT TAC CAA GAG CAG AGG	CAG ATT CCA GAT TCC AGA T
SAHH	NM_001034315	CAA TGT CAA TGA CTC TGT	CTT GAT GCC ATC TAT GAG
SARDH	NM_001193041	GAG GAG GTG TCA GAT GAG	GCA GAC TGT GGA CTT AAT C

BADH, Betaine aldehyde dehydrogenase; *BHMT*, Betaine homocysteine methyltransferase; *BHMT2*, Betaine homocysteine methyltransferase 2; *CBS*, Cystathionine β-synthase; *CDO*, cysteine dioxygenase; *CEPT1*, Choline/ethanolamine phosphotransferase 1; *CHDH*, Choline dehydrogenase; *CHKA*, choline kinase A; *CHKB*, choline kinase B; *CSAD*, Cysteine sulfinic acid decarboxylase; *DMGDH*, Dimethylglycine dehydrogenase; *GCLC*, Glutamate-cysteine ligase, catalytic subunit; *GSR*, Glutathione reductase; *GSS*, Glutathione synthase; *MAT1A*, Methionine adenosyltransferase 1A; *MAT2A*, Methionine adenosyltransferase 2A; MTHFR, Methylenetetrahydrofolate reductase; *MTR*, 5-methyltetrahydrofolate-homocysteine methyltransferase; *PCYT1A*, Phosphate cytidylyltransferase 1A; *PCYT1B*, Phosphate cytidylyltransferase 1B; *PEMT*, Phosphatidylethanolamine methyltransferase; *SAHH*, S-adenosylhomocysteine hydrolase; *SARDH*, Sarcosine dehydrogenase.

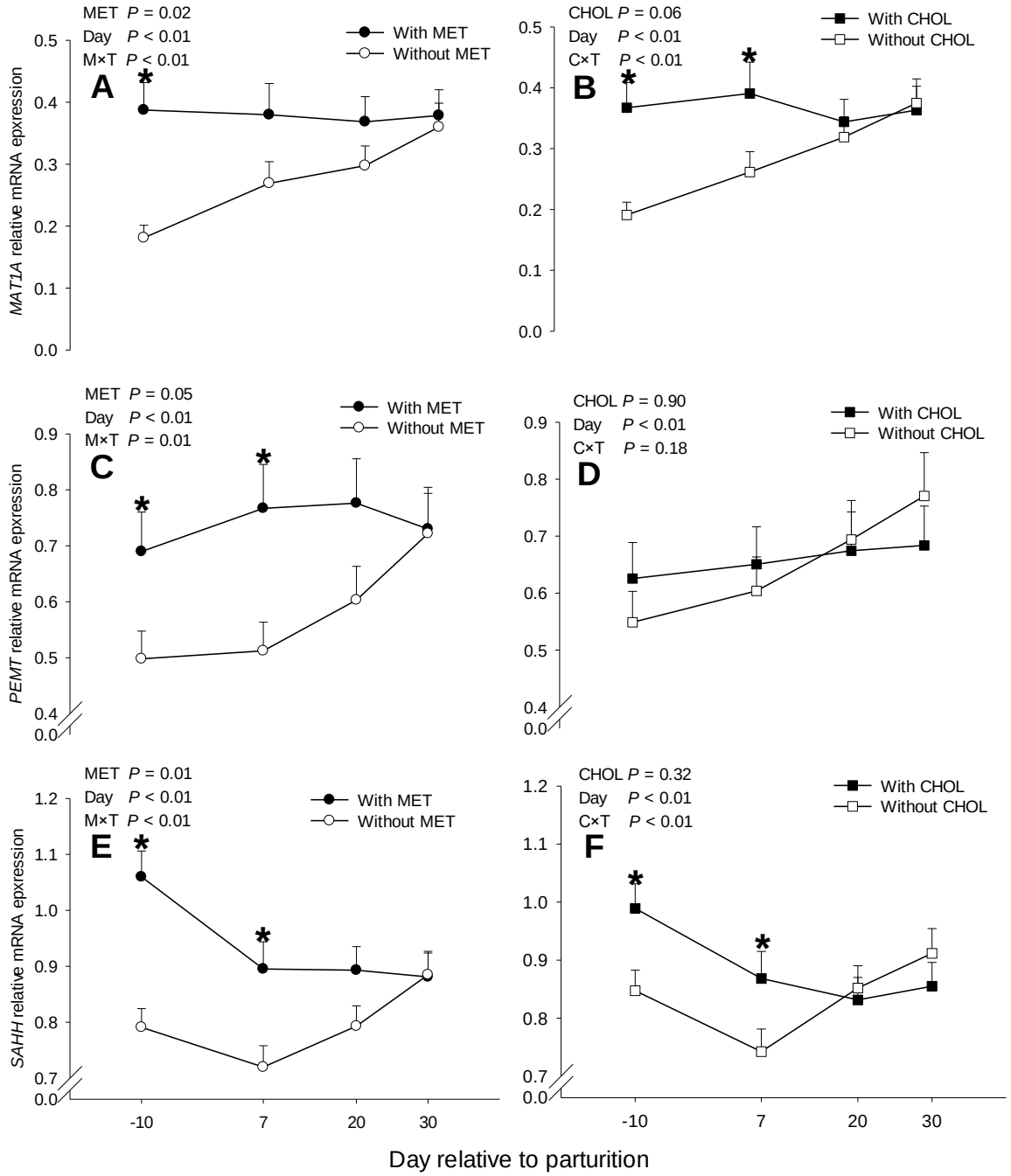
Supplemental Figure 4.1. Hepatic mRNA expression of *BADH* (A, B), *CHDH* (C, D), and *DMGDH* (E, F) in cows that were unsupplemented or were supplemented with rumen-protected MET or CHOL for 51 d. Values are means + SEMs (n = 20). *Means at that time differ, $P < 0.05$. *BADH*, Betaine aldehyde dehydrogenase; *CHDH*, Choline dehydrogenase; *DMGDH*, Dimethylglycine dehydrogenase.



Supplemental Figure 4.2. Hepatic mRNA expression of *CHKA* (A, B), *PCYT1A* (C, D), and *PCYT1B* (E, F) in periparturient cows that were unsupplemented or were supplemented with rumen-protected MET or CHOL for 51 d. Values are means + SEMs (n = 20). *Means at that time differ, $P < 0.05$. *CHKA*, choline kinase A; *PCYT1A*, Phosphate cytidylyltransferase 1A; *PCYT1B*, Phosphate cytidylyltransferase 1B.



Supplemental Figure 4.3. Hepatic mRNA expression of *MAT1A* (A, B), *PEMT* (C, D), and *SAHH* (E, F) in periparturient cows that were unsupplemented or were supplemented with rumen-protected MET or CHOL for 51 d. Values are means + SEMs (n = 20). *Means at that time differ, $P < 0.05$. *MAT1A*, Methionine adenosyltransferase 1A; *PEMT*, Phosphatidylethanolamine methyltransferase; *SAHH*, S-adenosylhomocysteine hydrolase.



Supplemental Methods

RNA extraction and mRNA expression calculation

Liver RNA extraction. Liver tissue (30-50 mg) was homogenized in a TissueLyser II (Qiagen GmbH) for 2 x 2 min bursts at 30 Htz. Total RNA and DNA were extracted using a Qiagen AllPrep DNA/RNA mini kit (Qiagen GmbH) as per manufacturer's instructions and RNA was treated with DNase (Ambion DNA-free kit; Ambion Inc., Austin, TX).

mRNA expression calculation. Efficiency of qPCR amplification for each gene was calculated using the standard curve method [Efficiency = $10^{(-1/\text{slope})}$]. The mRNA expression of the measured genes was calculated as previously reported (Bionaz and Loor, 2008), using the inverse of PCR efficiency raised to ΔCt (gene abundance = $1/E^{\Delta Ct}$, where ΔCt = Ct of tested gene - geometric mean Ct of 3 internal control genes). Overall mRNA expression for each gene among all samples of the same liver tissue was calculated using the median ΔCt, and overall percentage of each mRNA as a proportion of measured genes was computed from the equation: 100 × mRNA abundance of each individual gene / sum of mRNA abundance of all the genes investigated.

qRT-PCR. The qPCR was performed in a MicroAmp Optical 384-Well Reaction Plate (Cat. #4309849, Applied Biosystems). Within each well, 4 μL of diluted cDNA combined with 6 μL of mixture composed of 5 μL 1×SYBR Green master mix (Cat. #4309155, Applied Biosystems), 0.4 μL each of 10 μM forward and reverse primers, and 0.2 μL of DNase/RNase-free water were added. Three replicates and a 6-point standard curve plus the nontemplate control (NTC) were run for each sample to test the relative expression level. qPCR was conducted in ABI Prism 7900 HT SDS instrument (Applied Biosystems) following the conditions below: 2 min at 50°C, 10

min at 95°C, 40 cycles of 15 s at 95°C (denaturation), and 1 min at 60°C (annealing + extension). The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures to 95°C for 15 s, then 65 °C for 15 s. The threshold cycle (Ct) data were analyzed and transformed using the standard curve with the 7900 HT Sequence Detection System Software (version 2.2.1, Applied Biosystems, CA). Data were then normalized with the geometric mean of the three Internal Control Genes (ICG).

Enzyme activity assays

***BHMT activity assay*.** All liver samples were weighed and homogenized immediately in 4 vol of 50 mM potassium phosphate buffer (pH 7.4) containing 2 mM EDTA and 1 mM mercaptoethanol. The homogenates was centrifuged at 4 °C for 45 min at 18,000 × g. The supernatant fraction was used for enzyme assay and protein concentration determination via the Bradford assay. DL-homocysteine was prepared fresh daily from homocysteine thiolactone hydrochloride (15.4 mg) in 400 µL 2 N sodium hydroxide and 600 µL saturated mono potassium phosphate. The standard assay contained 5 mM DL-Hcy, 2 mM betaine (0.05-0.1 µCi), and 50 mM Tris-HCl (pH = 7.5). The final reaction volume was 0.5 mL. Reaction tubes were capped with rubber stoppers and kept in ice water until the assay was started by transferring the tubes into a 37 °C water bath. After 1 h incubation, the reaction was terminated by adding 2.5 mL of ice-cold water into each tube. The samples were applied to a Dowex 1-X4 (OH-; 200-400 mesh) column (0.6 × 3.75 cm), and the unreacted betaine was washed off the column with 15 mL water. Dimethylglycine and methionine were eluted into scintillation vials with 3 mL of 1.5 N HCl. Seventeen milliliters of scintillation mixture (Ecolume, ICN) were added to each vial and counted. Blanks contained all of the reaction components except enzyme, and their values were subtracted from the sample values. All samples were assayed in duplicate.

MTR activity assay. All liver samples were weighed and homogenized immediately in 4 vol of 50 mM potassium phosphate buffer (pH 7.5) containing 2 mM EDTA. The homogenates was centrifuged at 4 °C for 45 min at 18,000 × g. The supernatant fraction was used for enzyme assay and protein concentration determination by the Bradford assay. The standard assay contained 1 M potassium phosphate buffer (pH 7.2), 0.5 M DTT, 3.8 mM S-adenosylmethionine, 100 mM DL-Hcy, 5 mM hydroxocobalamin, and 5 mM methyl-tetrahydrofolate (0.1 μCi). The final reaction vol was 0.5 mL. Reaction tubes were capped with rubber stoppers and kept in ice water until the assay was started by transferring the tubes into a 37 °C water bath. After 0.5 h incubation, the reaction was terminated by adding 0.5 mL of ice-cold water into each tube. The samples were applied to an AG 1X8 column, and elute was collected also from additional 2 mL of ice-cold water applied to the column. Seventeen milliliters of scintillation mixture (Ecolume, ICN) were added to each vial and counted. Blanks contained all of the reaction components except enzyme, and their values were subtracted from the sample values. All samples were assayed in duplicate.

CBS activity assay. All liver samples were weighed and homogenized immediately in 4 vol of 50 mM potassium phosphate buffer (pH 7.4) containing 2 mM EDTA and 1 mM mercaptoethanol. The homogenates was centrifuged at 4 °C for 45 min at 18,000 × g. The supernatant fraction was used for enzyme assay and protein concentration determination by the Bradford assay. The standard assay mixture (400 μL) contained 0.38 $mmol \cdot L^{-1}$ S-adenosylmethionine, 7.5 mM L-Hcy, 10 mM ^{14}C-Ser (0.07 μCi), and 50 μL cow liver extract. Reaction tubes were capped with rubber stoppers and kept in ice water until the assay was started by transferring the tubes into a 37 °C water bath. After 1 h incubation, the reaction was terminated by adding 0.4 mL 10% trichloroacetic acid and 2.2 mL ice-cold water into each tube. The samples were applied to an

AG 50 W X8 column, 8 mL of water was applied to the column, and ^{14}C-Ser was eluted by applying 24 mL of 0.6M hydrochloric acid to the column. Following an additional wash with 16 mL water, ^{14}C-cystathionine was eluted by applying 5 mL of 3M ammonium hydroxide to the column and collected in scintillation vials. Seventeen milliliters of scintillation mixture (Ecolume, ICN) were added to each vial and counted. Blanks contained all of the reaction components except enzyme, and their values were subtracted from the sample values. All samples were assayed in duplicate.

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CHAPTER 5

METHIONINE AND CHOLINE SUPPLEMENTATION DURING THE PERIPARTURIENT PERIOD ALTER PLASMA AMINO ACID PROFILES TO VARIOUS EXTENTS: POTENTIAL ROLE ON HEPATIC METABOLISM AND ANTIOXIDANT STATUS

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INTRODUCTION

Around parturition, the increased demand for nutrients to sustain fetal growth and lactation coupled with depressed dry matter intake (**DMI**) impose tremendous metabolic stress on dairy cows. Consequently, health problems and compromised production efficiency likely occur not only due to negative energy balance-induced increases in circulating free fatty acids, but a negative AA balance (Goff and Horst, 1997, Bell et al., 2000, Zhou et al., 2016d). For instance, increased mobilization of tissue protein is often observed due to inadequate availability of AA substrates for gluconeogenesis as well as synthesis of protein in liver and mammary gland (Overton, 1998). In fact, a moderate net loss of carcass protein was observed even in animals fed to predicted metabolizable protein requirements around parturition (McNeill et al., 1997, Bell et al., 2000), indicating suboptimal AA profile may be the limiting factor for AA utilization during this period. In line with this assumption, improving AA profile through balancing limiting AA in the diet or supplementing rumen-protected limiting AA has achieved various benefits in terms of lactation performance and health status of periparturient dairy cows (Lee et al., 2012, Osorio et al., 2013, Paz et al., 2013, Osorio et al., 2014a, Zhou et al., 2016f, Zhou et al., 2016g).

Methionine (**Met**) is an essential sulfur-containing AA engaged in various key physiologic events. Previous research has underscored the importance of Met as a limiting AA for milk protein synthesis in many diets (Schwab et al., 1992, Pisulewski et al., 1996). Apart from its apparent key role in mammary gland and liver protein synthesis, Met also serves as substrate for sulfur-containing antioxidants, namely glutathione (**GSH**) and taurine (Brosnan and Brosnan, 2006). Additionally, as a key component of one-carbon metabolism, hundreds of methylation reactions acquire methyl groups from Met via S-adenosyl methionine (**SAM**) (Ulrey et al., 2005). Furthermore, as a gluconeogenic AA, a portion of Met may be taken up by liver to sustain the abrupt increase in demand for glucose at the onset of lactation. In line with the various demands for Met, its deficiency has often been reported in cows around parturition (Zhou et al., 2016d). In fact, circulating Met concentration decreased markedly through parturition and was not restored to prepartum level until 28 d postpartum (Zhou et al., 2016d).

An unfavorable circulating AA profile around parturition likely occurs due to 1) increased production of positive acute-phase proteins (**APP**) and immune related proteins induced by oxidative stress and inflammation; 2) enhanced carcass protein mobilization to provide AA for gluconeogenesis; and/or 3) limited uptake of other AA and increased N excretion due to lack of Met. Recent research with periparturient dairy cows has demonstrated benefits to overall health and production performance in response to MET supplementation (Osorio et al., 2014a, Zhou et al., 2016f, Zhou et al., 2016g). However, how plasma AA and downstream products of their metabolism respond to periparturient MET supplementation is lacking.

Although CHOL is not an AA, it may regulate AA metabolism by altering AA requirements, especially Met, around parturition. For instance, Met can be regenerated when homocysteine receives a methyl group from CHOL through betaine (Wong and Thompson,

1972b, Li and Vance, 2008a), suggesting a potential reduction around parturition in Met requirement with CHOL supplementation. Additionally, accumulation of fat in liver can induce inflammation and oxidative stress, which almost certainly leads to increased AA requirements for production of positive APP and other immune function-related proteins (Pinotti et al., 2002). As precursor for hepatic very low-density lipoprotein (**VLDL**) assembly, CHOL has a crucial role in the export of triacylglycerol to prevent fatty liver by promoting phosphatidylcholine synthesis via the Kennedy pathway instead of sequential methylation using Met derived SAM, which may also spare a portion of Met around parturition.

Few studies have attempted to characterize the profile of circulating AA and their derivatives around parturition in cows (Verbeke et al., 1972, Meijer et al., 1995b, Doepel et al., 2002, Zhou et al., 2016d). To our knowledge, this is the first study profiling AA and their derivatives in dairy cows in response to MET or CHOL supplementation around parturition. Considering that periparturient MET supplementation leads to greater feed intake, increased milk yield, and better overall cow health while CHOL cows did not achieve similar benefits (Zhou et al., 2016g), our hypothesis was that MET and CHOL supplementation result in different alterations in AA metabolism-associated events which ultimately contribute to their distinct role in the overall health and production efficiency of the animal.

MATERIALS AND METHODS

Experimental design and treatments

All procedures for this study (protocol no. 13023) were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Illinois. Details of the experimental design have been described previously (Zhou et al., 2016g). Briefly, the experiment

was conducted as a randomized, complete, unbalanced, block design with 2×2 factorial arrangement of MET (Smartamine M, Adisseo NA) and CHOL (ReaShure, Balchem Inc) level (with or without). Cows within each block were balanced for parity, previous lactation milk yield, and BCS before the close-up diet groups were assigned. A total of 81 cows were used. Treatments were control (CON, n = 20), with no MET or CHOL supplementation; Smartamine (SMA, n = 21), CON plus MET at a rate of 0.08% of DM; Reashure (REA, n = 20), CON+CHOL at 60 g/d; or Smartamine and Reashure (MIX, n = 20), CON+MET+CHOL. Dosage of MET was based on Osorio et al. (2013) (Osorio et al., 2013), whereas CHOL was supplemented following the manufacturer's recommendations. Per IACUC guidelines only a subset of 40 multiparous cows (10 cows/treatment) were used for this portion of the study. All cows received the same far-off diet from -50 to -22 d before expected parturition, close-up diet from -21 d to expected parturition, and lactation diet from parturition through 30 days in milk (**DIM**). The MET and CHOL supplements were both top-dressed from -21 ± 2 to 30 DIM once daily at the AM feeding using approximately 50 g of ground corn as carrier for all treatments. Supplementation of SMA (0.08% DM of TMR offered) was calculated daily for each cow. Smartamine M was supplied as small beads containing a minimum of 75% DL-Met, physically protected by a pH sensitive coating, which is considered to have a Met bioavailability of 80% (Graulet et al., 2005); therefore, per 10 g of SMA, the cows received 6 g of metabolizable Met. The REA supplement is reported to contain 28.8% choline chloride and is protected by microencapsulation. The product is considered to have CHOL bioavailability of 72% (Benoit, 2009); therefore, per 60 g of REA, the cows received 12.4 g of metabolizable choline chloride. To our knowledge, neither SMA nor REA have specific characteristics that may affect palatability of diets.

Animal management

Dry cows were housed in a ventilated enclosed barn during the dry period and fed individually once daily at 0630 h using an individual gate system (American Calan Inc., Northwood, NH). After parturition, cows were housed in a tie-stall barn and were fed a common lactation diet once daily. Feed offered was adjusted daily to achieve ~10% refusals.

Blood Sample Collection and Analyses of Plasma AA and their Derivatives

Blood was sampled from the coccygeal vein on -30 and -10 d relative to expected parturition date and on 4, 14 and 28 d relative to actual parturition date before the morning feeding. Samples were collected into evacuated tubes (BD Vacutainer; BD and Co., Franklin Lakes, NJ) containing lithium heparin for isolation of plasma.

Plasma was used to analyze the concentrations of free arginine (**Arg**), histidine (**His**), isoleucine (**Ile**), leucine (**Leu**), lysine (**Lys**), methionine (**Met**), phenylalanine (**Phe**), threonine (**Thr**), tryptophan (**Trp**), valine (**Val**), asparagine (**Asn**), aspartate (**Asp**), alanine (**Ala**), glutamate (**Glu**), glutamine (**Gln**), glycine (**Gly**), proline (**Pro**), serine (**Ser**), tyrosine (**Tyr**), citrulline (**Cit**), carnosine, ornithine (**Orn**), sarcosine (**Sar**), cystathionine, cystine, homocystine, taurine, α-aminoadipic acid, α-aminobutyric acid, β-alanine, γ-aminobutyric acid (**GABA**), hydroxylysine, hydroxyproline, phosphoserine, 1-methyl histidine, and 3-methyl histidine at the University of Missouri Agriculture Experiment Station Chemical Laboratories (Columbia, MO) using high performance liquid chromatography (Deyl et al., 1986, Fekkes, 1996). Plasma total GSH was measured using a commercial kit (Cat. No. NWH-GSH01; Northwest Life Science Specialties LLC, Vancouver, WA). The EAA pool included Arg + His + Ile + Leu + Lys + Met + Phe + Thr + Trp + Val; the NEAA pool included Asn + Asp + Ala + Gln + Glu + Gly + Pro +

Ser + Tyr; total AA was the sum of EAA and NEAA; the total sulfur-containing compounds (**TSC**) included Met + cystine + cystathionine + homocystine + taurine + GSH.

Liver Sample Collection and Quantitative RT-PCR (qPCR)

Liver was sampled via puncture biopsy (Dann et al., 2005) from cows under local anesthesia at approximately 0800 h on d -10, 7, 20, and 30 d relative to parturition. Liver was frozen immediately in liquid nitrogen and stored until analysis. The qPCR was performed in liver samples as describe previously (Osorio et al., 2014a).

Statistical analysis

Data were analyzed using PROC MIXED of SAS (SAS Institute Inc., Cary, NC) according to the following model:

$$y_{ijklm} = \mu + b_i + M_j + C_k + MC_{jk} + T_l + TM_{jl} + TC_{kl} + TMC_{jkl} + A_{m:ijk} + \varepsilon_{ijklm}$$

where Y_{ijklm} is the dependent, continuous variable; μ is the overall mean; b_i is the random effect of the ith block; M_j is the fixed effect of MET (j = with or without); C_k is the fixed effect of CHOL (k = with or without); T_l is the fixed effect of time (day or week) of the experiment; A_m is the random effect of the *m*th animal (cow); ε_{ijklm} is the residual error. The covariate of parity (2^{nd} vs. 3^{rd} lactation and above) and concentrations obtained at -30 d for various AA and derivatives were maintained in the model for all variables when significant ($P < 0.05$). Plasma AA and derivatives and hepatic gene expression were analyzed at various time points that were not equally spaced. Therefore, the first order ante-dependence covariance structure ANTE (1) were used for repeated measures. Variables were assessed for normality of distribution using Shapiro-Wilk test. When normality assumption was rejected, data were log-transformed before

statistical analysis. Back transformed data were reported in tables and figures for the ease of interpretation. Least square means separation between time points was performed using the PDIFF statement. Statistical differences were declared significant at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

RESULTS

Essential AA

Main effects of MET, CHOL, time, and interactions for essential AA are presented in **Table 5.1**. Overall, plasma concentrations of most EAA (Arg, His, Ile, Leu, Lys, Thr, Trp, Val) decreased soon after parturition (4 d) except for Met and Phe, levels of which were increased and unchanged at 4 d, respectively (**Figure 5.1**, **Figure 5.2**, **Figure S5.1,** and **Figure S5.2**). As expected, MET-supplemented cows had greater plasma Met concentration ($P < 0.01$, **Figure 5.1A**) compared with cows without MET. In fact, proportion of Met in EAA ($P < 0.01$, **Figure 5.1D**) and TAA ($P < 0.01$, **Figure 5.1F**) also was greater in response to MET supplementation. In contrast, similar plasma Met ($P > 0.10$, **Figure 5.1B**) and Met%EAA ($P > 0.10$) levels were observed regardless of CHOL supplementation. MET main effect ($P = 0.02$) also was observed for Lys, the other most-limiting AA for milk production, mainly due to greater plasma concentrations at -10 d and 20 d (M×D $P < 0.05$, **Figure 5.2E**). Similarly, plasma Arg concentration also was greater ($P = 0.02$) in MET cows owing to greater concentrations at -10 d and 20 d (M×D $P < 0.05$, **Figure 5.2B**), but not at 4 d postpartum. Although greater Thr was observed in MET cows at -10 d (M×D $P < 0.05$, **Figure S5.2**), Thr concentration was similar regardless of MET supplementation, thus, only a tendency ($P = 0.09$) was detected in these cows. In addition to greater ($P < 0.01$) Trp in MET cows, the main effect of CHOL also was observed for Trp ($P = 0.04$, **Figure S5.1. G and H**). Despite the lack of main effects of MET for plasma

His, Phe and BCAA (Val, Leu, Ile) concentrations, the EAA tended to be greater ($P = 0.06$, **Figure 5.1C**) in response to MET mainly owing to greater Met, Lys, Arg, Thr, and Trp.

Non-essential AA

Main effects of MET, CHOL, time, and interactions for non-essential proteinogenic AA are presented in **Table 5.1**. Similar to EAA, plasma concentrations for the majority of the NEAA (Asp, Ala, Glu, Gln, Tyr) decreased soon after parturition (**Figure S5.3** and **Figure S5.4**). In contrast, plasma concentrations of Asn, Gly, Pro, and Ser increased at 4 d compared with -10 d (**Figure S5.3** and **Figure S5.4**). Although total NEAA did not change in response to MET or CHOL ($P > 0.10$), TAA in MET-supplemented cows were greater ($P = 0.03$) as a result of an overall tendency towards greater EAA together with greater Asn, Asp, Ala, and Pro ($P < 0.05$, **Figure 5.1C, Figure S5.3,** and **Fig S5.4**) as well as a tendency towards greater Glu ($P = 0.10$ **Figure S5.3**).

It is noteworthy that similar to Arg and Lys, plasma Ala concentrations were already greater (M×D $P < 0.05$, **Figure 5.2A** and **Figure 5.2C**) at -10 d prepartum in MET cows. After parturition, plasma Arg, Lys, and Ala were similar in cows regardless of MET supplementation at 4 d but were again greater at 14 d (M×D $P < 0.05$, **Figure 5.2**) in MET cows. Unlike Met, only a tendency towards greater ($P = 0.07$) Asp was detected in response to CHOL.

Sulfur-containing compounds

Main effects of MET, CHOL, time, and interactions for sulfur-containing compounds are presented in **Table 5.2**. As expected, MET supplementation increased plasma concentrations of all sulfur-containing AA ($P < 0.05$, **Figure 5.2** and **Figure 5.3**), which account for the greater overall plasma TSC level at all time points evaluated (M×D $P < 0.05$, **Figure 5.3C**). Similar to

Ala, Arg, and Lys, greater ($P < 0.01$) cystine and cystathionine concentrations were also detected in response to MET due to greater plasma level at -10, 14, and 28 d (M×D $P < 0.05$, **Figure 5.2C** and **Figure 5.2D**). In addition to increased circulating sulfur-containing compounds, MET also increased the proportion of Met in TSC ($P = 0.01$, **Figure 5.3D**). In contrast, although plasma cystine concentration was greater ($P = 0.02$) in response to CHOL supplementation. a decreased proportion of Met in TSC was observed in CHOL cows around parturition ($P = 0.03$, **Figure S5.8**).

Non-proteinogenic AA and derivatives

Main effects of MET, CHOL, time, and interactions for essential AA are presented in **Table 5.2**. Although no main effect of MET or CHOL was observed for ornithine, greater Cit ($P < 0.01$, **Figure 5.4E**) and a tendency ($P = 0.07$) towards greater urea were observed in MET-supplemented cows in addition to greater Arg. Besides taurine, plasma concentrations of carnosine, another antioxidant, was also greater ($P = 0.02$, **Figure 5.4D**) in MET cows. Although no main effect of CHOL was observed for carnosine, greater (C×D $P < 0.05$) concentrations were detected at -10 d and 28 d. Both α-aminobutyric acid and γ-aminobutyric acid were greater ($P < 0.05$ **Figure 5.4C** and **Figure 5.4G**) in MET cows; however, only greater γ-aminobutyric acid plasma concentration was observed in CHOL cows ($P = 0.02$ **Figure 5.4H**). The indicator of protein mobilization, 3-methylhistidine was lower in CHOL cows ($P = 0.02$ **Figure 5.4B**). Similarly, plasma 1-methylhistidine concentration also was lower in these cows ($P = 0.04$ **Figure 5.4A)**.

Pyruvate carboxylase and phosphoenolpyruvate caboxykinase 1 expression

Main effects of MET, CHOL, time, and interactions for expression of pyruvate carboxylase (***PC***) and phosphoenolpyruvate caboxykinase 1 (***PCK1***) are presented in **Table 5.3**. Main effects of MET and CHOL or interactions were not detected for the mRNA expression of *PC* and *PCK1*.

DISCUSSION

A brief summary of production performance and immunometabolic status of the cows is presented in Table 5.4 to facilitate interpretation of the results in this study. Detailed discussion of those data have been published elsewhere (Zhou et al., 2016a, Zhou et al., 2016g).

Supplemental MET improved plasma AA profiles

MET and Lys in metabolizable protein are the most-limiting AA in a wide-range of diets for dairy cows due to their low concentrations in feed protein relative to their concentrations in milk (NRC, 2001a). During the periparturient period, the decreased feed intake coupled with increased AA requirements to sustain fetal growth and lactation lead to greater tissue protein mobilization evidenced in the present study by the highest plasma 3-methyl histidine at 4 d regardless of treatments. Although AA released from tissue mobilization can partly mitigate the demand for AA, cow body tissue protein is relatively low in Met and Lys (O'Connor et al., 1993). Consequently, this physiological stage is characterized by an overall decrease in circulating AA, especially the most-limiting AA (e.g. Met and Lys). Apart from limiting milk production, inadequate Met availability also could potentially limit the utilization of other circulating AA according to von Liebig's hypothesis which is commonly described with the analogy of the water barrel with broken staves (Mitchell and Block, 1946). Strategies to increase

circulating Met are, therefore, expected to improve overall circulating AA profiles and utilization during the periparturient period.

The fact that various proteinogenic circulating EAA (Arg, Lys, Met, Thr, and Trp), NEAA (Ala, Asn, Asp, Glu, Gln, and Pro) and non-proteinogenic AA (Cit) were greater in response to rumen-protected MET supplementation, apart from greater plasma Met, are indications of a better circulating AA profile during the periparturient period. However, it cannot be ignored that MET cows had greater DMI both prepartum and postpartum (Zhou et al., 2016g). Therefore, the DMI increase in MET-supplemented cows might have contributed to the observed increase in circulating EAA and TAA. However, it is noteworthy that despite concomitant increases in circulating Met, EAA and TAA, the proportion of Met in EAA and TAA (Met%EAA and Met%TAA) as well as the proportion of Lys in TAA were increased in response to MET supplementation, indicating a better AA profile at least in regards to limiting AA, i.e. Met and Lys.

Considering the overall greater milk yield, milk protein %, and milk protein yield (Zhou et al., 2016g) (Table 5.4), it is reasonable to speculate that MET supplementation contributes to better lactation performance both by increasing intake and proportion of Met and Lys in the circulating AA pool. Although CHOL can potentially achieve similar benefits by promoting *in vivo* synthesis of Met, the lack of change in circulating AA does not seem to support such hypothesis.

Utilization of circulating AA close to parturition

Around parturition, the demand for AA for glucose and protein synthesis increases abruptly and results in greatly increased AA uptake from the circulation (Larsen and Kristensen,

2013). The few published reports have revealed that circulating AA concentrations in dairy cows generally reach a nadir close to parturition mainly as a result of increased demand for milk protein synthesis and gluconeogenesis coupled with insufficient dietary intake (Verbeke et al., 1972, Meijer et al., 1995a, Doepel et al., 2002, Zhou et al., 2016d). In agreement with these reports, we detected the lowest circulating concentrations of most AA at 4 d relative to parturition regardless of treatment.

Although circulating concentrations of multiple AA (Ala, Arg, Lys) and peptides (cystine, cystathionine) in MET-supplemented cows were already greater at -10 d compared with cows without MET supplementation and regained greater levels at 14 d postpartum, the circulating concentrations of all AA and peptides at 4 d were similar regardless of MET supplementation. Whether the lack of difference in circulating concentrations of these AA at 4 d in MET cows was due to inadequate supply in the circulation (e.g. AA from intake and tissue mobilization) (Komaragiri and Erdman, 1997) or enhanced utilization (e.g. greater liver and mammary uptake) remains unknown. However, the fact that average DMI at d 4 remained greater (+3.33 kg/d more) in MET-supplemented cows (Zhou et al., 2016g), together with similar 3-methylhistidine in cows with or without MET supplementation, seem to support an overall greater, rather than lower, AA supply at 4 d in response to MET supplementation. In line with the increase in AA supply, MET-supplemented cows regained greater concentrations of these AA at 14 d.

In terms of AA utilization, a previous study detected the greatest liver uptake of EAA at 4 d postpartum in dairy cows (Larsen and Kristensen, 2013). Similarly, hepatic uptake of total NEAA, especially Ala, also was substantially greater in the periparturient period, indicating this AA may act as a precursor for glucose synthesis (Kuhla et al., 2011, Larsen and Kristensen,

2013). The greater (+4.10 kg/d more) milk yield in MET-supplemented cows at 4 d (Zhou et al., 2016g) (and Table 5.4) indicated that the greater uptake of these AA by mammary gland soon after parturition accounted for the lower AA concentration in the circulation compared with -10 d. Presumably, greater mammary availability of AA accounted for the greater milk protein % (Zhou et al., 2016g).

It is also noteworthy that a reduction in net flux of carbon from volatile fatty acids across the liver and an increase in glucose release was detected early postpartum in a previous report (Larsen and Kristensen, 2013), indicating that the greatest flux through citric acid cycle occurs soon after parturition. Considering the severe negative energy balance and depressed DMI soon after parturition, the increased demand by the liver for energy and glucose synthesis likely accounted for such increase. Whether periparturient MET supplementation enhanced gluconeogenesis by promoting AA flux through the citric acid cycle is unknown, but does not seem to be related to regulation at the transcriptional level as evidenced by unchanged mRNA abundance of the key gluconeogenic genes *PC* and *PCK1*.

Sulfur-containing compound pool and metabolism

Met and cysteine are the two sulfur-containing AA that are incorporated into proteins. Apart from their well-known role in contributing sulfur bonds during protein synthesis, Met and cysteine are precursors for downstream functional compounds (homocysteine, cystathionine, and taurine) and, thus, are considered the principal components *in vivo* of the sulfur-containing compound pool (Brosnan and Brosnan, 2006). The fact that circulating concentrations of all sulfur-containing compounds measured (except GSH) were greater in MET-supplemented cows during the periparturient period indicate an enriched sulfur-containing compound pool.

It is noteworthy that other than Met, all other plasma sulfur-containing compounds measured are components of the transsulfuration pathway. Considering plasma homocystine concentrations are highly dependent on intracellular homocysteine metabolism in liver (Schalinske and Smazal, 2012), and cystathionine is a sensitive marker of changes in flux through the transsulfuration pathway (Guttormsen et al., 2004), the greater homocystine and cystathionine indicate increased hepatic flux through this pathway in response to MET supplementation. In addition to the involvement of sulfur-containing compounds, the fact that circulating concentrations of α-aminobutyric acid also was greater in MET-supplemented cows indicates increased flux through the transsulfuration pathway (Stabler et al., 2009). Considering the key role of Met as the most-limiting AA for milk protein synthesis, the fact that it is the major precursor for sulfur-containing compounds in the transsulfuration pathway indicates that such increase may result in depletion of Met which could potentially give rise to unfavorable lactation performance. However, despite the increased flux through the transsulfuration pathway, the sustained greater circulating concentration of Met and proportion of Met in TSC during the periparturient period indicates that sufficient Met was available in MET-supplemented cows even at 4 d. In contrast, although greater circulating cystine was detected in response to CHOL supplementation, the proportion of Met in TSC was decreased, indicating enhanced flux through the transsulfuration pathway at the expense of Met. Whether the decrease of plasma Met proportion in TSC in CHOL-supplemented cows contributed to the lack of benefit in performance is unknown; however, it does not seem to support the hypothesis that CHOL can promote Met synthesis *in vivo* in periparturient dairy cows.

Around parturition, the increased demand for nutrients and energy leads to an increase in the production reactive oxygen metabolites (**ROM**), the accumulation of which could deplete

antioxidants and give rise to oxidative stress that may cause substantial tissue damage and render cows more susceptible to various health disorders (Bernabucci et al., 2005, Castillo et al., 2005). Among the sulfur-containing compounds measured, taurine and GSH are potent intracellular antioxidants due to their marked ability to scavenge ROM and free radicals (Young and Woodside, 2001). Hence, hepatic concentrations of GSH and taurine have been widely-used as oxidative stress biomarkers (Romeu et al., 2010, Vetrani et al., 2013, Saharan and Mandal, 2014). It is noteworthy that liver is the main site of taurine synthesis and releases taurine into plasma (Garcia and Stipanuk, 1992), but not for transport to other tissues (Hayes and Sturman, 1981). Therefore, the greater overall circulating taurine in response to MET supplementation indicates greater hepatic taurine availability and, hence, potentially less oxidative stress in these cows. However, although previous results from our group revealed greater total and reduced hepatic GSH in MET-supplemented cows (Osorio et al., 2014c, Zhou et al., 2016f), plasma GSH was barely detectable and did not respond to MET. Considering that concentration of GSH in whole blood was 200-fold higher than in plasma due to high concentration in erythrocytes, small amounts of hemolysis may lead to great variations in plasma GSH concentration (Pocius et al., 1981). Therefore, plasma GSH may not be a reliable oxidative stress biomarker for periparturient dairy cows.

AA derivatives

AA derivatives have unique metabolic properties and, thus, many have been adopted as biomarkers for metabolic status. For instance, because 3-methylhistidine is released from the catabolism of actin and myosin in skeletal muscle and is not further metabolized in the body, it has been regarded as a reliable marker for tissue protein mobilization (Harris and Milne, 1981). The overall lower plasma 3-methylhistidine in response to CHOL supplementation during the

periparturient period indicates less degree of muscle catabolism in these cows. Although lower circulating 1-methylhistidine concentration also was observed in response to CHOL, the postpartum pattern of a gradual increase with time indicates that it is not a suitable indicator of muscle catabolism considering that it was at its greatest right after parturition as indicated by 3-methylhistidine.

The antioxidant activity of carnosine in non-ruminants has been demonstrated both in terms of reducing oxidative damage and improving the enzymatic and non-enzymatic activity of other antioxidants (Boldyrev et al., 2013). The fact that supplementation of carnosine in non-ruminants was able to rescue prooxidant-antioxidant balance by restoring depleted levels of blood GSH and activities of antioxidant enzymes (Aydin et al., 2010) indicates the potential decrease in circulating carnosine in events of oxidative stress. Although the contribution of carnosine to restore prooxidant-antioxidant balance under physiologic (without carnosine supplementation) conditions remains unknown, the overall greater circulating carnosine together with greater circulating taurine as well as hepatic GSH reported previously (Zhou et al., 2016a) indicate a less pronounced oxidative stress status in response to periparturient MET supplementation.

As a neurotransmitter distributed in both neural and non-neural tissue, GABA has various physiologic functions including feed intake regulation (Cheng et al., 2014). In rats, GABA-B agonist administration increased intake by attenuating satiety signals (Higgs and Barber, 2004). In lactating dairy cows, increased DMI in response to rumen-protected GABA supplementation during mid-lactation has been reported (Wang et al., 2013, Cheng et al., 2014). The greater circulating GABA in response to periparturient MET supplementation along with the greater DMI (Zhou et al., 2016g) seem to suggest a role of GABA in mediating DMI regulation by

MET. However, considering that CHOL supplementation failed to increase periparturient DMI yet it led to a similar increase in circulating GABA, this response is likely unrelated to MET or CHOL supplementation and may not play a regulatory role in the control of intake.

CONCLUSIONS

The greater Ala, Arg, and Lys together with cystine and cystathionine concentrations at -10 d and 14 d, but not at 4 d, in cows fed MET indicated greater utilization of these AA and peptides at the time of most-severe negative AA balance. The enriched circulating sulfur-containing compound pool together with greater α-aminobutyric acid revealed enhanced transsulfuration pathway activity in response to MET supplementation. Despite the greater circulating cystine in CHOL cows, the lower proportion of Met in TSC indicates enhanced flux through the transsulfuration pathway at the expense of Met. Whether the decrease in the proportion Met was due to insufficient choline supplementation remains unknown. As a precursor for Met synthesis in vivo, it is possible that increasing periparturient choline supplementation could mitigate a decrease in Met. Although plasma GSH did not differ in response to MET due to barely detectable concentration in plasma, the greater circulating taurine and carnosine indicate less oxidative stress in MET supplemented cows. In conclusion, the overall better health and production performance reported previously in MET cows was due, at least in part, to a better plasma AA profile and overall lower oxidative stress status. Hence, the lack of change in AA profiles could be one of the reasons preventing CHOL-fed cows from achieving comparable performance and health benefits during the periparturient period.

TABLES AND FIGURES

Table 5.1. Plasma proteinogenic AA concentrations during the periparturient period in cows supplemented with or without rumen-protected MET and CHOL.

	MET		CHOL			Day				*P-value*[2]				
AA (μM)	Without	With	Without	With	SEM[1]	-10	4	14	28	MET[3]	CHOL[4]	Time	M×T[5]	C×T[6]
Essential AA														
Arginine	54.75	60.43	57.99	57.05	1.75	58.58^{ab}	48.47^{c}	63.29^{a}	60.92^{ab}	0.02	0.69	<0.01	0.05	0.96
Histidine	52.38	54.55	54.04	52.87	1.18	57.28^{a}	53.53^{b}	52.30^{b}	50.92^{b}	0.19	0.49	<0.01	0.13	0.17
Isoleucine	95.58	103.61	100.64	98.40	4.42	98.18	95.54	106.87	97.83	0.19	0.72	0.18	0.07	0.36
Leucine	153.31	161.79	152.70	162.43	6.69	151.35^{b}	153.51^{b}	173.18^{a}	152.92^{b}	0.35	0.31	0.03	0.27	0.31
Lysine	60.99	68.74	63.47	66.06	2.45	66.75^{ab}	57.56^{c}	70.47^{a}	64.91^{b}	0.02	0.47	<0.01	0.03	0.85
Methionine	18.65	28.95	24.04	23.55	0.83	22.75^{c}	25.25^{a}	24.92^{ab}	22.27^{c}	<0.01	0.68	<0.01	0.14	0.17
Phenylalanine	46.64	47.09	45.88	47.86	1.02	45.00^{b}	47.64^{b}	51.16^{a}	43.97^{b}	0.75	0.17	<0.01	0.26	0.92
Threonine	72.55	78.67	75.93	75.30	2.50	71.87	72.22	78.70	79.66	0.09	0.86	0.07	0.01	0.52
Tryptophan	22.26	25.04	22.68	24.61	0.64	24.56^{ab}	19.44^{c}	25.08^{a}	25.50^{a}	<0.01	0.04	<0.01	0.42	0.63
Valine	226.77	244.54	229.16	241.99	9.32	230.11	223.02	249.41	240.25	0.17	0.33	0.08	0.26	0.31
BCAA	476.69	510.97	483.71	503.55	19.87	480.67	473.17	530.28	491.93	0.21	0.48	0.08	0.19	0.27
EAA	827.12	896.20	849.22	874.1	25.54	848.26^{bc}	816.78^{bc}	921.2^{a}	860.39^{ab}	0.06	0.50	0.03	0.08	0.32
Met%EAA	2.26	3.18	2.73	2.64	0.09	2.59^{b}	3.04^{a}	2.62^{b}	2.52^{b}	<0.01	0.41	<0.01	0.11	0.23
Non-essential AA														
Alanine	187.39	206.37	198.37	195.39	5.55	188.83^{b}	175.45^{c}	211.30^{a}	211.95^{a}	0.02	0.71	<0.01	0.05	0.32
Asparagine	34.90	39.63	37.78	36.61	1.48	27.82^{c}	34.08^{b}	45.67^{a}	44.19^{a}	0.02	0.56	<0.01	0.22	0.42
Aspartate	4.21	4.79	4.24	4.76	0.21	4.91^{a}	3.78^{b}	4.80^{a}	4.57^{a}	0.04	0.07	<0.01	0.59	0.14
Glutamate	35.93	38.34	36.44	37.81	1.04	44.24^{a}	32.17^{d}	37.84^{b}	35.24^{c}	0.10	0.36	<0.01	0.54	0.38
Glutamine	246.22	259.53	257.92	247.82	6.18	278.17^{a}	252.84^{b}	242.52^{b}	237.96^{b}	0.15	0.25	<0.01	0.21	0.29
Glycine[*]	426.83	406.23	427.39	405.67	15.10	236.99^{c}	456.97^{b}	536.19^{a}	435.98^{b}	0.35	0.31	<0.01	0.92	0.22
Proline	72.30	78.24	74.03	76.50	2.02	59.19^{c}	67.52^{b}	90.21^{a}	84.15^{a}	0.04	0.39	<0.01	0.35	0.74
Serine	86.62	88.92	88.67	86.87	2.28	73.88^{c}	90.85^{b}	100.78^{a}	85.57^{b}	0.48	0.58	<0.01	0.16	0.49
Tyrosine	40.82	43.39	40.78	43.43	1.53	42.03^{a}	36.26^{b}	46.32^{a}	43.80^{a}	0.24	0.24	<0.01	0.08	0.94
NEAA	1114.59	1151.58	1153.58	1112.59	24.79	946.13^{c}	1136.83^{b}	1301.27^{a}	1177.00^{b}	0.29	0.24	<0.01	0.36	0.23
TAA	1926.95	2052.97	1995.87	1982.22	41.32	1781.41^{d}	1947.89^{bc}	2215.47^{a}	2035.97^{b}	0.03	0.82	<0.01	0.10	0.53
Met%TAA	0.94	1.37	1.15	1.13	0.04	1.20^{a}	1.25^{a}	1.06^{b}	1.05^{b}	<0.01	0.64	<0.01	0.14	0.23
Lys%TAA	3.22	3.47	3.26	3.42	0.10	3.84^{a}	3.02^{c}	3.23^{bc}	3.29^{b}	0.08	0.28	<0.01	0.13	0.85

[*]Significant ($P < 0.05$) parity effect observed.

[1]Greatest SEM.

[2]MET × CHOL interaction was included in the supplemental file.

[3]Overall effect of MET supplementation.

Table 5.1 (Continued)

[4]Overall effect of CHOL supplementation.

[5]Interaction of MET × time.

[6]Interaction of CHOL × time.

Table 5.2. Plasma non-proteinogenic AA and AA derivatives concentrations during the periparturient period in cows supplemented with or without rumen-protected MET and CHOL.

	MET		CHOL			Day				*P-value*[2]				
Parameter (μM)	Without	With	Without	With	SEM[1]	-10	4	14	28	MET[3]	CHOL[4]	Time	M×T[5]	C×T[6]
AA and derivatives[#]														
1-methyl histidine	15.36	16.56	16.79	15.15	0.57	12.82^{c}	12.58^{c}	18.38^{b}	21.85^{a}	0.12	0.04	<0.01	0.36	0.50
3-methyl histidine	6.44	6.48	6.97	5.99	0.29	5.21^{c}	11.00^{a}	6.91^{b}	4.40^{d}	0.92	0.02	<0.01	0.12	0.75
α-aminoadipic acid	7.37	8.15	8.03	7.48	0.36	6.09^{d}	6.99^{c}	8.65^{b}	9.81^{a}	0.12	0.27	<0.01	0.91	0.30
α-aminobutyric acid	15.64	19.78	18.13	17.07	0.81	8.94^{c}	23.60^{a}	22.77^{a}	19.93^{b}	<0.01	0.32	<0.01	0.61	0.44
β-alanine	9.06	8.87	8.81	9.11	0.32	8.97	8.69	9.24	8.94	0.68	0.50	0.54	0.96	0.92
γ-aminobutyric acid	2.42	3.49	2.46	3.45	0.29	1.88^{b}	3.08^{a}	3.44^{a}	3.41^{a}	0.01	0.02	<0.01	0.27	0.75
Carnosine[*]	15.88	19.92	16.60	19.07	1.29	16.74^{c}	14.20^{d}	18.71^{b}	22.51^{a}	0.02	0.14	<0.01	0.30	0.02
Citrulline	71.19	83.47	79.08	75.14	2.92	69.61^{c}	63.69^{c}	82.86^{b}	96.11^{a}	<0.01	0.30	<0.01	0.99	0.60
Glutathione	4.68	4.73	4.84	4.57	0.27	4.60^{b}	6.11^{a}	4.35^{bc}	3.76^{c}	0.89	0.50	<0.01	0.50	0.74
Hydroxylysine	0.32	0.28	0.30	0.29	0.04	0.33	0.29	0.33	0.25	0.37	0.79	0.21	0.61	0.70
Hydroxyproline	15.19	15.36	15.42	15.13	0.48	12.80^{c}	18.10^{a}	16.31^{b}	13.89^{c}	0.81	0.67	<0.01	0.94	0.12
Ornithine	29.14	32.00	30.48	30.60	1.35	39.37^{a}	22.11^{d}	30.04^{c}	33.25^{b}	0.13	0.95	<0.01	0.12	0.45
Phosphoserine	6.22	6.18	6.07	6.33	0.18	5.53^{c}	5.91^{bc}	6.72^{ab}	6.74^{a}	0.88	0.30	<0.01	0.71	0.91
Sarcosine	10.65	11.43	11.50	10.58	0.56	8.32^{b}	11.31^{a}	12.03^{a}	12.50^{a}	0.33	0.27	<0.01	0.76	0.37
Urea	4121.63	4442.94	4323.55	4235.16	123.89	4104.53^{bc}	4317.26^{b}	4036.81^{c}	4687.49^{a}	0.07	0.60	<0.01	0.55	0.62
Sulfur-containing compounds														
Cystathionine	1.60	2.12	1.84	1.89	0.07	2.30	1.51	1.71	1.92	<0.01	0.61	<0.01	0.08	0.36
Cystine	7.56	9.58	7.65	9.48	34.5	11.20^{a}	6.41^{d}	7.73^{c}	8.92^{b}	<0.01	0.02	<0.01	0.01	0.17
Homocystine	4.17	5.10	4.52	4.70	0.30	4.80^{a}	3.99^{b}	4.80^{a}	4.92^{a}	0.02	0.65	0.01	0.69	0.28
Taurine	33.23	47.04	39.43	40.84	1.28	34.51	40.49	40.06	45.48	<0.01	0.44	<0.01	0.16	0.92
TSC[7]	70.65	97.37	82.47	85.95	1.93	80.52	84.03	83.93	87.57	<0.01	0.44	0.08	0.07	0.48
Met%TSC	28.44	31.4	31.09	28.74	0.72	29.65^{b}	32.35^{ab}	31.15^{b}	26.51^{c}	0.01	0.03	<0.01	0.74	0.24

[*]Significant ($P < 0.05$) parity effect observed. [#]β-aminobutyric acid undetectable

[1]Greatest SEM.

[2]MET × CHOL interaction was included in the supplemental file.

[3]Overall effect of MET supplementation.

[4]Overall effect of CHOL supplementation.

[5]Interaction of MET × time.

[6]Interaction of CHOL × time.

[7]Total sulfur-containing AAs and derivatives (methionine, cystathionine, cystine, homocystine, and taurine).

Table 5.3. Hepatic relative *PC*[#] and *PCK1* mRNA expression during the periparturient period in cows supplemented with or without rumen-protected MET and CHOL.

	MET		CHOL			Day				*P-value*[2]				
Genes	Without	With	Without	With	SEM[1]	-10	7	20	30	MET[3]	CHOL[4]	Time	M×T[5]	C×T[6]
PC	2.12	2.02	2.09	2.05	0.07	1.49^{c}	2.88^{a}	2.23^{b}	1.93^{d}	0.33	0.70	<0.01	0.28	0.34
PCK1	2.27	2.43	2.39	2.31	0.10	1.57^{c}	2.62^{ab}	2.74^{a}	2.69^{ab}	0.27	0.55	<0.01	0.48	0.46

[*]Significant ($P < 0.05$) parity effect observed. [#]*PC*=pyruvate carboxylase; *PCK1*=Phosphoenolpyruvate carboxykinase 1.

[1]Greatest SEM.

[2]MET × CHOL interaction was included in the supplemental file.

[3]Overall effect of MET supplementation.

[4]Overall effect of CHOL supplementation.

[5]Interaction of MET × time.

[6]Interaction of CHOL × time.

Table 5.4. Performance and immunometabolic status of multiparous Holstein cows during the periparturient period in cows supplemented with or without rumen-protected MET and CHOL.

	MET		CHOL			*P*-value		
Variables	With	Without	With	Without	SEM[1]	MET[2]	CHOL[3]	Time
Prepartum DMI (kg/d)	14.3[a]	13.2[b]	14.0	13.5	0.3	0.02	0.28	<0.01
Postpartum DMI (kg/d)	19.2[a]	17.2[b]	18.1	18.3	0.6	0.02	0.79	<0.01
Milk yield (kg/d)	44.2[a]	40.4[b]	41.4	43.2	1.2	0.02	0.27	<0.01
Milk protein (%)	3.32[a]	3.14[b]	3.27	3.19	0.04	<0.01	0.19	<0.01
Plasma albumin (g/L)	36.55	35.53	35.87	36.21	0.35	0.04	0.50	0.37
Plasma haptoglobin (g/L)	0.35	0.47	0.41	0.41	0.05	0.08	0.94	<0.01
Plasma paraoxonase	93.09	84.54	89.18	88.45	3.20	0.07	0.87	<0.01
Hepatic glutathione	62.83	23.32	39.61	46.55	10.59	0.01	0.64	<0.01
Neutrophil phagocytosis (%)	61.05	54.69	58.14	57.60	1.67	0.01	0.81	0.10
Neutrophil oxidative burst (%)	57.27	49.28	54.34	52.20	2.63	0.03	0.54	0.45

[1]Greatest SEM.

[2]Overall effect of MET supplementation.

[3]Overall effect of CHOL supplementation.

Figure 5.1.

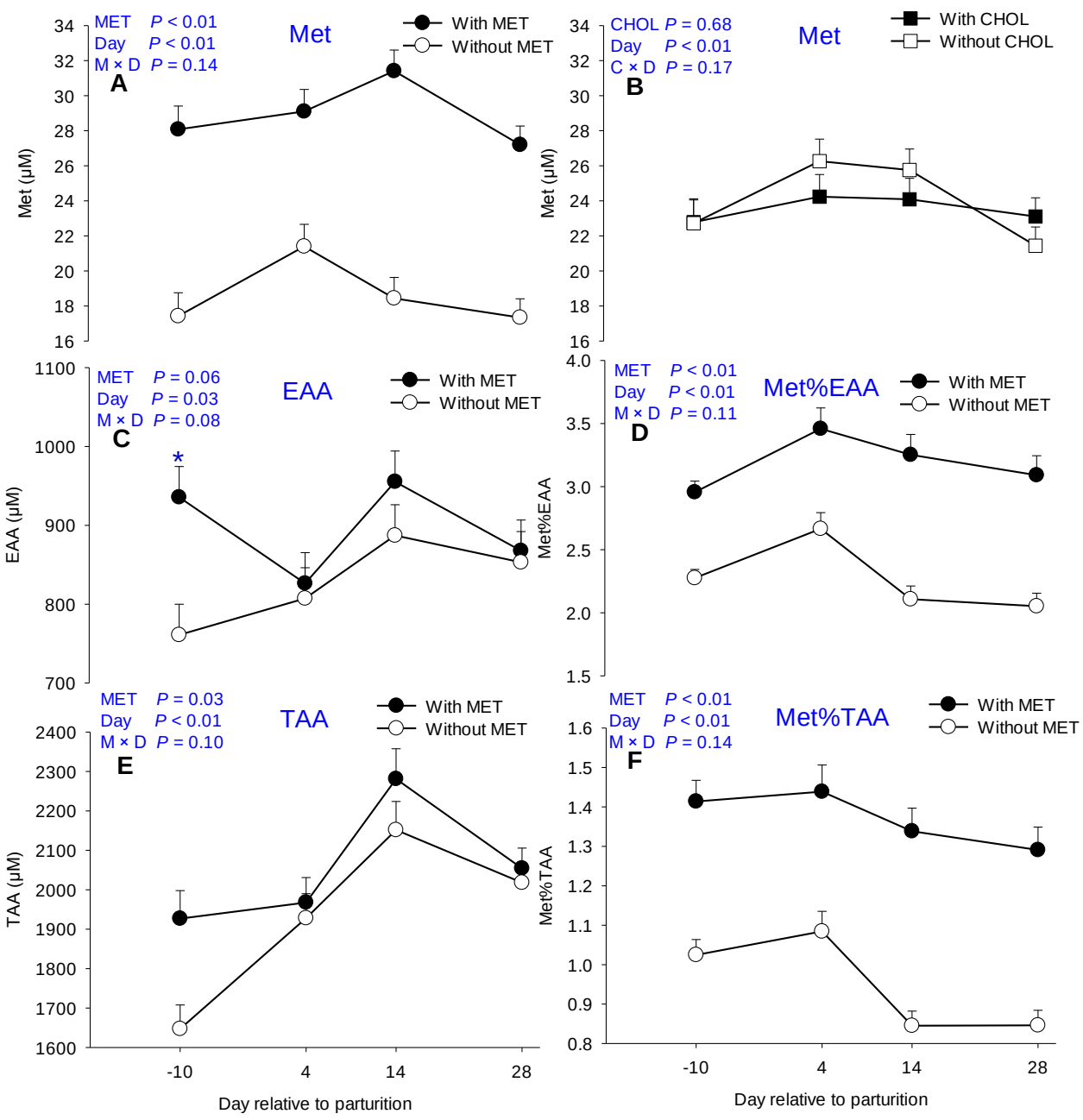

MET P < 0.01
Day P < 0.01
M × D P = 0.14
Met
With MET
Without MET
A
Met (μM)
CHOL P = 0.68
Day P < 0.01
C × D P = 0.17
Met
With CHOL
Without CHOL
B
MET P = 0.06
Day P = 0.03
M × D P = 0.08
EAA
C
*
EAA (μM)
MET P < 0.01
Day P < 0.01
M × D P = 0.11
Met%EAA
D
MET P = 0.03
Day P < 0.01
M × D P = 0.10
TAA
E
TAA (μM)
MET P < 0.01
Day P < 0.01
M × D P = 0.14
Met%TAA
F
-10
4
14
28
Day relative to parturition

Figure 5.2.

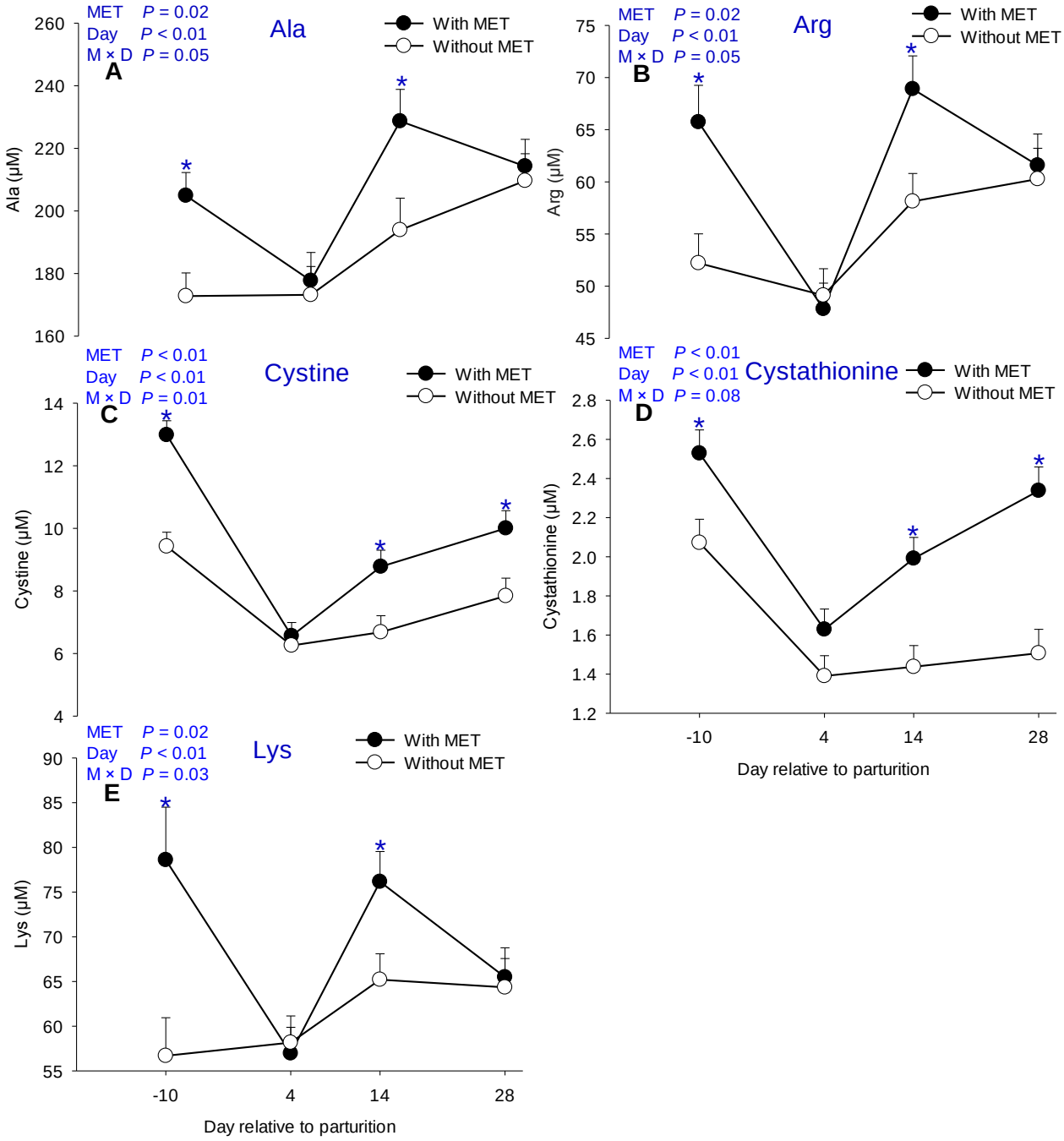

MET P = 0.02
Day P < 0.01
M × D P = 0.05
A
Ala
With MET
Without MET
Ala (μM)
260
240
220
200
180
160
MET P = 0.02
Day P < 0.01
M × D P = 0.05
B
Arg
With MET
Without MET
Arg (μM)
75
70
65
60
55
50
45
MET P < 0.01
Day P < 0.01
M × D P = 0.01
C
Cystine
With MET
Without MET
Cystine (μM)
14
12
10
8
6
4
MET P < 0.01
Day P < 0.01
M × D P = 0.08
D
Cystathionine
With MET
Without MET
Cystathionine (μM)
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
MET P = 0.02
Day P < 0.01
M × D P = 0.03
E
Lys
With MET
Without MET
Lys (μM)
90
85
80
75
70
65
60
55
-10
4
14
28
Day relative to parturition
-10
4
14
28
Day relative to parturition

Figure 5.3.

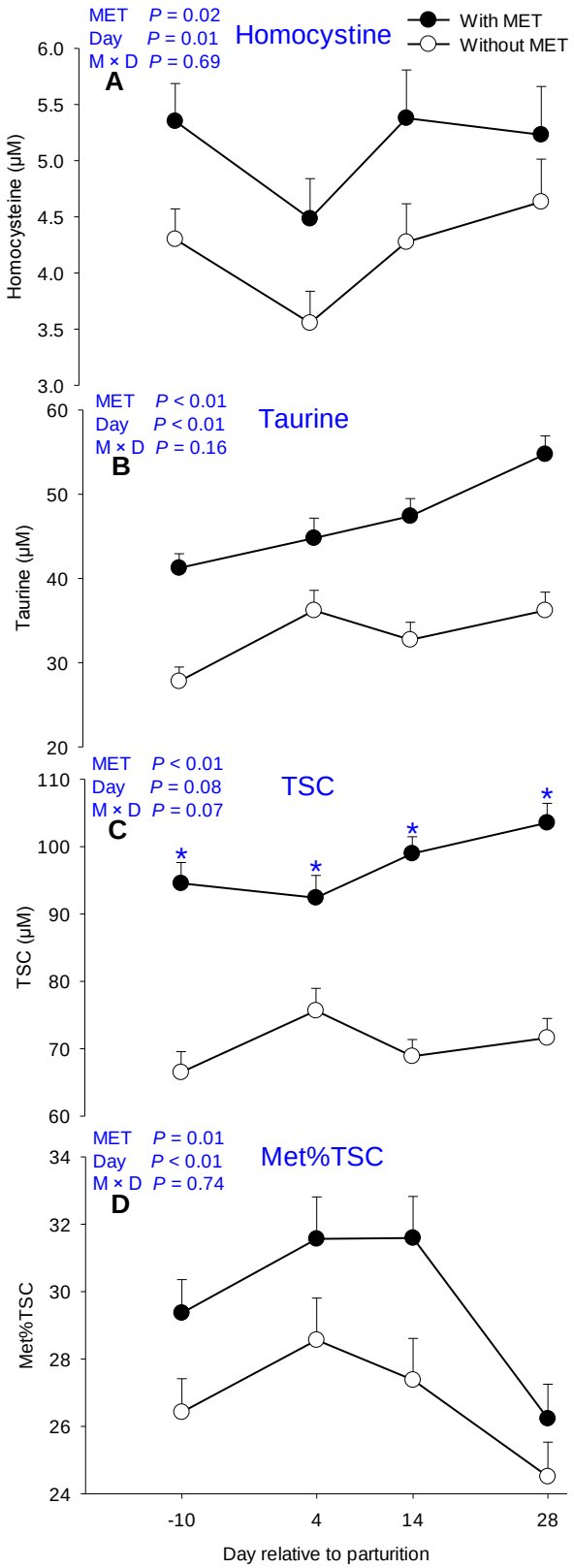

MET P = 0.02
Day P = 0.01
M × D P = 0.69
Homocystine
With MET
Without MET
A
Homocysteine (µM)
6.0
5.5
5.0
4.5
4.0
3.5
3.0
MET P < 0.01
Day P < 0.01
M × D P = 0.16
Taurine
B
Taurine (µM)
60
50
40
30
20
MET P < 0.01
Day P = 0.08
M × D P = 0.07
TSC
C
TSC (µM)
110
100
90
80
70
60
*
MET P = 0.01
Day P < 0.01
M × D P = 0.74
Met%TSC
D
Met%TSC
34
32
30
28
26
24
-10
4
14
28
Day relative to parturition

Figure 5.4.

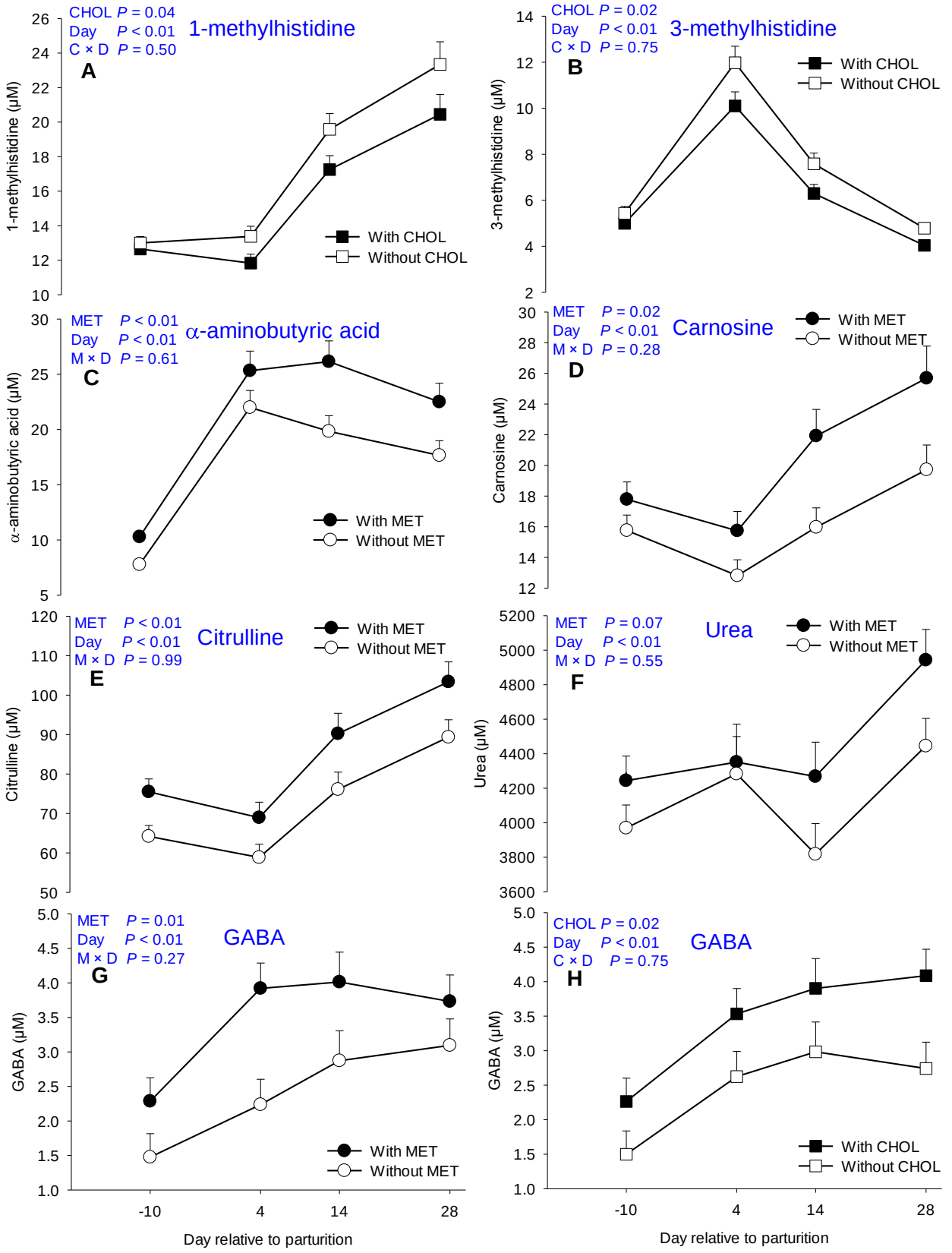

CHOL P = 0.04
Day P < 0.01
C × D P = 0.50
1-methylhistidine
A
1-methylhistidine (μM)
With CHOL
Without CHOL
CHOL P = 0.02
Day P < 0.01
C × D P = 0.75
3-methylhistidine
B
3-methylhistidine (μM)
With CHOL
Without CHOL
MET P < 0.01
Day P < 0.01
M × D P = 0.61
α-aminobutyric acid
C
α-aminobutyric acid (μM)
With MET
Without MET
MET P = 0.02
Day P < 0.01
M × D P = 0.28
Carnosine
D
Carnosine (μM)
With MET
Without MET
MET P < 0.01
Day P < 0.01
M × D P = 0.99
Citrulline
E
Citrulline (μM)
With MET
Without MET
MET P = 0.07
Day P < 0.01
M × D P = 0.55
Urea
F
Urea (μM)
With MET
Without MET
MET P = 0.01
Day P < 0.01
M × D P = 0.27
GABA
G
GABA (μM)
With MET
Without MET
CHOL P = 0.02
Day P < 0.01
C × D P = 0.75
GABA
H
GABA (μM)
With CHOL
Without CHOL
-10
4
14
28
Day relative to parturition
Day relative to parturition

Figure 5.1. Effects of supplementing multiparous Holstein cows during the periparturient period with rumen-protected methionine (MET; Smartamine M, Adisseo NA) or rumen-protected choline (CHOL; ReaShure, Balchem Inc.) on circulating methionine concentrations and proportion of methionine in total AA (TAA) and essential AA (EAA). Values are means, with standard errors represented by vertical bars.

Figure 5.2. Effects of supplementing multiparous Holstein cows during the periparturient period with rumen-protected methionine (MET; Smartamine M, Adisseo NA) on circulating alanine, arginine, cystine, cystathionine, and lysine concentrations. Values are means, with standard errors represented by vertical bars.

Figure 5.3. Effects of supplementing multiparous Holstein cows during the periparturient period with rumen-protected methionine (MET; Smartamine M, Adisseo NA) on circulating sulfur-containing compound concentrations and proportion of methionine in total sulfur-containing compound (TSC). Values are means, with standard errors represented by vertical bars.

Figure 5.4. Effects of supplementing multiparous Holstein cows during the periparturient period with rumen-protected methionine (MET; Smartamine M, Adisseo NA) or rumen-protected choline (CHOL; ReaShure, Balchem Inc.) on circulating citrulline and AA derivative concentrations. GABA = γ-aminobutyric acid Values are means, with standard errors represented by vertical bars.

Supplemental Table 5.1. Plasma proteinogenic AA concentrations during the transition period in cows supplemented with or without rumen-protected MET and CHOL.

	Treatments					*P*-value	
AA (μM)	CON[1]	SMA	REA	MIX	SEM[2]	M×C[3]	M×C×T[4]
Essential AA							
Arginine	54.61	61.5 8	54.89	59.31	2.52	0.61	0.31
Histidine	52.94	55.17	51.83	53.94	1.69	0.98	0.17
Isoleucine	97.84	103.50	93.36	103.72	6.28	0.69	0.99
Leucine	151.85	153.56	154.77	170.47	7.55	0.46	0.69
Lysine	59.28	67.94	62.74	69.55	3.56	0.75	0.82
Methionine	18.88	29.21	18.42	28.68	1.19	0.97	0.46
Phenylalanine	47.41[b]	44.40[b]	45.87[b]	49.94[a]	1.50	0.02	0.56
Threonine	75.65	76.20	69.46	81.13	3.56	0.12	0.52
Tryptophan	21.56	23.80	22.95	26.28	0.91	0.55	0.49
Valine	225.27	233.12	228.27	256.52	13.80	0.45	0.95
BCAA	476.13	491.42	477.22	531.31	29.33	0.49	0.91
EAA	831.48	866.95	822.75	925.45	35.85	0.08	0.32
Met%EAA	2.26	3.30	2.26	3.07	0.14	0.40	0.63
Non-essential AA							
Alanine	191.99	204.74	182.78	208.00	7.89	0.43	0.28
Asparagine	36.39	39.22	33.46	40.05	2.11	0.33	0.21
Aspartate	3.85	4.68	4.62	4.90	0.30	0.26	0.01
Cysteine/Cystine[*]	6.56	8.75	8.54	10.41	0.43	0.70	0.97
Glutamate	34.91	38.05	36.99	38.64	1.47	0.58	0.02
Glutamine	251.94	263.91	240.5	255.15	9.01	0.88	0.38
Glycine	434.39	420.4	419.27	392.03	21.53	0.76	0.95
Proline	72.63	75.44	71.97	81.04	2.88	0.29	0.26
Serine	90.31	87.02	82.94	90.81	3.23	0.09	0.20
Tyrosine	41.82[ab]	39.75[b]	39.82[b]	47.04[a]	2.17	0.04	0.10
NEAA	1141.09	1181.91	1104.44	1140.62	35.63	0.96	0.38
TAA	1959.00	2049.14	1910.98	2075.87	60.09	0.51	0.31
Met%TAA	0.94	1.39	0.94	1.34	0.06	0.67	0.69
Lys%TAA	3.07	3.43	3.34	3.48	0.15	0.13	0.99

[*]Significant ($P < 0.05$) parity effect observed.

[1]CON = control; SMA = Smartamine M (0.08% of DM); REA = ReaShure (60 g/d); MIX = SMA+REA.

[2]Greatest SEM.

[3]Interaction of MET × CHOL.

[4]Interaction of MET × CHOL × Time.

Supplemental Table 5.2. Plasma non-proteinogenic AA and AA derivatives concentrations during the transition period in cows supplemented with or without rumen-protected MET and CHOL.

	Treatments					*P*-value	
Parameter (μM)	CON[1]	SMA	REA	MIX	SEM[2]	M×C[3]	M×C×T[4]
AA and derivatives[#]							
1-methyl histidine	16.30	17.29	14.48	15.86	0.82	0.73	0.50
3-methyl histidine	6.85	7.09	6.06	5.92	0.42	0.63	0.90
α-aminoadipic acid	7.57	8.52	7.18	7.80	0.53	0.78	0.90
α-aminobutyric acid	15.99	20.56	15.31	19.04	1.22	0.78	0.61
β-alanine	9.08	8.54	9.04	9.19	0.45	0.45	0.71
γ-aminobutyric acid	1.86	3.07	2.99	3.91	0.42	0.73	0.59
Carnosine[*]	14.62	18.84	17.25	21.08	1.27	0.78	0.64
Citrulline	73.50	85.08	68.96	81.88	4.13	0.79	0.71
Glutathione	4.48	5.20	4.88	4.26	0.40	0.09	0.43
Hydroxylysine	0.34	0.27	0.30	0.28	0.06	0.62	0.90
Hydroxyproline	15.35	15.50	15.04	15.22	0.71	0.98	0.76
Ornithine	29.60	31.38	28.69	32.64	1.95	0.56	0.63
Phosphoserine	5.91	6.23	6.55	6.13	0.27	0.15	0.19
Sarcosine	11.35	11.65	9.95	11.20	0.83	0.54	0.88
Urea	4169.91	4483.16	4073.91	4402.78	176.54	0.95	0.73
Sulfur containing AA and derivatives							
Cystathionine	1.55	2.12	1.65	2.12	0.10	0.57	0.64
Homocystine	4.22	4.85	4.13	5.36	0.45	0.49	0.26
Taurine	30.57[c]	48.29[a]	35.89[b]	45.80[a]	1.81	0.04	0.53
TSAA[5]	63.05	92.02	69.16	93.04	2.63	0.35	0.16
Met%TSAA	30.27	31.92	26.61	30.88	1.06	0.21	0.94

[*]Significant ($P < 0.05$) parity effect observed. [#]β-aminobutyric acid undetectable

[1]CON = control; SMA = Smartamine M (0.08% of DM); REA = ReaShure (60 g/d); MIX = SMA+REA.

[2]Greatest SEM.

[3]Interaction of MET × CHOL.

[4]Interaction of MET × CHOL × Time.

[5]Total sulfur-containing AAs and derivatives (methionine, cystathionine, cystine, homocysteine, and taurine).

Supplemental Table 5.3. Hepatic short chain acyl coenzyme A (CoA) concentrations and relative *PC*[#] and *PCK1* mRNA expression during the transition period in cows supplemented with or without rumen-protected MET and CHOL.

	Treatments					*P*-value	
Parameter	CON[1]	SMA	REA	MIX	SEM[2]	M×C[3]	M×C×T[4]
CoAs (nmol/g)							
Acetyl-CoA[*]	1.46	0.91	0.47	0.45	0.45	0.49	0.34
Propionyl-CoA	4.14	1.34	3.02	1.34	0.92	0.46	0.40
Succinyl-CoA[*]	67.68^{cd}	216.24^{a}	121.89^{b}	91.57^{bc}	28.38	<0.01	0.13
Total CoA[*]	95.60^{b}	226.15^{a}	129.12^{b}	94.30^{b}	29.40	<0.01	0.08
Gene							
PC	2.13	2.05	2.11	1.99	0.11	0.87	0.77
PCK1	2.39	2.39	2.16	2.46	0.14	0.26	0.10

[*]Significant ($P < 0.05$) parity effect observed. [#]PC=pyruvate carboxylase; PCK1=Phosphoenolpyruvate carboxykinase 1.

[1]CON = control; SMA = Smartamine M (0.08% of DM); REA = ReaShure (60 g/d); MIX = SMA+REA.

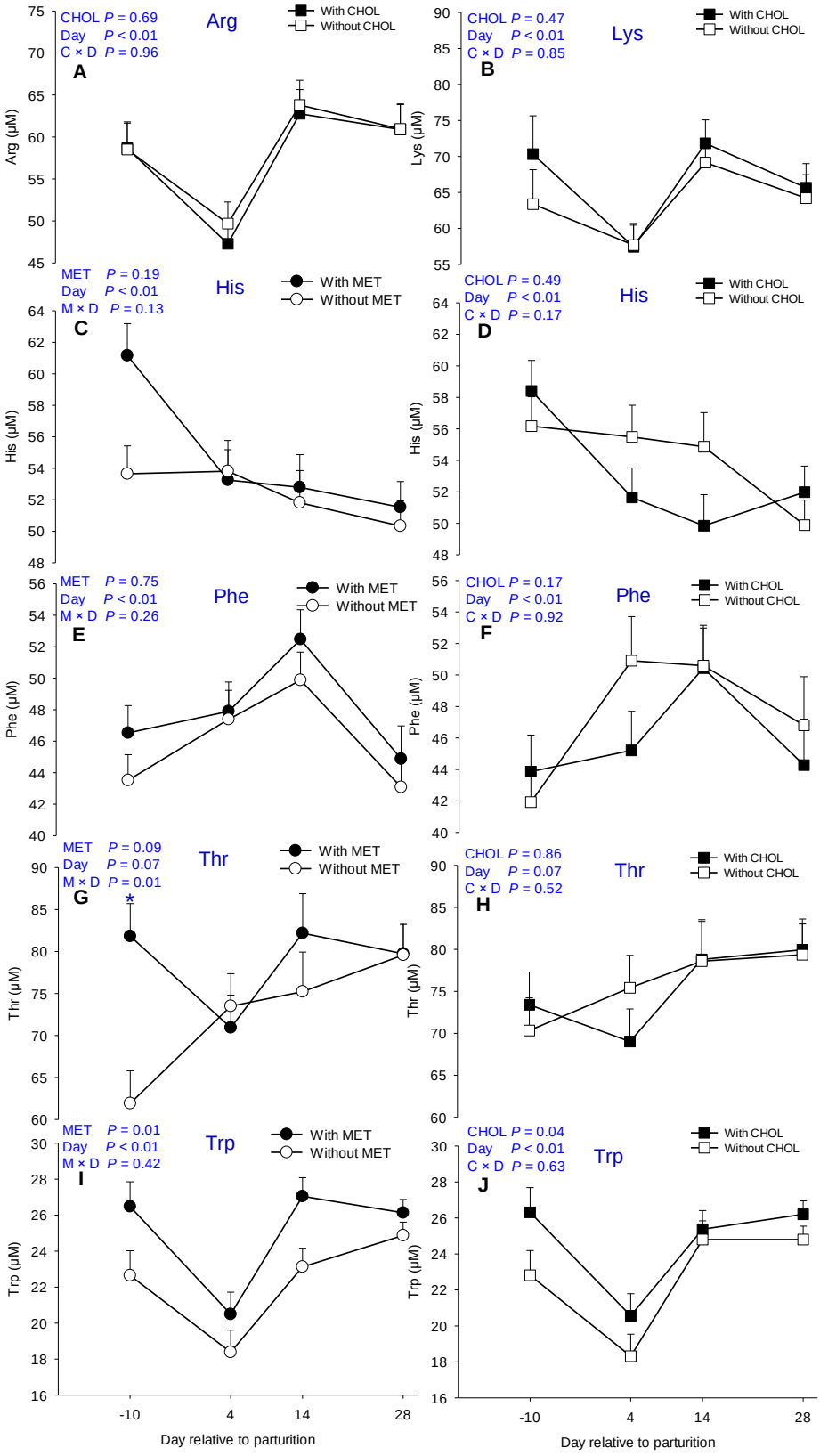
[2]Greatest SEM.

[3]Interaction of MET × CHOL.

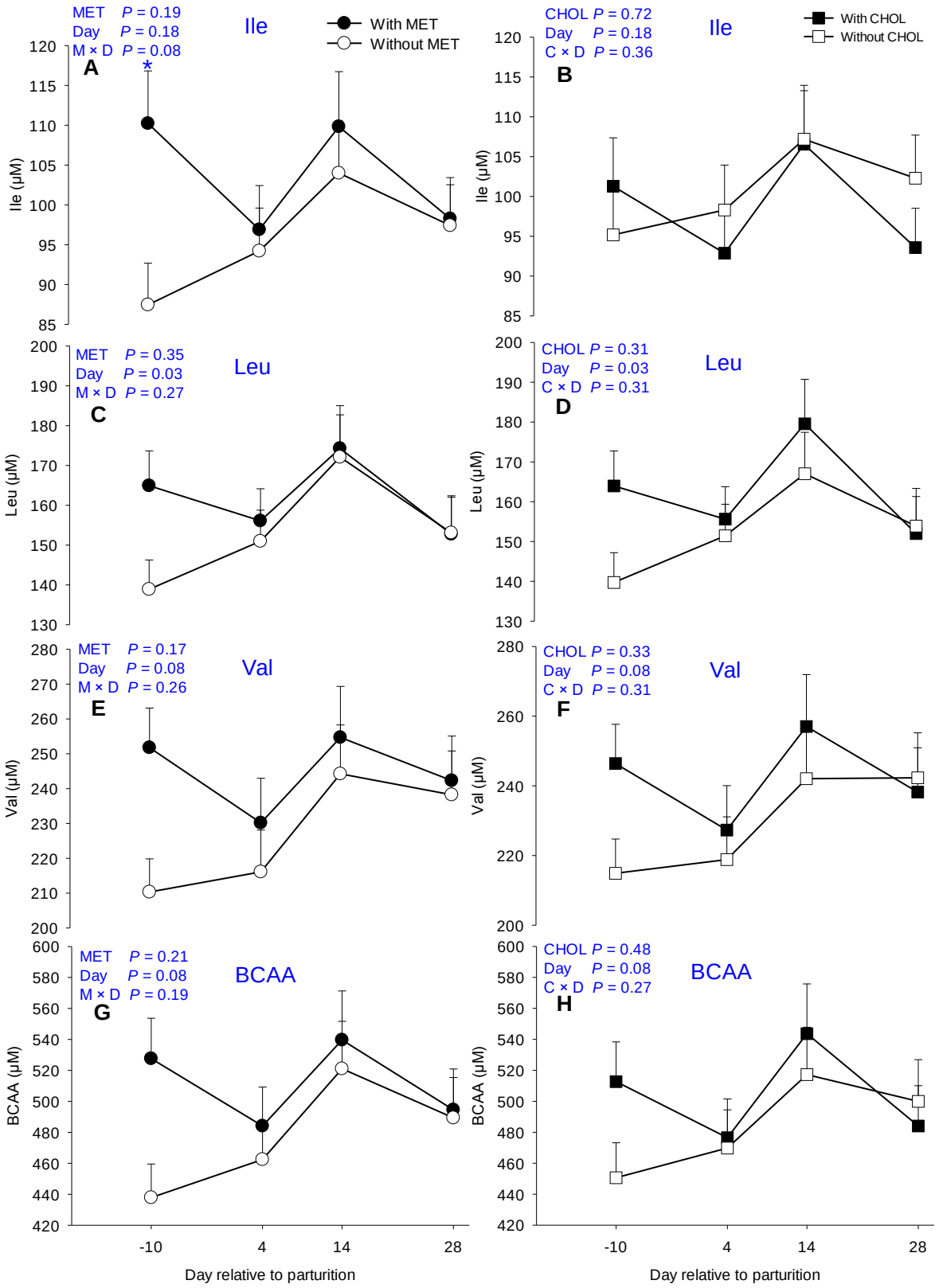
[4]Interaction of MET × CHOL × Time.

[5]Total sulfur-containing AAs and derivatives (methionine, cystathionine, cystine, homocysteine, and taurine).

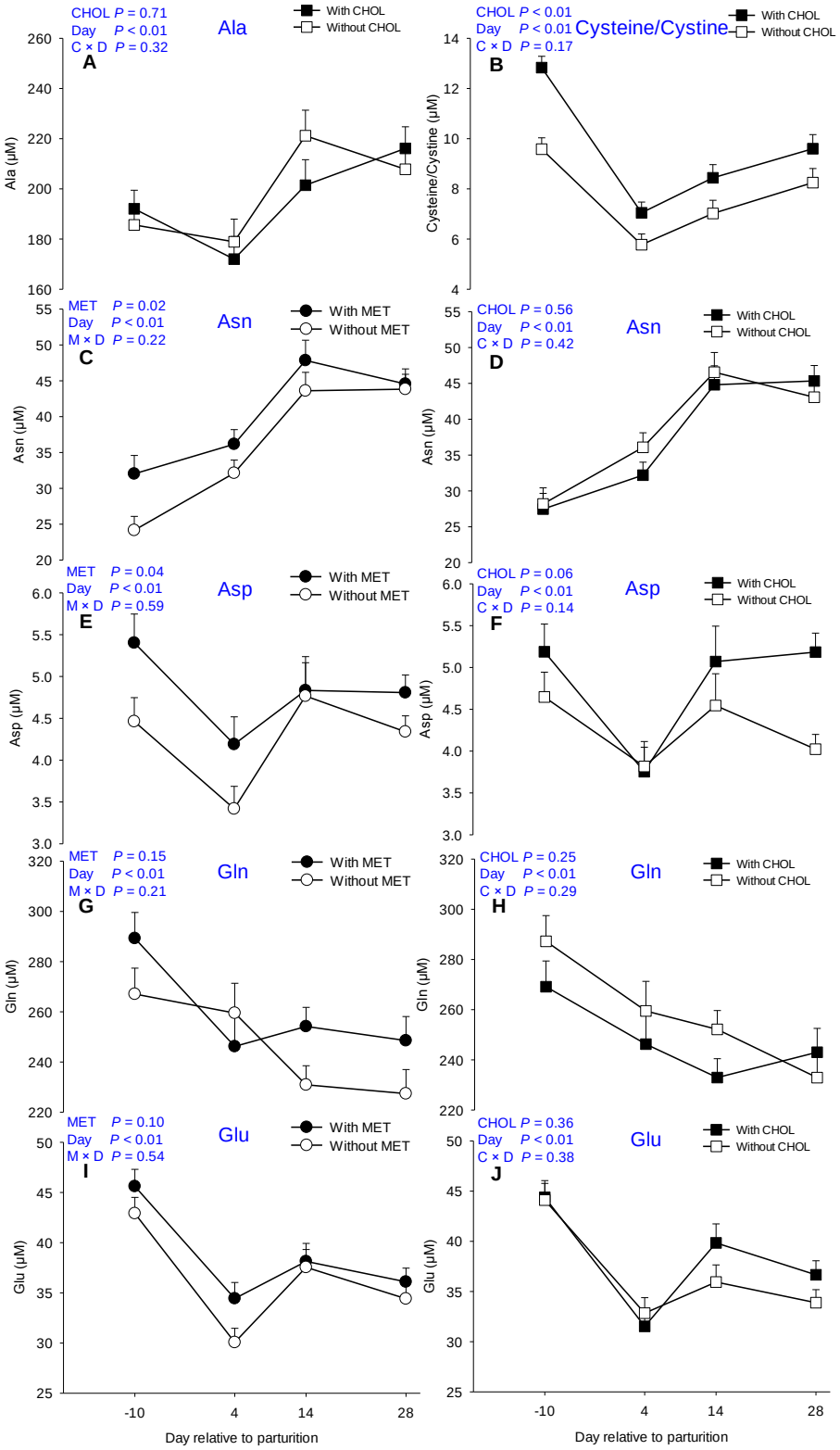
Supplemental Figure 5.1



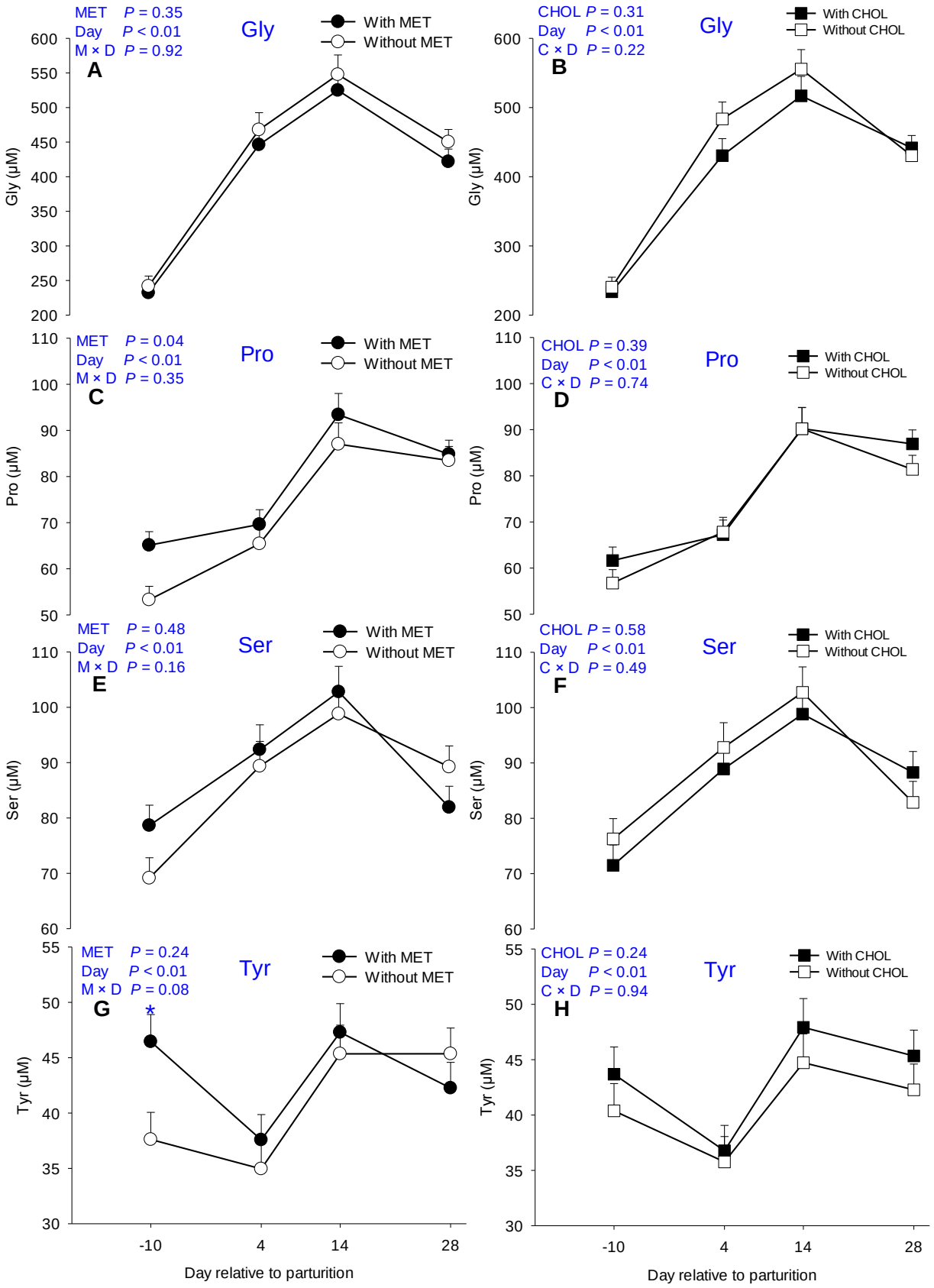
Supplemental Figure 5.2



Supplemental Figure 5.3



Supplemental Figure 5.4



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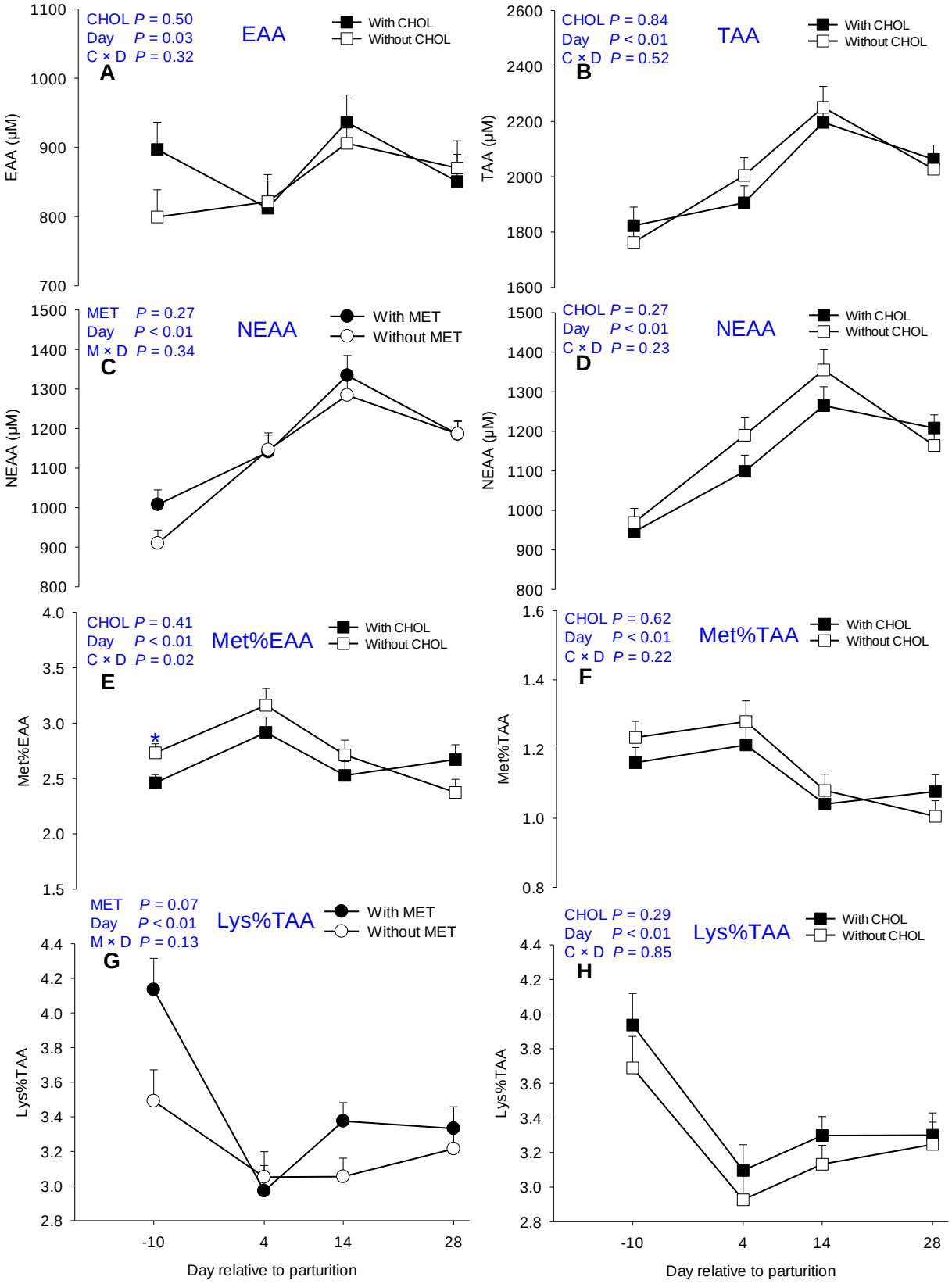
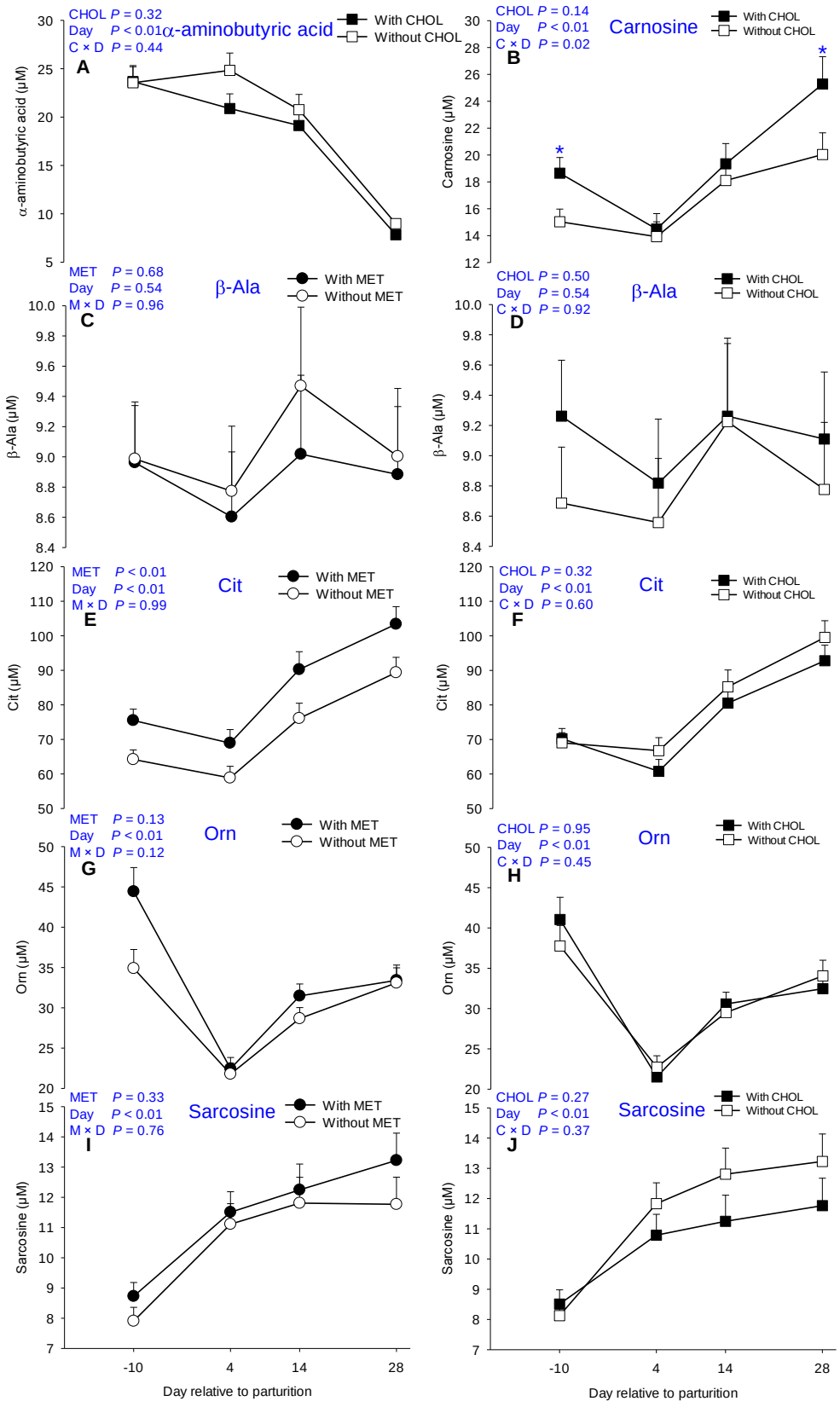
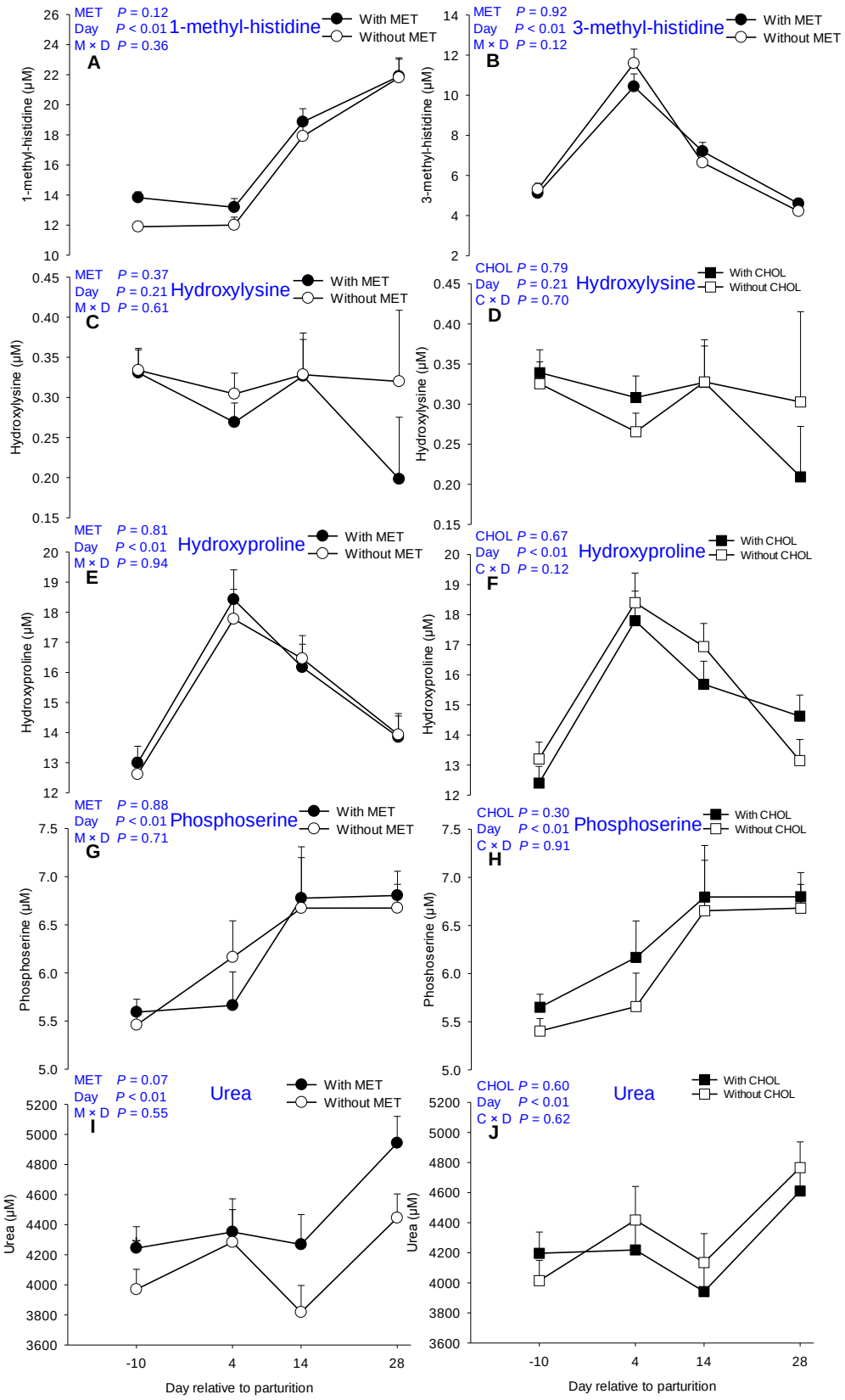


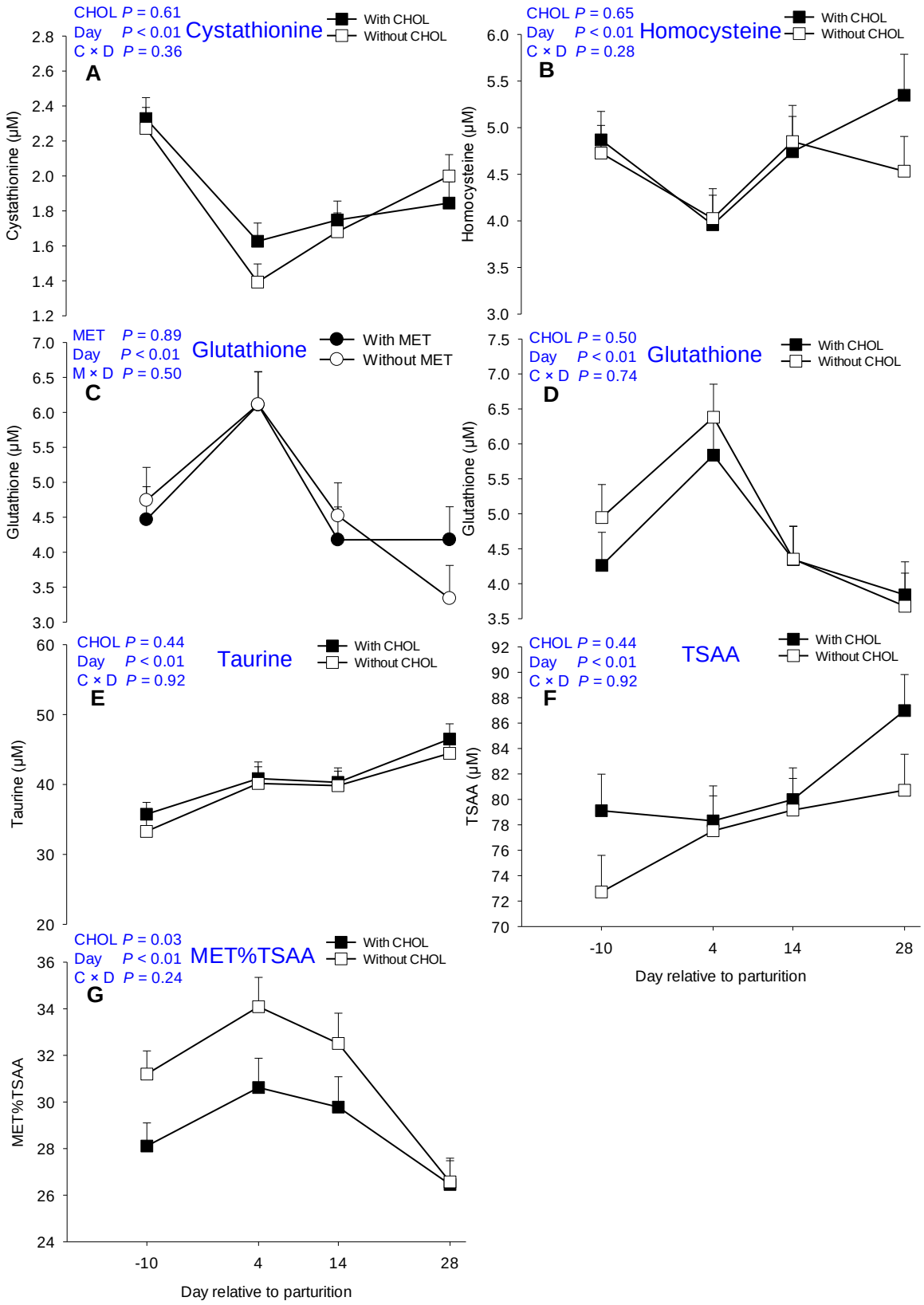
Supplemental Figure 5.6



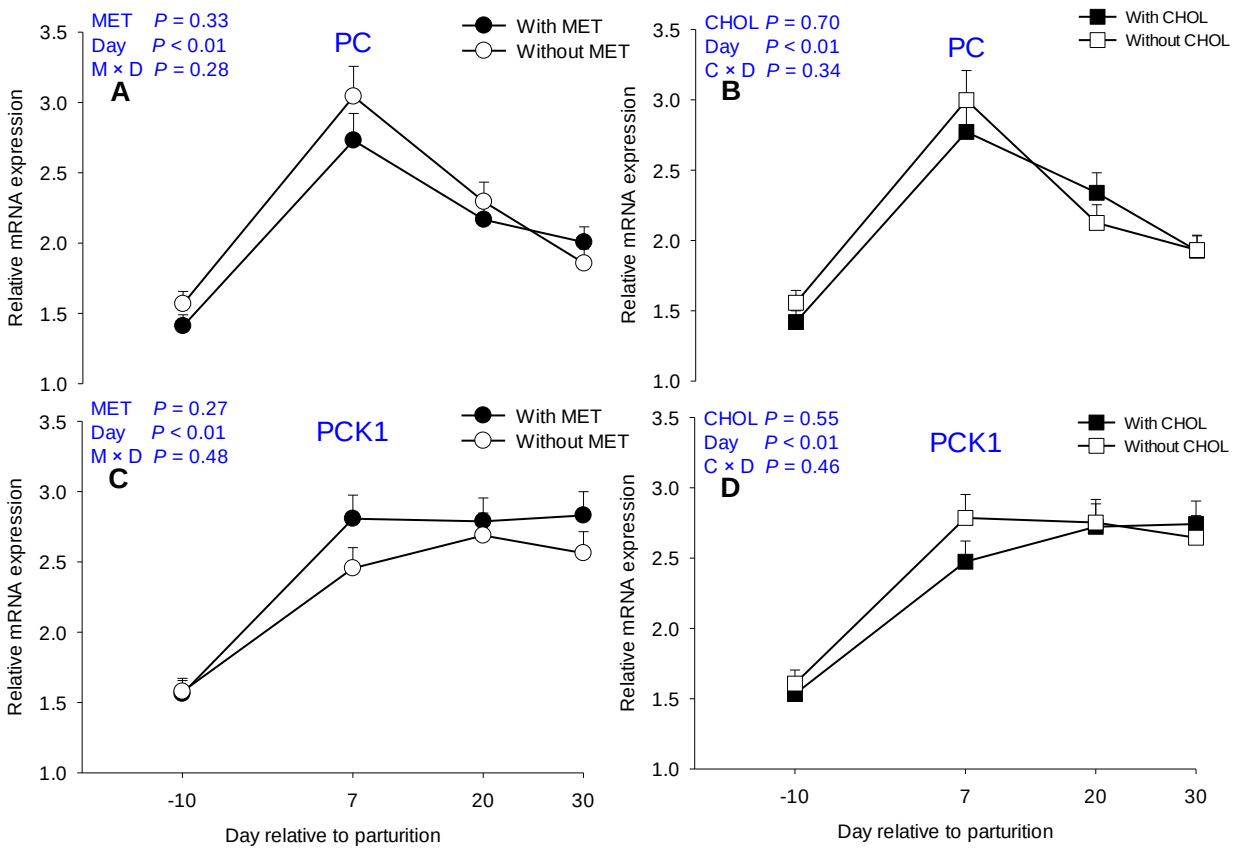
Supplemental Figure 5.7



Supplemental Figure 5.8



Supplemental Figure 5.9



Supplemental Figure 5.1. Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected methionine (MET; Smartamine M, Adisseo NA) or rumen-protected choline (CHOL; ReaShure, Balchem Inc.) on circulating arginine, lysine, histidine, phenylalanine, threonine, and tryptophan. Values are means, with standard errors represented by vertical bars.

Supplemental Figure 5.2. Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected methionine (MET; Smartamine M, Adisseo NA) on circulating valine, leucine, isoleucine, and branch-chained amino acids (BCAA) concentrations. Values are means, with standard errors represented by vertical bars.

Supplemental Figure 5.3. Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected methionine (MET; Smartamine M, Adisseo NA) on circulating alanine, cysteine/cystine, asparagine, aspartate, glutamine, and gluatamate concentrations. Values are means, with standard errors represented by vertical bars.

Supplemental Figure 5.4. Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected methionine (MET; Smartamine M, Adisseo NA) or rumen-protected choline (CHOL; ReaShure, Balchem Inc.) on circulating glycine, proline, serine, and tyrosine concentrations. Values are means, with standard errors represented by vertical bars.

Supplemental Figure 5.5. Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected methionine (MET; Smartamine M, Adisseo NA) or rumen-protected choline (CHOL; ReaShure, Balchem Inc.) on circulating essential amino acids (EAA), non-essential amino acids (NEAA), total amino acids (TAA) concentrations, and percentage of methionine in EAA, percentage of methionine in TAA, and percentage of lysine in TAA. Values are means, with standard errors represented by vertical bars.

Supplemental Figure 5.6. Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected methionine (MET; Smartamine M, Adisseo NA) or rumen-protected choline (CHOL; ReaShure, Balchem Inc.) on circulating α-amino butyric acid, β-alanine, carnosine, citrulline, and ornithine concentrations. Values are means, with standard errors represented by vertical bars.

Supplemental Figure 5.7. Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected methionine (MET; Smartamine M, Adisseo NA) or rumen-protected choline (CHOL; ReaShure, Balchem Inc.) on circulating 1-methyl histidine, 3-methyl histidine, hydroxylysine, hydroxyproline, phosphoserine, and urea concentrations. Values are means, with standard errors represented by vertical bars.

Supplemental Figure 5.8. Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected methionine (MET; Smartamine M, Adisseo NA) or rumen-protected choline (CHOL; ReaShure, Balchem Inc.) on circulating cystathionine, homocystine, glutathione, taurine, total sulfur-containing amino acids (TSAA) concnetrantions, and percentage of methionine in TSAA. Values are means, with standard errors represented by vertical bars.

Supplemental Figure 5.9. Effects of supplementing multiparous Holstein cows during the peripartal period with rumen-protected methionine (MET; Smartamine M, Adisseo NA) or rumen-protected choline (CHOL; ReaShure, Balchem Inc.) on relative mRNA expression of *pyruvate carboxylase (PC)* and *phosphoenolpyruvate carboxykinase 1 (PCK1)*. Values are means, with standard errors represented by vertical bars.

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CHAPTER 6

DIFFERENCES IN LIVER FUNCTIONALITY INDICES IN PERIPARTAL DAIRY COWS SUPPLEMENTED WITH RUMEN-PROTECTED METHIONINE OR CHOLINE ARE ASSOCIATED WITH PERFORMANCE, OXIDATIVE STRESS STATUS, AND PLASMA AA PROFILES

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INTRODUCTION

Around parturition, the metabolic stress inflicted by negative energy and nutrient balance contributes greatly to the occurrence of most metabolic and infectious diseases of dairy cows during the first few weeks of lactation (Goff and Horst, 1997). In fact, various diseases manifested at later stages of lactation also are related to metabolic insults during the peripartal period (Bertoni et al., 2008). It is noteworthy that metabolic stress around parturition may impair production and reproduction performance of dairy cows even in the absence of serious infections or other pathologies (Bionaz et al., 2007). Hence, assessing metabolic status around parturition can help diagnose nutritional management-related issues in dairy farms.

Although metabolic profiling can be a useful diagnostic tool to prevent metabolic disorders, it has not been routinely adopted owing to cost and insensitivity to nutritional inadequacies (Bertoni and Trevisi, 2013). In contrast, a composite index such as the liver functionality index (**LFI**) using changes in plasma concentrations of several blood biomarkers (i.e. albumin, cholesterol, and bilirubin) has promise for diagnosis and correction of management and nutritional problems on dairy farms (Bertoni and Trevisi, 2013). In fact, results from previous work have demonstrated the applicability of the LFI to assess immune and

inflammatory status as well as metabolic profiles in the periparturient period (Calamari et al., 2014, Zhou et al., 2016b). For instance, a low LFI (**LLFI**) is indicative of a pronounced inflammatory response and less favorable circulating AA profile, which together is suggestive of a more difficult transition from gestation to lactation. In contrast, a high LFI (**HLFI**) is suggestive of a smooth transition (Trevisi et al., 2012).

It is noteworthy that feed additives such as rumen-protected methionine (**MET**) and choline (**CHOL**) and ionophores are often supplemented to transition dairy cows in an effort to improve not only essential AA and gluconeogenic precursor availability but also hepatic fatty acid metabolism. As such, these approaches result in physiologic profiles associated with a smoother transition into lactation (Zahra et al., 2006, Swyers et al., 2014, Osorio et al., 2016). Although it is possible to assess changes in metabolic and inflammatory status in response to feed additive supplementation (Batistel et al., 2016, Osorio et al., 2016, Zhou et al., 2016a, Zhou et al., 2016c), the cost and time needed to obtain results make it a less desirable approach for evaluating effectiveness of feed additives. In addition, due to differences in nutrition and management strategies among farms, variation in effectiveness of such feed additives are commonly observed even among research conducted in well-controlled environments (Pinotti et al., 2003, Guretzky et al., 2006, Chen et al., 2011, Zom et al., 2011, Osorio et al., 2013, Leiva et al., 2015).

A composite index such as the LFI, which can be determined at lower cost without invasive procedures, can potentially help evaluate effectiveness of feed additives within a farm-specific environment. However, it is unknown whether feed additive-induced changes in metabolic and inflammatory profiles of transition dairy cows are manifested in the LFI. Our general hypothesis was that favorable changes in response to peripartal MET and CHOL

supplementation lead to higher HLFI, indicating a more favorable immunometabolic status around parturition. Therefore, the objectives of the present study were to determine 1) if MET and/or CHOL alter the LFI response in cows; and 2) if HLFI and LLFI cows have distinct oxidative stress status in addition to lactation performance and plasma AA profiles.

Materials and Methods

Experimental design and treatments

All procedures for this study (protocol no. 13023) were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Illinois. Details of the experimental design and the feeding regime (nutrient composition and evaluation) have been described previously (Zhou et al., 2016c). Briefly, the experiment was conducted as a randomized, complete, unbalanced, block design with 2×2 factorial arrangement of MET (Smartamine M, Adisseo NA, Alpharetta, GA) and CHOL (ReaShure, Balchem Inc, New Hampton, NY) level (with or without) using a total of 81 cows. Cows within each block were balanced for parity, previous lactation milk yield, and BCS before the close-up diet groups were assigned. Per IACUC guidelines, only half of the cows in each treatment were used for routine blood sampling. Thus, for the present study a total of 40 cows were used. Treatments were control (**CON**, n = 10), with no MET or CHOL supplementation; Smartamine (**SMA**, n = 10), CON plus MET at a rate of 0.08% of DM; Reashure (**REA**, n = 10), CON+CHOL at 60 g/d; or Smartamine and Reashure (**MIX**, n = 10), CON+MET+CHOL. Dosage of MET was based on Osorio et al. (2013), whereas CHOL was supplemented following the manufacturer's recommendations. All cows received the same far-off diet from -50 to -22 d before expected parturition, close-up diet from -21 d to expected parturition, and lactation diet from parturition through 30 days in milk (**DIM**). The MET and CHOL supplements were both top-dressed from -

21 ± 2 to 30 DIM once daily at the AM feeding using approximately 50 g of ground corn as carrier for all treatments. Supplementation of SMA (0.08% DM of TMR offered) was calculated daily for each cow. Smartamine M was supplied as small beads containing a minimum of 75% DL-Met, physically protected by a pH sensitive coating, which is considered to have a Met bioavailability of 80% (Graulet et al., 2005); therefore, per 10 g of SMA, the cows received 6 g of metabolizable Met. With MET supplementation, the amount of Met in prepartal diets increased from 1.86% to 2.37% of the MP. Similarly, the amount of Met in postpartal diets also increased from 1.79% to 2.30% of the MP. The REA supplement is reported to contain 28.8% choline chloride and is protected by microencapsulation. Although the product is considered to have CHOL bioavailability of 72% (Benoit, 2009), i.e. per 60 g of REA the cows should have received 12.4 g of metabolizable choline chloride, a recent study estimated the relative bioavailability (i.e. net absorption of choline in ReaShure from intestine) at 8% (de Veth et al., 2016). Thus, using that value to calculate the amount of choline that was bioavailable from ReaShure in our study gives 1.03 g free choline (60 g/d ReaShure × 28.8% choline-chloride (CC) content × 74.6% choline in CC × 8% bioavailability. Clearly, these discrepancies should be taken into account when evaluating the present data in the context of choline adequacy. To our knowledge, neither SMA nor REA have specific characteristics that may affect palatability of diets.

The LFI was determined for all cows based on plasma concentration of albumin, cholesterol and bilirubin as described in Bertoni and Trevisi (2013), and this used to rank the cows retrospectively. The LFI calculation was carried out in two steps; the first one considers the concentration values (V) of the three parameters detected on d 3 (V3) and changes in concentrations between d 3 and 28 (V28). The albumin and cholesterol index was calculated as

0.5*V3 + 0.5*(V28-V3), with albumin and cholesterol level on d 3 postpartum representing 50% and the reduction between d 3 and 28 the remaining 50% of the partial LFI index. Similarly, the bilirubin index was calculated using concentration value as 0.67V3 + 0.33 (V28-V3), with bilirubin level on d 3 postpartum representing 67% and the reduction between d 3 and 28 the remaining 33% of the partial LFI index. In the second step, the LFI was calculated according to the following formula: LFI = [(albumin index - 17.71)/1.08 + (cholesterol index - 2.57)/0.43-(bilirubin index - 6.08)/2.17] (Zhou et al., 2016b). Cows were ranked retrospectively according to LFI and assigned to a low LFI group (**LLFI**), and a high LFI group (**HLFI**) regardless of MET or CHOL supplementation. Cows with a positive LFI (31 cows, mean ± SEM: 2.04 ± 0.24) were considered as the HLFI group and cows with a negative LFI (9 cows, mean ± SEM: -3.63 ± 0.76) were considered as the LLFI group.

Animal management

Full details have been reported previously (Zhou et al., 2016c). Briefly, dry cows were housed in a ventilated enclosed barn during the dry period and fed individually once daily at 0630 h using an individual gate system (American Calan Inc., Northwood, NH). After parturition, cows were housed in a tie-stall barn and were fed a common lactation diet once daily. Feed offered was adjusted daily to achieve ~10% refusals.

Feed and Milk Sample Collection and Analyses

Full details have been reported previously (Zhou et al., 2016c). Briefly, dry matter intake and milk yield were recorded daily. Consecutive morning, midday, 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, MUN, and SCC by mid-infrared procedures in a commercial laboratory (Dairy Lab Services, Dubuque, IA).

Blood and Liver Sample Collection and Biomarker Analyses

Full details have been reported previously (Zhou et al., 2016a). Briefly, blood was sampled for biomarker analysis from the coccygeal vein on -10 d relative to expected calving date and on 4, 8, 20, and 30 d relative to actual calving date before the morning feeding. For plasma AA and derivative analyses, blood was sampled from the coccygeal vein on -30 and -10 d relative to expected parturition date and on 4, 14 and 28 d relative to actual parturition date before the morning feeding. Samples were collected into evacuated tubes (BD Vacutainer; BD and Co., Franklin Lakes, NJ) containing either clot activator or lithium heparin for serum and plasma, respectively. Liver was sampled via puncture biopsy from cows under local anesthesia at approximately 0800 h on d -10, 7, 20, and 30 d relative to parturition (Zhou et al., 2016a). Liver was frozen immediately in liquid nitrogen and stored until analysis.

Plasma was analyzed for creatinine, bilirubin, aspartate aminotransferase (**GOT**), gamma-glutamyl transferase (**GGT**), cholesterol, paraoxonase (**PON**), albumin, ceruloplasmin, haptoglobin, myeloperoxidase (**MPO**), reactive oxygen metabolites (**ROM**), and ferric reducing ability of plasma (**FRAP**) using kits purchased from Instrumentation Laboratory (Lexington, MA) following the procedures described previously (Jacometo et al., 2015) using the clinical auto-analyzer (ILAB 600, Instrumentation Laboratory). Bovine IL-1β (Cat. No. ESS0027; Thermo Scientific, Rockford, IL) plasma concentration was determined using commercial kits. Plasma total glutathione (GSH) as well as liver total and reduced GSH were measured using a commercial kit (Cat. No. NWH-GSH01; Northwest Life Science Specialties LLC, Vancouver, WA).

Analyses of Plasma AA and their Derivatives

Plasma was used to analyze the concentrations of free Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val, Asn, Asp, Ala, Glu, Gln, Gly, Pro, Ser, Tyr, Cit, carnosine, Orn, Sar, cystathionine, cystine, homocystine, taurine, α-aminoadipic acid, α-aminobutyric acid, β-alanine, γ-aminobutyric acid (**GABA**), hydroxylysine, hydroxyproline, phosphoserine, 1-methyl histidine, and 3-methyl histidine at the University of Missouri Agriculture Experiment Station Chemical Laboratories (Columbia, MO) using high-performance liquid chromatography (Deyl et al., 1986, Fekkes, 1996). The essential AA (**EAA**) pool included Arg + His + Ile + Leu + Lys + Met + Phe + Thr + Trp + Val; the non-essential AA (**NEAA**) pool included Asn + Asp + Ala + Gln + Glu + Gly + Pro + Ser + Tyr; total AA (**TAA**) was the sum of EAA and NEAA; the total sulfur-containing compounds (**TSC**) included Met + cystine + cystathionine + homocystine + taurine + GSH.

Statistical analysis

Data were assessed for normality of distribution using the Shapiro-Wilk test. When the normality assumption was rejected, data were log-transformed before statistical analysis. Back-transformed data were reported in Tables and Figures for ease of interpretation. Data were analyzed using PROC MIXED of SAS (SAS Institute Inc., Cary, NC). The model for data analysis contained LFI, day, and their interactions as fixed effects, and cow nested within LFI as random effect. For the analysis of all variables, parity was kept in the model as covariate when significant. For the analysis of various AA and derivatives, concentrations obtained at -30 d were maintained in the model as covariates when significant ($P < 0.05$). Amino acids and biomarkers in plasma and liver were analyzed at various time points that were not equally spaced. Therefore, the first order ante-dependence covariance structure ANTE (1) were used for repeated measures.

Frequency of MET- and CHOL-supplemented cows in the HLFI or LLFI group and health data were analyzed with PROC GLIMMIX (distribution = binary and link = logit) of SAS. Least square means separation between time points was performed using the PDIFF statement. Statistical differences were declared significant at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

RESULTS

Distribution of MET- and/or CHOL- supplemented cows in LFI groups

Distribution MET and/or CHOL supplemented cows in LLFI and HLFI groups is summarized in Table 6.1. Among the 20 MET-supplemented cows, only 2 cows had an LFI < 0. In contrast, 7 cows without MET supplementation during the peripartal period were in the LLFI. Hence, although the majority (65%) of cows without MET supplementation ended up with a HLFI, a tendency for a greater ($P = 0.06$) number of MET-supplemented cows in the HLFI was observed. In contrast, peripartal CHOL supplementation resulted in a similar number of cows in the LLFI (4 vs. 5) and HLFI (15 vs. 16).

Animal Health and Performance

Health-related problems that occurred during the experiment and production performance are summarized in Table 6.2 and Table 6.3, respectively. As expected, cows with LLFI had greater incidence of health-related problems, as indicated by more cases of ketosis ($P < 0.01$) and a tendency for more cases of displaced abomasum ($P = 0.06$, 1 vs. 0). Both prepartal ($P < 0.01$; Figure 6.1A) and postpartal ($P < 0.01$; Figure 6.1B) DMI was greater in cows with HLFI. Similarly, compared with LLFI cows, overall milk yield ($P < 0.01$; Figure 6.1C), milk protein yield ($P < 0.01$), and milk fat yield ($P = 0.02$) were also greater in cows with HLFI.

Biomarkers of muscle mass, inflammation, liver function, and oxidative stress

Effects of LFI on blood and liver biomarkers are summarized in Table 6.4. Although plasma BHBA did not differ between LLFI and HLFI cows, a tendency ($P = 0.09$) for lower fatty acid concentrations was observed in HLFI cows. Greater ($P = 0.05$; Figure 6.2A) circulating 3-methyl histidine concentration was observed in LLFI cows. In agreement with 3-methyl histidine, a tendency for lower ($P = 0.06$; Figure 6.2B) creatinine concentration was also shown in plasma of LLFI cows. As expected, greater albumin ($P < 0.01$; Figure 6.2C) and cholesterol ($P < 0.01$; Figure 6.2D) as well as lower bilirubin ($P = 0.05$; Figure 6.2E) were observed in HLFI cows. In addition, overall plasma haptoglobin concentration also was lower ($P = 0.02$; Figure 6.2F) in HLFI cows. Although overall circulating concentration did not differ between LLFI and HLFI cows, an LFI×day interaction ($P < 0.01$) was observed for ceruloplasmin, which increased to a greater extent in cows with LLFI on d 30 postpartum.

Among the oxidative stress biomarkers determined in the present study, HLFI cows had greater ($P < 0.01$; Figure 6.2G) overall circulating PON concentrations compared with LLFI cows. In agreement with PON, overall hepatic total ($P = 0.05$) and reduced ($P = 0.05$; Figure 6.2H) GSH levels in HLFI cows were doubled compared with cows in the LLFI group. Although overall plasma FRAP values did not differ between HLFI and LLFI cows, an LFI×day interaction ($P < 0.01$) was observed due to a greater level in LLFI cows on d 30 postpartum.

AA and derivatives

Effects of LFI on plasma AA and derivatives are summarized in Table 6.5 and Table 6.6, respectively. Although similar ($P > 0.05$) levels of circulating EAA were observed in LLFI and HLFI cows, HLFI cows had greater circulating His ($P = 0.02$) and Trp ($P = 0.03$) as well as a tendency ($P = 0.06$) for greater plasma Ile. Similarly, plasma Met also was greater ($P = 0.01$; Figure 6.3D) in cows with HLFI owing to more ($P = 0.06$) MET-supplemented cows in this

group. Although overall plasma Arg did not differ between HLFI and LLFI cows, an LFI×day interaction ($P = 0.10$) was observed due to greater level of Arg on d 4 in HLFI cows. In contrast, an LFI×day interaction ($P = 0.02$) was observed due to a lower level of Phe on d 28 in HLFI cows.

Unlike EAA, circulating concentrations of most NEAA were either greater (Ala, Asp, Gln, Pro, and Ser, $P < 0.05$) or tended to be greater (Tyr, $P = 0.09$) in HLFI cows. In fact, despite similar circulating levels of EAA between LLFI and HLFI cows, overall circulating TAA was greater ($P = 0.01$; Figure 6.3A) in HLFI cows owing mainly to greater ($P = 0.01$; Figure 6.3B) overall circulating NEAA concentrations in these cows. It is noteworthy that HLFI cows also tended to have greater ($P = 0.08$) overall percentage of Met in the total AA pool (Met%TAA). In contrast, an LFI×day interaction was observed due to a lower ($P = 0.03$) percentage of Lys in the total AA pool (Lys%TAA) on d 4 in HLFI cows.

As expected, greater ($P < 0.01$; Figure 6.3F) homocystine and a tendency ($P = 0.07$; Figure 6.3E) for greater cystathionine were observed in HLFI cows likely due to more MET-supplemented cows in this group. Although overall taurine concentration was similar between HLFI and LLFI cows, an LFI×day interaction was observed due to a greater ($P = 0.03$) concentration of taurine on d -10 in HLFI cows (Figure 6.4). In addition, the proportion of Met in the TSC also was greater ($P = 0.05$; Figure 6.3C) in the HLFI group due to greater circulating Met in these cows. Consequently, overall circulating level of TSC also was greater in HFLI compared with LLFI cows.

Besides taurine, concentration of carnosine, another antioxidant, also was greater ($P = 0.01$) in cows with HLFI. Although overall plasma α-aminobutyric acid and γ-aminobutyric acid concentrations were similar between LLFI and HLFI cows, an LFI×day interaction was observed

due to higher concentrations of α-aminobutyric acid ($P = 0.01$) and γ-aminobutyric acid ($P = 0.03$) on d 14 in HLFI cows. Although a main effect of LFI for Orn was not observed, greater Cit ($P < 0.01$) and an interaction ($P = 0.10$) for greater circulating urea on 28 d were observed in HLFI cows in addition to greater circulating concentration of Arg on 4 d in HFLI cows.

DISCUSSION

Health and performance in cows with HLFI and LLFI

Compared with LLFI (LFI < 0), cows with HLFI (LFI > 0) are considered to be at a lesser risk of health problems during the peripartal period (Bertoni and Trevisi, 2013, Zhou et al., 2016b). The fact that none of the 31 cows in the HLFI group were ketotic or experienced displaced abomasum confirmed the usefulness, in the present study, of LFI in identifying cows at risk even when metabolically important feed additives such as MET or CHOL are supplemented around parturition. Considering MET supplementation led to a lower incidence of ketosis (Zhou et al., 2016c), and that more MET-supplemented cows were in the HLFI group, the overall lower incidence of ketosis observed in HLFI cows was likely due to MET supplementation.

In agreement with better lactation performance benefits observed in HLFI cows, recent results from our group have demonstrated greater pre- and post-partum DMI, milk fat yield, and milk protein yield in response to peripartal MET but not CHOL supplementation (Zhou et al., 2016c). Considering only a tendency for greater milk yield was detected in a previous LFI study that did not involve MET or CHOL supplementation (Zhou et al., 2016b), the differences observed between LLFI and HLFI cows were due in large part to peripartal MET supplementation.

Inflammation and oxidative stress in cows with HLFI and LLFI

Acute phase proteins (**APP**) of liver origin serve as useful biomarkers to evaluate chronic inflammation mainly due to the decrease of circulating concentrations of negative APP (e.g. albumin) and the contemporary increase of positive APP (e.g. haptoglobin) (Bertoni et al., 2008, Ceciliani et al., 2012). Therefore, the substantially greater plasma concentration of albumin (2.1 g/L) and lower haptoglobin (0.12 g/L) in HLFI cows was suggestive of a less pronounced inflammatory status around parturition.

Inflammatory biomarkers in plasma are increased in the circulation of ketotic cows around parturition (Abuajamieh et al., 2016). Hence, the fact that none of the cows in the HLFI group were ketotic may have greatly contributed to the differences in inflammation status observed between the two cohorts of cows. In addition, rodent research revealed that limiting MET and CHOL in ketogenic diets increased expression of fatty acid oxidation and inflammatory genes, supporting a potential role of MET and CHOL supplementation on ketosis-related inflammation (Pissios et al., 2013). In agreement with this, MET-supplemented cows experienced fewer cases of ketosis and had greater plasma concentrations of albumin, cholesterol (index of lipoproteins) and lower haptoglobin (Zhou et al., 2016a, Zhou et al., 2016c), suggesting MET supplementation had direct bearing on the lower inflammatory status in HLFI cows. In contrast, CHOL supplementation was not associated with changes in the incidence of ketosis or plasma albumin, cholesterol, and haptoglobin concentrations.

Apart from disease-induced inflammation around parturition, the lack of proper antioxidant defenses renders oxidative stress as another significant contributing factor to systemic inflammation (Sordillo et al., 2009). The fact that a hepatic intracellular antioxidant (GSH), a circulating antioxidant enzyme (PON), FRAP/ROM ratio, and a circulating dipeptide (carnosine) with antioxidant properties were all greater in HLFI cows indicated an overall less

pronounced state of oxidative stress (Bionaz et al., 2007, Boldyrev et al., 2013). From a mechanistic standpoint, the fact that in vivo synthesis of GSH ultimately requires sulfur from MET, support the view that the greater hepatic GSH and reduced GSH concentrations in HLFI cows were a result of the greater number of MET-supplemented cows in this group. In agreement with this, as a non-sulfur-containing compound, peripartal CHOL supplementation did not seem to increase hepatic sulfur-containing antioxidant synthesis through the transsulfuration pathway (Zhou et al., 2017a). In fact, an increase in plasma cystine in response to peripartal CHOL supplementation was likely achieved at the expense of MET (Zhou et al., 2017b). As such, we speculate that peripartal MET but not CHOL supplementation led to less pronounced oxidative stress status in HLFI cows by enhancing sulfur-containing antioxidant synthesis through the transsulfuration pathway.

It is noteworthy that although differences in plasma bilirubin and cholesterol concentrations indicate a less-than-optimal liver function in LLFI cows, the similar plasma GOT and GGT concentrations between LLFI and HLFI cows and the fact these values were within the range considered normal for healthy cows (Bertoni et al., 2008) are indicative of the absence of liver damage. Similarly, although LLFI cows had lower hepatic GSH and plasma PON suggesting a less-than-optimal oxidative stress status, the fact that concentrations of FRAP, MPO, and ROM in LLFI cows were similar to HLFI cows indicates that they were not suffering from severe systemic oxidative stress. Thus, although biomarker analysis in the present study indicated that LLFI cows were in a more challenging physiologic state, they were still in better overall health than those reported in previous research design to test the validity of the LFI index (Bionaz et al., 2007; Bertoni et al., 2008).

AA profile in cows with HLFI and LLFI

Around parturition the decrease in DMI coupled with the increase in AA requirements to sustain fetal growth and lactation lead to greater tissue protein mobilization evidenced in part by the highest plasma 3-methyl histidine at 4 d regardless of LFI. However, the lower degree of tissue catabolism indicated by lower 3-methyl histidine along with the greater muscle mass suggested by greater circulating creatinine also revealed a lesser degree of tissue mobilization around parturition in HLFI cows. Whether such response was due to a more desirable circulating AA profile or an endocrine response induced by differences in AA is unknown. We speculate that the greater concentrations of various proteinogenic circulating EAA (His, Ile, Met, and Trp), NEAA (Ala, Asn, Gln, Pro, Ser, and Tyr), and non-proteinogenic AA (Cit) in HLFI cows not only reflect the greater pre- and post-partum DMI but also less reliance on skeletal muscle to provide substrates for milk protein synthesis and gluconeogenesis. It remains to be determined at a more mechanistic levels what adaptations might have been induced in the skeletal muscle when MET was supplemented. In addition, the fact that concentration of circulating AA was lower in LLFI cows supports recent data in sheep demonstrating greater consumption of AA during inflammatory events (Hoskin et al., 2016).

It is noteworthy that the proportion of Met in TAA (Met%TAA) was greater in HLFI cows. Concomitantly, the overall greater cystathionine and homocystine in addition to Met were largely responsible for the greater circulating total sulfur-containing compounds. Considering that an increase in sulfur-containing compounds in a previous LFI study without MET supplementation was not observed (Zhou et al., 2016b), and that more cows with MET supplementation ended up in the HLFI group, the increase in circulating sulfur-containing compounds were likely the result of MET supplementation. Although CHOL could potentially achieve similar outcomes by promoting *in vivo* synthesis of Met, recent data from our group have

demonstrated that *in vivo* CHOL supplementation did not seem to promote MET synthesis in liver, a response that could have been due to a limited level of sulfur-containing substrate around parturition (Zhou et al., 2017a).

CONCLUSIONS

Overall, the lower incidence of health complications, better lactation performance, and more favorable biomarker and plasma AA profiles in HLFI cows indicate a better immunometabolic status during the peripartal period. As more MET-supplemented cows were in the HLFI group, the differences observed between the two cohorts were due in large part to peripartal MET supplementation. Although CHOL supplementation is expected to achieve similar benefits as MET in terms of immunometabolic status, the fact that CHOL supplementation failed to improve LFI, at least in the present study, did not support this notion. However, if as reported recently the choline bioavailability from ReaShure meant that only a small fraction of the targeted dose was available to tissues, it is still possible that greater CHOL levels are able to impact LFI in a positive fashion. The LFI was able to differentiate effectiveness between MET and CHOL feeding, hence, supporting its broader application in the management of transition cows. Further studies on the applicability of these biomarkers to monitor transition success appears warranted.

TABLES AND FIGURES

Table 6.1. Frequency of peripartal rumen protected methionine (MET) and/or choline (CHOL) supplemented multiparous Holstein cows in low and high liver functionality index (LFI) groups.

	MET		CHOL		*P*-value	
LFI	+MET	-MET	+CHOL	-CHOL	MET	CHOL
Low LFI	2	7	5	4	0.06	0.71
High LFI	18	13	15	16		
Low LFI (%)	10 %	35 %	25 %	20 %		
High LFI (%)	90 %	65 %	75 %	80 %		

Table 6.2. Frequency of occurrence of health problems during the peripartal period in multiparous Holstein cows retrospectively grouped into low and high liver functionality index (LFI).

Variable	Low LFI	High LFI	Low LFI (%)	High LFI (%)	*P*-value
Cows	9	31	-	-	-
Ketosis[1]	6	0	67 %	0 %	< 0.01
Displaced abomasum	1	0	11 %	0 %	0.06
Retained placenta[2]	2	2	22 %	7 %	0.17
Mastitis	0	1	0 %	3 %	0.59

[1]Defined as cows having moderate (~40 mg/dL) or large ketone concentrations (>80 mg/dL) in urine and treated by veterinarians with oral propylene glycol or intravenous dextrose.

[2]Defined as fetal membranes retained >24 h after calving.

Table 6.3. DMI and milk production in peripartal Holstein cows retrospectively grouped into low and high liver functionality index (LFI).

	LFI			*P*-value		
Parameter (kg/d)	LLFI	HLFI	SEM[1]	LFI	Day	LFI×Day
Prepartum						
DMI	11.59	14.62	0.74	< 0.01	< 0.01	0.68
Postpartum						
DMI	16.14	20.21	0.96	< 0.01	< 0.01	0.21
Milk yield	36.24	46.30	2.20	< 0.01	< 0.01	0.07
Milk fat yield	1.35	1.59	0.08	0.02	< 0.01	0.34
Milk protein yield	1.09	1.48	0.07	< 0.01	< 0.01	0.06

[1]Greatest SEM.

Table 6.4. Blood and liver biomarkers in peripartal Holstein cows retrospectively grouped into low and high liver functionality index (LFI).

	LFI			*P*-value		
Parameter	LLFI	HLFI	SEM	LFI	Day	LFI×Day
Muscle body mass						
Creatinine (µmol/L)	86.35	91.91	2.42	0.06	< 0.01	0.28
3-methyl histidine	7.23	6.25	0.45	0.05	< 0.01	0.71
Liver function markers						
Bilirubin (µmol/L)	3.83	2.51	0.71	0.05	< 0.01	0.61
AGT (U/L)	97.78	98.91	4.57	0.83	< 0.01	0.36
Cholesterol (mmol/L)	2.60	3.35	0.14	< 0.01	< 0.01	< 0.01
GGT (U/L)	25.07	22.72	2.34	0.36	< 0.01	0.93
Inflammation and APP						
Albumin (g/L)	34.36	36.46	0.48	< 0.01	0.64	< 0.01
Ceruloplasmin (µmol/L)	2.71	2.67	0.15	0.84	< 0.01	< 0.01
Haptoglobin (g/L)	0.37	0.25	0.05	0.02	< 0.01	0.17
IL-1β (pg/mL)	5.49	4.67	1.08	0.48	< 0.01	0.17
Oxidative stress						
Ferric reducing ability of plasma (µmol/L)	131.81	132.59	4.28	0.87	0.08	0.05
Myeloperoxidase (U/L)	441.57	396.56	28.99	0.16	0.21	0.60
Paraoxonase (U/mL)	71.49	90.35	4.32	< 0.01	< 0.01	< 0.01
Reactive oxygen metabolites (mg H_2O_2/100mL)	13.65	12.97	0.67	0.37	< 0.01	0.19
Liver total glutathione (µmol/g protein)	12.26	25.11	4.27	0.05	< 0.01	0.97
Liver reduced glutathione (µmol/g protein)	11.75	24.35	4.21	0.05	< 0.01	0.98

[a,b,c,d] Mean values with different superscripts differ ($P < 0.05$).

[1]Greatest SEM.

Table 6.5. Plasma proteinogenic AA concentrations in peripartal Holstein cows retrospectively grouped into low and high liver functionality index (LFI).

	LFI			*P*-value		
AA (μM)	LLFI	HLFI	SEM[1]	LFI	Day	LFI×Day
Essential AA						
Arginine	54.95	58.29	2.47	0.26	< 0.01	0.10
Histidine	50.93	55.14	1.58	0.02	0.01	0.28
Isoleucine	89.39	102.66	5.56	0.06	0.30	0.24
Leucine	147.88	160.40	9.20	0.26	0.15	0.52
Lysine	62.74	65.34	3.57	0.53	0.03	0.14
Methionine	19.28	25.11	1.95	0.01	0.13	0.27
Phenylalanine	48.98	46.26	1.63	0.14	< 0.01	0.02
Threonine	70.68	77.04	3.73	0.14	0.09	0.88
Tryptophan	21.49	24.27	1.05	0.03	< 0.01	0.48
Valine	219.67	240.28	12.90	0.19	0.18	0.42
BCAA[2]	458.86	504.08	27.44	0.18	0.13	0.40
EAA[3]	789.06	858.75	36.42	0.12	0.04	0.31
Met%EAA	2.42	2.77	0.18	0.11	0.01	0.54
Lys%EAA	7.71	8.07	0.28	0.26	0.23	0.11
Non-essential AA						
Alanine	177.09	202.63	7.74	0.01	< 0.01	0.62
Asparagine	34.28	40.70	1.78	< 0.01	< 0.01	0.59
Aspartate	4.41	4.52	0.30	0.75	< 0.01	0.30
Glutamate[*]	36.94	37.17	1.36	0.88	< 0.01	0.11
Glutamine	235.01	258.06	9.06	0.03	< 0.01	0.76
Glycine	369.88	393.41	19.26	0.30	< 0.01	0.72
Proline	69.25	77.02	2.96	0.03	< 0.01	0.99
Serine	81.69	89.54	3.24	0.04	< 0.01	0.45
Tyrosine	38.47	43.16	2.34	0.09	< 0.01	0.10
NEAA[4]	1076.99	1175.86	31.12	0.01	< 0.01	0.70
TAA[5]	1884.92	2054.00	54.12	0.01	< 0.01	0.86
Met%TAA	1.01	1.18	0.08	0.08	< 0.01	0.54
Lys%TAA	3.45	3.31	0.16	0.45	< 0.01	0.03

[a,b,c,d] Mean values with different superscripts differ ($P < 0.05$).

[*]Significant ($P < 0.05$) parity effect observed.

[1]Greatest SEM.

[2]Branched-chain AA

[3]Essential AA

[4]Non-essential AA

[5]Total AA

Table 6.6. Plasma amino acid derivative concentrations in peripartal Holstein cows retrospectively grouped into low and high liver functionality index (LFI).

	LFI			*P*-value		
Parameter (μM)	LLFI	HLFI	SEM	LFI	Day	LFI×Day
AA and derivatives[#]						
1-methyl histidine	15.79	17.10	0.96	0.24	< 0.01	0.86
α-amino adipic acid	7.09	7.95	0.48	0.14	< 0.01	0.03
α-aminobutyric acid	16.06	18.06	1.20	0.18	< 0.01	0.01
β-alanine	8.79	8.71	0.47	0.87	0.94	0.60
γ-aminobutyric acid	2.33	3.13	0.49	0.16	< 0.01	0.03
Carnosine[*]	14.36	18.93	1.29	0.01	< 0.01	0.05
Citrulline	67.12	80.24	3.43	< 0.01	< 0.01	0.88
Hydroxylysine	0.35	0.31	0.04	0.35	0.76	0.75
Hydroxyproline	15.87	15.10	0.70	0.34	< 0.01	0.01
Ornithine	29.45	30.86	1.87	0.52	< 0.01	0.15
Phosphoserine	5.93	6.28	0.27	0.28	0.02	0.80
Sarcosine	9.31	9.92	0.74	0.49	< 0.01	0.60
Urea	4246.54	4412.14	176.69	0.41	0.01	0.10
Sulfur-containing compounds						
Cystathionine	1.64	1.93	0.13	0.07	< 0.01	0.11
Cystine	8.18	8.68	0.63	0.49	< 0.01	0.71
Glutathione	4.25	4.11	0.38	0.76	0.03	0.56
Homocystine	3.77	5.30	0.43	< 0.01	0.02	0.64
Taurine	37.07	41.02	2.74	0.21	< 0.01	0.04
TSC[*2]	75.22	86.57	4.92	0.05	0.06	0.29
Met%TSC	26.04	28.83	1.21	0.05	0.01	0.01

[a,b,c,d] Mean values with different superscripts differ ($P < 0.05$).

[1]Greatest SEM.

[2]Total sulfur-containing compounds. TSC included Met + cystine + cystathionine + homocystine + taurine + glutathione.

Figure 6.1

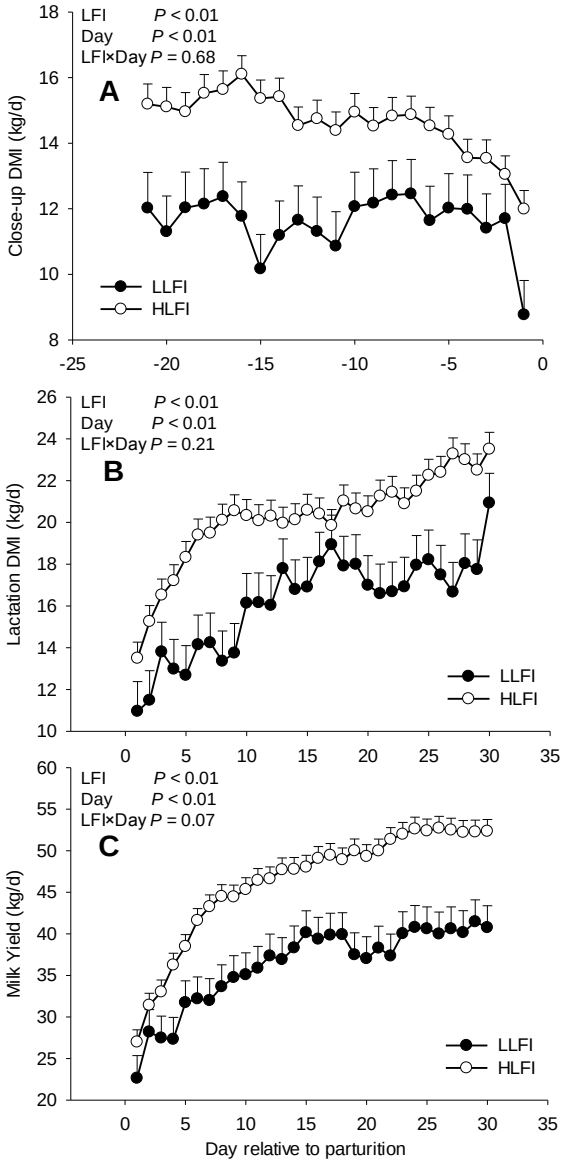
LFI P < 0.01
Day P < 0.01
LFI×Day P = 0.68
A
Close-up DMI (kg/d)
LLFI
HLFI
LFI P < 0.01
Day P < 0.01
LFI×Day P = 0.21
B
Lactation DMI (kg/d)
LLFI
HLFI
LFI P < 0.01
Day P < 0.01
LFI×Day P = 0.07
C
Milk Yield (kg/d)
LLFI
HLFI
Day relative to parturition

Figure 6.2

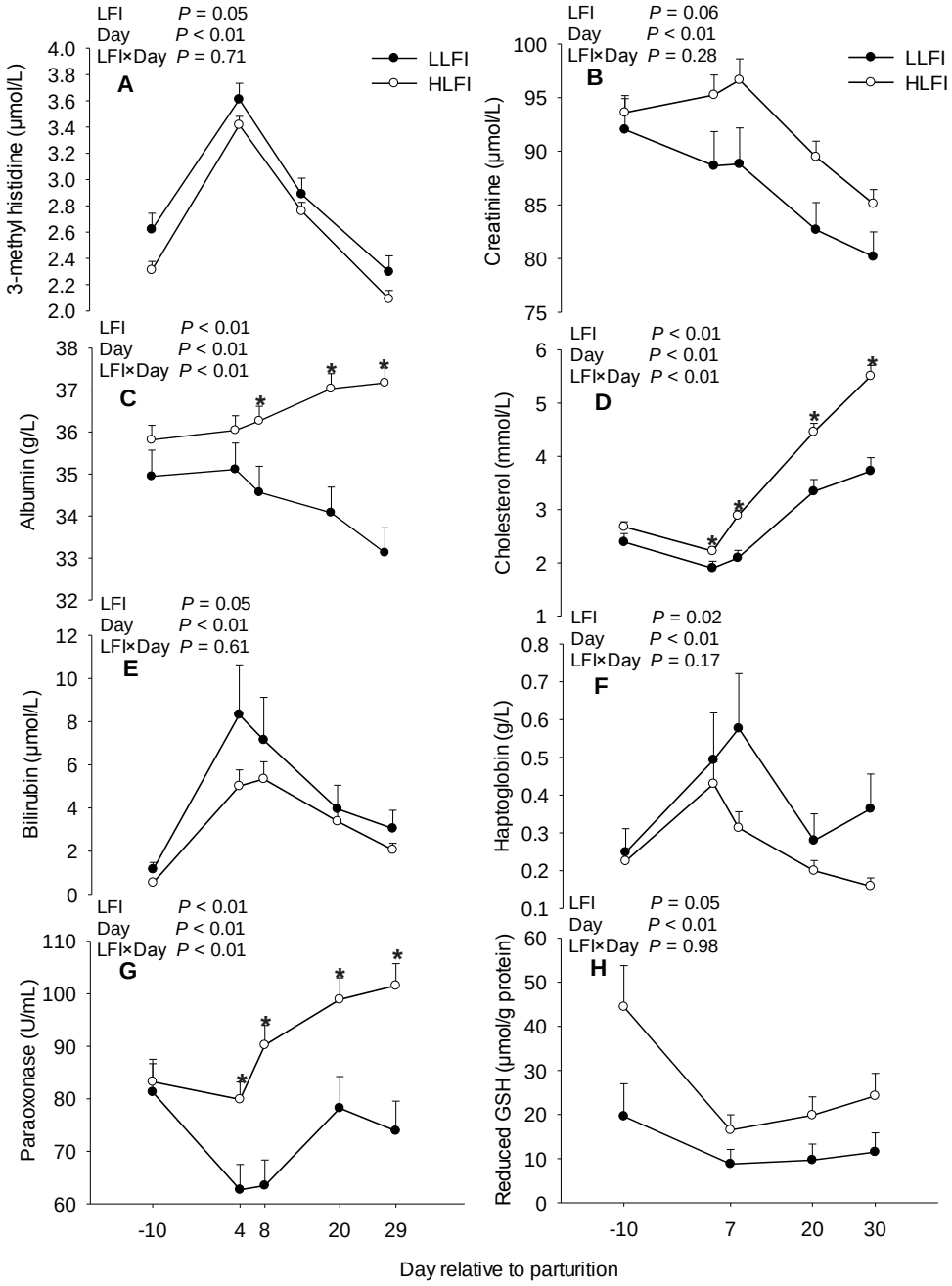
LFI P = 0.05
Day P < 0.01
LFI×Day P = 0.71
A
LLFI
HLFI
3-methyl histidine (µmol/L)
LFI P = 0.06
Day P < 0.01
LFI×Day P = 0.28
B
Creatinine (µmol/L)
LFI P < 0.01
Day P < 0.01
LFI×Day P < 0.01
C
Albumin (g/L)
LFI P < 0.01
Day P < 0.01
LFI×Day P < 0.01
D
Cholesterol (mmol/L)
LFI P = 0.05
Day P < 0.01
LFI×Day P = 0.61
E
Bilirubin (µmol/L)
LFI P = 0.02
Day P < 0.01
LFI×Day P = 0.17
F
Haptoglobin (g/L)
LFI P < 0.01
Day P < 0.01
LFI×Day P < 0.01
G
Paraoxonase (U/mL)
LFI P = 0.05
Day P < 0.01
LFI×Day P = 0.98
H
Reduced GSH (µmol/g protein)
-10
4
8
20
29
7
30
Day relative to parturition

Figure 6.3

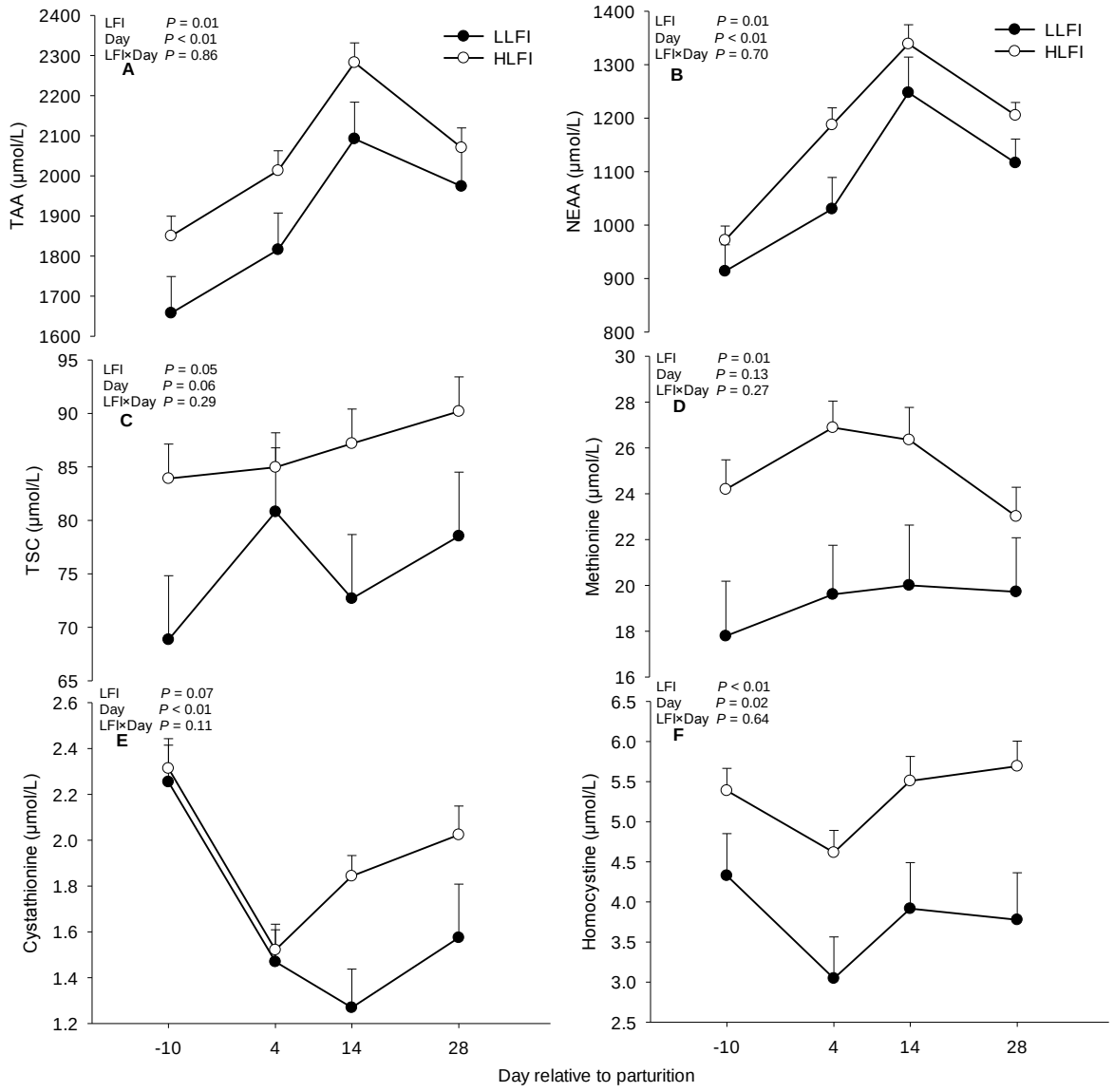
LFI P = 0.01
Day P < 0.01
LFI×Day P = 0.86
A
LLFI
HLFI
TAA (µmol/L)
LFI P = 0.01
Day P < 0.01
LFI×Day P = 0.70
B
NEAA (µmol/L)
LFI P = 0.05
Day P = 0.06
LFI×Day P = 0.29
C
TSC (µmol/L)
LFI P = 0.01
Day P = 0.13
LFI×Day P = 0.27
D
Methionine (µmol/L)
LFI P = 0.07
Day P < 0.01
LFI×Day P = 0.11
E
Cystathionine (µmol/L)
LFI P < 0.01
Day P = 0.02
LFI×Day P = 0.64
F
Homocystine (µmol/L)
-10
4
14
28
Day relative to parturition

Figure 6.1. Dry matter intake and milk yield during the periparturient period in cows retrospectively grouped into high (H) and low (L) liver functionality index (LFI; HLFI, LLFI).

Figure 6.2. Plasma concentrations of 3-methyl histidine, creatinine, albumin, cholesterol, bilirubin, haptoglobin, paraoxonase, and hepatic reduced glutathione during the periparturient period in cows retrospectively grouped into high (H) and low (L) liver functionality index (LFI; HLFI, LLFI). *LSM differ by $P < 0.05$ (LFI×Day).

Figure 6.3. Plasma concentrations of total AA (TAA), non-essential AA (NEAA), total sulfur-containing compounds (TSC), Methionine, cystathionine, and homocystine during the periparturient period in cows retrospectively grouped into high (H) and low (L) liver functionality index (LFI; HLFI, LLFI).

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