

## ABSTRACT

Title of dissertation: SUPPLEMENTAL CHOLINE AND METHIONINE FOR DAIRY CATTLE: EFFECTS ON PERFORMANCE, LIVER FAT CONTENT AND GENE EXPRESSION, AND PLASMA AMINO ACID AND CHOLINE METABOLITE CONCENTRATIONS

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The objectives of this dissertation were to 1) determine markers of post-ruminal choline supply so that bioavailability of rumen-protected choline (RPC) sources can be calculated in lactating cows and 2) investigate the production performance and metabolic effects of supplemental choline, methionine, or both on periparturient dairy cows.

Observations from Experiment 1 indicated that of the 26 choline metabolites investigated, including 16 species of phosphatidylcholine (PC) and 4 species of lysophosphatidylcholine, free choline and betaine in blood and milk were most responsive to post-ruminal choline supplied via abomasal infusion. However, RPC did not elicit changes in blood or milk choline metabolites, even at the very high doses tested in Experiment 2. These results suggest that choline supplied as RPC is absorbed differently than choline supplied via abomasal infusion, that RPC is over-protected such that choline

supplied in this form is not available to the cow, or that responses to RPC vary depending physiological state of the cow.

Results from Experiment 2 indicated that primi- and multiparous cows respond differently to supplemental choline and methionine fed during the periparturient period. Feeding RPC to primiparous cows increased milk yield, while feeding rumen-protected methionine (RPM) had minimal effects on production. In contrast, RPM improved milk components and fat-corrected milk yield for multiparous cows. These observations suggest that primi- and multiparous cows have different methionine and choline requirements in the periparturient period.

Investigation into the specific metabolic effects of choline and methionine fed to periparturient cows in Experiment 3 suggested that both RPC and RPM modify choline metabolism. The milk and blood PC profile was altered by both RPC and RPM. In line with this observation, RPC increased hepatic expression of the gene that encodes the enzyme responsible for catalyzing the rate-limiting step of PC synthesis via the CDP-choline pathway. The RPC-induced increase in hepatic *betaine-homocysteine methyltransferase* expression provided additional support for the connection between choline and methionine metabolism via one-carbon metabolism. Modification of postpartum plasma lactate concentrations by RPC for both primi- and multiparous cows, in conjunction with alterations in pre- or postpartum body condition, also suggest a choline-induced modification of tissue mobilization.

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Dissertation submitted to the Faculty of the Graduate School of the  
University of Maryland, College Park in partial fulfillment  
of the requirements for the degree of  
Doctor of Philosophy  
2019

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## ACKNOWLEDGEMENTS

I would first like to thank Dr. Richard Erdman for serving as my mentor. I am very grateful for his confidence in my abilities, teaching, patience, advice, and encouragement throughout my program. I would like to extend my thanks to Dr. Rick Kohn, Dr. Erin Connor, Dr. Nishanth Sunny, and Dr. Thomas Castonguay for serving on my committee and providing helpful suggestions, guidance, and support. I also would like to thank Dr. Kasey Moyes for allowing me to use space in her laboratory to conduct my analyses. I am also grateful for the opportunity to have studied under Dr. Bahram Momen, who passed away unexpectedly during my program.

Many thanks to the staff at the Central Maryland Research and Education Center Dairy Unit, especially Mike Dwyer and Brian Spielman, for their assistance and care of the animals that I used during my studies. I would also like to acknowledge Tim Shellum, Claudia Gomez, and Emily Davis for their assistance with data collection and animal care. I sincerely appreciate my fellow graduate students whom I have had the privilege to know and learn from during my program, especially Cynthia Scholte, Marie Iwaniuk, Kristen Brady, and Latisha Judd. I am very grateful for their research assistance, thought-provoking discussions, encouragement, and camaraderie.

Without the support of my family, I would not have been able to complete this program. I am grateful to my mother for her unwavering encouragement and support. I would also like to acknowledge other members of my family, especially Katie, Hayley, and Nancy, for providing me with advice, perspective, and reassurance whenever I needed it. Lastly, I am forever grateful to my husband for his reassurance and understanding, and for sharing his love for the dairy industry with me.

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## LIST OF ABBREVIATIONS

AA	Amino acids
ACC	Acetyl-CoA carboxylase
ACS	Acyl-CoA synthase
AdoHcy	S-adenosyl-homocysteine
apoB100	Apolipoprotein B100
BCAA	Branched-chain amino acids
BCS	Body condition score
BET	Betaine
BHBA	$\beta$ -hydroxybutyric acid
BHMT	Betaine-homocysteine methyltransferase
BW	Body weight
CDP-choline	Cytidine diphosphate-choline
CH <sub>3</sub> -THF	CH <sub>3</sub> -tetrahydrofolate
Cho	Free choline
ChoCl	Choline chloride
CK	Choline kinase
CLA	Conjugated linoleic acid
CPT1A	Carnitine palmitoyltransferase 1
CT	Cytidine triphosphate:phosphocholine cytidyltransferase
CTP	CDP-choline:1,2-diacylglycerol cholinephosphotransferase
DA	Displaced abomasum
DGAT1	Diacylglycerol acyl transferase 1
DHIA	Dairy herd improvement association
DIM	Days in milk
DMI	Dry matter intake
EAA	Essential amino acids
ER	Endoplasmic reticulum
ETC	Electron transport chain
FA	Fatty acid
FA-CoA	Fatty acyl-Coenzyme A
FATP	Fatty acid transport protein
FCM	Fat-corrected milk
GH	Growth hormone
GPC	Glycerophosphocholine
Hcy	Homocysteine
HMG-CoA	$\beta$ -hydroxy- $\beta$ -methylglutaryl-CoA
HSL	Hormone sensitive lipase
IFG-1	Insulin-like growth factor 1
LDL	Low-density lipoprotein

LPC	Lysophosphatidylcholine
Lys	Lysine
Met	Methionine
MTTP	Microsomal triglyceride transport protein
MTR	5-methyltetrahydrofolate-homocysteine methyltransferase
NDF	Neutral detergent fiber
NEB	Negative energy balance
NEFA	Non-esterified fatty acids
NFC	Non-fiber carbohydrate
PC	Phosphatidylcholine
PCho	Phosphocholine
PCYT1a	Phosphate cytidyltransferase 1 choline, $\alpha$
PE	Phosphatidylethanolamine
PEMT	Phosphatidylethanolamine <i>N</i> -methyltransferase
PPAR	Peroxisome proliferator-activated receptors
ROS	Reactive oxygen species
RPC	Rumen-protected choline
RPM	Rumen-protected methionine
SAM	S-adenosylmethionine
SM	Sphingomyelin
TCA	Tricarboxylic acid
TG	Triglyceride
TMR	Total mixed ration
VFA	Volatile fatty acid
VLDL	Very low density lipoproteins

## **CHAPTER 1: INTRODUCTION**

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The periparturient period is a challenging time for the dairy cow as she transitions from pregnancy to lactation. During this time, there are several metabolic adaptations and challenges that ensue, making the cow vulnerable to clinical and subclinical health disorders that impede optimal performance. Several nutritional and management strategies have been examined, implemented, and shown to be effective in promoting cow health, production, and reproduction. One strategy is to optimize the diet by adding supplemental nutrients that are presumed to be limiting during this time. Due to the classic elevation in adipose tissue mobilization and subsequent hepatic lipid synthesis and very low density lipoprotein (VLDL) export that occurs during the early postpartum period, researchers have investigated the potential for lipotropes, such as choline and methionine, to improve health and performance. Furthermore, the importance of transmethylation reactions in metabolism and gene expression has also prompted interest in balancing diets to provide sufficient choline, methionine, and B-vitamins to support one-carbon metabolism.

Both choline and methionine (in the form of rumen-degradable protein) are degraded by rumen microbes (NRC, 2001); thus, dietary supplementation of these nutrients is accomplished through feeding them in a rumen-protected form, allowing for passage to the lower gut with minimal microbial degradation. While the protection from ruminal degradation of rumen-protected choline (RPC) and rumen-protected methionine (RPM) have been well-established (Deuchler et al., 1998; Bach et al., 2000), it is important to quantify the availability of these nutrients once they reach the small intestine. For RPM, this has been accomplished by measuring the change in methionine concentrations in the blood and estimates of the bioavailability for RPM sources have been reported (Bach et al.,



2000; Südekum et al., 2004). However, bioavailability estimates for RPC sources are lacking. Deuchler et al. (1998) showed that post-ruminal choline supplied via an abomasal infusion increased total choline concentrations in milk. More recently, de Veth et al. (2016) showed that specific choline metabolites such as choline, betaine, and phosphocholine in blood and milk, not just total choline, were responsive to post-ruminal choline supplied via an abomasal infusion. However, similar, significant responses for cows fed RPC were not observed (de Veth et al., 2016), which complicates the task of estimating choline bioavailability from RPC sources. It is important, both from a ration balancing standpoint, to identify potential markers that will allow for bioavailability estimates of RPC to be reliably calculated.

Results from several studies in dairy cattle have shown production and health benefits to providing supplemental choline or methionine during the periparturient period (Zom et al., 2011; Osorio et al., 2013; Zhou et al., 2016; Zenobi et al., 2018a). Feeding RPC to feed-restricted dry cows reduces liver triglyceride accumulation (Cooke et al., 2007; Zenobi et al., 2018b), although this effect has not been consistently reported during the periparturient period in cows fed ad libitum (Zenobi et al., 2018a; Zhou et al., 2016). Despite this, RPC can elicit positive effects on milk production during the periparturient period (Zenobi et al., 2018a; Zom et al., 2011). Feeding RPM to periparturient cows generally increases fat-corrected milk yield, milk fat yield, milk protein percentage, and feed intake (Osorio et al., 2013; Zhou et al., 2016). Furthermore, as recently observed by (Osorio et al., 2014; Vailati-Riboni et al., 2017), RPM can have positive effects on immune function in periparturient cows.

Based on gene expression (Goselink et al., 2013; Zhou et al., 2017; Osorio et al., 2014), enzyme activity (Zhou et al., 2017), and protein abundance (Zhou et al., 2018) analyses, it is clear that both choline and methionine can affect hepatic lipid and one-carbon metabolism in dairy cows. However, further research into the mechanisms by which these nutrients elicit their effects is necessary to enhance their usefulness as nutritional tools to support cow health and production in the postpartum period. Because both choline and methionine metabolism are related through one-carbon metabolism and phosphatidylcholine synthesis, further exploration is also necessary to enrich our understanding of how these nutrients interact with each other in ruminant animals. To date, only one study has investigated this interaction in periparturient dairy cows (Zhou et al., 2016).

The two central hypotheses of this work are that 1) choline metabolites in blood and milk are responsive to RPC feeding such that they can be used to facilitate bioavailability calculations; and 2) dietary supplementation of RPC and RPM to periparturient cows has positive effects on production and liver metabolism and each nutrient influences hepatic lipid, choline, and methionine metabolism.

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## **CHAPTER 2: LITERATURE REVIEW**

The periparturient period is a challenging time for the dairy cow and is characterized by substantial alterations in lipid, glucose, and protein metabolism in order to support the onset of copious milk production. A cow's success during this time can have a lasting influence on her entire lactation. It is crucial to understand the metabolic adaptations and challenges that occur during this time, as well as strategies that can be implemented to help cows excel after calving. Providing supplemental nutrients, such as choline and methionine, is a strategy that has been explored to promote cow performance during this time. As such, it is important to understand the basic metabolism and functions of these nutrients.

### **THE METABOLIC STATE OF THE PERIPARTURIENT COW**

The transition period, defined as 21 d before calving through the first 21 d of lactation (Grummer, 1995), is a critical time for the dairy cow. Nutrient requirements increase substantially as the cow transitions from gestation to lactation, but feed intake does not increase as rapidly, resulting in negative energy balance (NEB). Occurrence of metabolic diseases, such as fatty liver, ketosis, milk fever, and retained placenta is high during the periparturient period (Markusfeld, 1987) and these diseases are correlated with NEB (Collard et al., 2000). Incidence of metabolic disease can have major impacts on productive performance, reproductive success, lifetime efficiency, animal welfare, and ultimately, profitability of the dairy cow. The success with which a cow transitions from the dry period to early lactation will impact her performance during the subsequent lactation.

The fetal growth rate is much greater during the last third of gestation than the first two trimesters (Bauman and Currie, 1980). This increases the dam's nutritional requirements even before initiation of lactation. Grummer (2008) estimated that between 60 d prepartum and 1 d prepartum, energy requirements for the growing fetus and gravid uterus increase from 2.9 to 3.7 Mcal NE<sub>L</sub>/d, representing a 6% increase in total energy requirements after energy required for maintenance was considered. At the same time, physiological changes that occur around the time of calving cause dry matter intake (DMI) to decline, such that between 21 d prepartum and the day of calving, it is reduced by ~30-40% (Vazquez-Añon et al., 1994; Grummer, 1995; Douglas et al., 2006). This decline in DMI prior to calving likely contributes to the occurrence of NEB that begins at calving (Grummer, 2008). Four days before parturition, a 680 kg cow requires ~14 Mcal NE<sub>L</sub>/d; the same cow will require ~33 Mcal NE<sub>L</sub>/d to produce 30 kg of 4% fat milk/d at 11 days in milk (DIM; NRC, 2001). Thus, between 4 d prepartum and 11 d postpartum, energy requirements increase by nearly 136%, but DMI only increases by ~30-50% during the first week postpartum (Roche et al., 2013). Drackley (1999) estimated that the postpartum dairy cow only consumes enough energy to meet ~74% of requirements by d 4 postpartum. After calving, DMI will recover and eventually surpass that of the prepartum period (Ingvarsten and Anderson, 2000; Douglas et al., 2006; Grummer, 2008), but the rate at which nutrient intake increases is not sufficient to meet the rapid increases in nutrient demands associated with the increase in milk production during the early postpartum period, which perpetuates the NEB that was initiated immediately prepartum.

The magnitude of DMI depression prepartum correlates positively with risk for metabolic disease in early lactation (Grummer, 2008). Over consumption of nutrients

during the dry period may negatively impact DMI postpartum (Douglas et al., 2006). Douglas et al. (2006) restricted DMI or allowed ad libitum DMI during the dry period. When compared with feed-restricted cows, ad libitum-fed cows gained body weight (BW) and body condition throughout the dry period and experienced greater DMI depression around parturition (Douglas et al., 2006). Results reported by Douglas et al. (2006) are consistent with the fact that body condition score (BCS) correlates negatively with DMI (Roche et al., 2009). Over-conditioned cows at calving are more likely to suffer from metabolic disease in the postpartum period than cows that calve in average condition (Roche et al., 2009). Furthermore, Hartwell et al. (2000) observed that cows that calved with a BCS  $\geq 3.75$  had increased liver triglyceride (TG) accumulation between 1 and 28 d postpartum than cows that calved with BCS  $< 3.75$ . Therefore nutrient intake and BCS should be monitored closely during the dry period to prevent over-conditioning prior to calving in order to mitigate the magnitude of DMI depression and reduce the occurrence of periparturient disease.

Reasons for the decline in DMI prior to parturition and the slow rate at which nutrient intake increases postpartum are not fully understood, but endocrine changes associated with parturition probably contribute to periparturient hypophagia (Ingvarsten and Anderson, 2000). In their review of potential mechanisms that influence DMI of the transition cow, Ingvarsten and Anderson (2000) suggested that various hormones, metabolites, and gut peptides are likely to have a strong influence over DMI during the periparturient period. Plasma estrogen levels are high during the prepartum period and begin to decrease immediately after calving (Ingvarsten and Anderson, 2000; NRC, 2001), and estrogen has been shown to elicit a hypophagic response in dairy cattle (Grummer et

al., 1990; Bremmer et al., 1999). Plasma insulin levels also increase during the prepartum period but begin to decline prior to parturition and remain low throughout the postpartum period (Veenhuizen et al., 1991; Ingvarsten and Anderson, 2000). Although insulin reduces DMI in dairy cows (Leury et al., 2003), it probably contributes little to the intake depression observed around calving because plasma concentrations of insulin are very low just prior to calving when the most drastic drop in DMI occurs, and concentrations remain low during the postpartum period (Ingvarsten and Anderson, 2000; NRC, 2001). Reasons for insufficient DMI immediately before calving and during the early postpartum period could also be related to a delay in rumen adaptation to a typical high energy, low neutral detergent fiber (NDF) lactation ration (Goff and Horst, 1997; NRC, 2001), oxidation of fuels by the liver (Allen et al., 2009), rumen fill, or other unknown mechanisms.

The process of tissue mobilization to support requirements for lactation is a normal physiological process that occurs in all mammals. However, excessive mobilization caused by severe NEB can be detrimental to cow health and productivity. Negative energy balance correlates positively with metabolic diseases, such as hepatic lipidosis and ketosis, and depresses immune function (Goff and Horst, 1997; Bobe et al., 2004). These health problems may result in an even greater energy deficit by further reducing DMI, which can lead to additional health problems such as displaced abomasum (Shaver, 1997).

### ***Changes in Lipid and Carbohydrate Metabolism***

During the transition period, homeorhetic responses cause significant changes in lipid and carbohydrate metabolism such that nutrients are partitioned to the mammary gland to support the rapid increase in milk production. Mobilization of adipose tissue



during the early postpartum period is crucial for the cow to meet unfulfilled energy requirements of the mammary gland. Bell (1995) estimated that within 4 d after calving, the mammary gland requires 4.5 times more fatty acids (FA) than the gravid uterus during late gestation. In addition, glucose requirements increase from ~1100 g/d during the prepartum period to ~2500 g/d during early lactation (Drackley et al., 2001).

### *Lipid Metabolism*

The postpartum period is marked by extensive mobilization of body fat stores that is synchronized by endocrine changes that occur around the time of calving. Mobilization of adipose tissue results in the release of FA and glycerol into the blood. Glycerol can be used as a substrate for gluconeogenesis by the liver and non-esterified FA (NEFA) are used for lipogenesis in the mammary gland, oxidized as fuel by body tissues, or metabolized by the liver (Bell, 1995; Roche et al., 2013). The decline of plasma insulin just prior to calving promotes lipolysis in adipose tissue and spares glucose to support the final stages of gestation, colostrogenesis, and subsequent high levels of milk production (Bell, 1995; Goff and Horst, 1997). In addition to reduced insulin concentration, high blood growth hormone (GH) concentration also plays a role in nutrient partitioning during early lactation (Butler et al., 2003).

Loss of hepatocyte GH sensitivity during the periparturient period (Boisclair et al., 2006; Vijayakumar et al., 2010) reduces the rate of insulin-like growth factor I (IGF-1) production by the liver, resulting in high blood GH levels and partitioning of nutrients to the mammary gland (Butler et al., 2003). For postpartum cows, insulin may play a major role in dictating hepatocyte GH sensitivity and serve to regulate blood GH concentrations.

Butler et al. (2003) examined early lactation cows under hyperinsulinemic conditions and observed an increase in hepatic GH receptor mRNA and a decrease in plasma GH. They suggested that higher insulin levels restored hepatic GH sensitivity and thereby decreased circulating GH concentrations (Butler et al., 2003). For the hyperinsulinemic treatment, both plasma NEFA concentration and milk yield were reduced, indicating a decreased rate of adipose tissue mobilization which reduced the energy supply for the mammary gland (Butler et al., 2003).

Hormone sensitive lipase (HSL) catalyzes the release of FA from TG and diacylglycerol, and is the rate limiting enzyme in the release of FA from adipose tissue (Holm, 2003). Hormone sensitive lipase is stimulated by glucagon and catecholamines, such as epinephrine and norepinephrine, and is inhibited by insulin (Holm, 2003). There is also evidence for GH to promote HSL activity by activating  $\beta$ -adrenergic receptors which are responsible for activation of HSL (Vijayakumar et al., 2010). Thus elevated plasma GH during the periparturient period likely serves to indirectly enhance lipolysis in adipose tissue. These results suggest that reduced blood insulin concentration, followed by a subsequent elevation of GH, is critical in the regulation of adipose mobilization during the transition period.

In contrast to depressed adipocyte lipogenesis, mammary lipogenesis is enhanced during the postpartum period. During established lactation, ~50% of milk FA originate from de novo synthesis (<C16:0) in the mammary gland, while the other 50% of milk FA are from preformed sources (>C16:0), such as the diet and adipose tissue (Bauman and Griinari, 2003). During established lactation when cows are in positive energy balance, of the 50% of FA from preformed sources, only ~10% are derived from adipose tissue

(Bauman and Griinari, 2003). However, during early lactation extensive adipose mobilization supplies the mammary gland with large quantities of NEFA resulting in a higher proportion of milk FA derived from preformed sources (Kay et al., 2005). Stoop and coworkers (2009) determined that the proportion of preformed FA in milk fat correlates positively with the degree of NEB ( $r=0.7$ ). These findings coincide with the increased rate of lipolysis that occurs during early lactation when cows are in NEB.

### *Carbohydrate Metabolism*

During late pregnancy, fetal growth rate increases rapidly which is paralleled by greater nutrient requirements (Bauman and Currie, 1980). As reviewed by Bell (1995), the growing fetus requires ~2.3 Mcal/d at 250 d of gestation, with ~30% of the energy demands met through oxidation of glucose or lactate. In fact, glucose demands for cows during the prepartum period are as high as 1100 g/d (~4.4 Mcal/d; Drackley et al., 2001). The high demand for glucose and amino acids during the last 40-60 d of gestation to support fetal growth and metabolism as well as colostrum synthesis require the cow to make substantial changes in the rate of hepatic gluconeogenesis (Bell, 1995; Drackley et al., 2001). Although glucose demands for the final weeks of gestation are high, requirements during the early lactation period are even greater, increasing from 1100 g/d during the prepartum period to ~2500 g/d during early lactation, representing a 127% increase (Drackley et al., 2001). Osmotic pressure is the main driver of milk volume, and lactose contributes to ~50% of the osmotic gradient for fluid transport in the mammary gland (Cai et al., 2018). Because lactose production requires 1 unit of glucose and 1 unit of galactose (Linzell and Peaker, 1971), the mammary gland must have an adequate supply of glucose in order to maximize milk production. To accommodate these changes in glucose and other nutrient

demands during the transition period, the metabolic activity of the liver nearly doubles during the transition period (Drackley et al., 2001) and the rate of hepatic gluconeogenesis is elevated substantially. Reynolds et al. (2003) observed an average liver glucose production rate of ~1200 g/d at 19 d prepartum; this rate was increased by 150% at 21 d postpartum (>3000 g/d).

Glucose absorption by the ruminant digestive tract is low because rumen microbes metabolize most of the glucose derived from the fermentation of more complex carbohydrates (Bergman, 1990). Instead, dairy cattle rely on volatile fatty acids (VFAs) as substrates for gluconeogenesis, with propionate being the main glucose precursor (Seal and Reynolds, 1993). Diets fed to early lactation cows usually contain high proportions of fermentable carbohydrates, such as corn grain, that increase ruminal propionate production (Bergman, 1990). However, total glucose precursor requirements cannot be met simply through ruminal propionate production, especially during early lactation when DMI is low and glucose requirements are high. Between 32-73% of glucose is derived from ruminal propionate, so the remaining 27-68% of glucose is derived from other glucogenic substrates, such as amino acids from dietary protein or muscle catabolism, lactate, and glycerol (Drackley et al., 2001). At 4 wk postpartum, Reynolds et al. (1988) determined that the maximum contributions of propionate, lactate, and amino acids to hepatic gluconeogenesis were 58, 21, and 15%, respectively. Bell (1995) estimated that at 4 d postpartum, 65% of mammary glucose requirements could be met through absorbed propionate and amino acids and 20% of glucose requirements could be met through glycerol resulting from TG catabolism; the remaining 15% of glucose likely originates from amino acids derived from body tissues. In a more recent study, glucogenic precursors,

including propionate, lactate, alanine, and glycerol, contributed to 73% and 84% of the glucose produced by the liver at 9 d pre- and 11 d postpartum, respectively (Reynolds et al., 2003). This implies that other glucogenic substrates, such as amino acids from muscle, account for 27-16% of the glucose produced in the liver. Between 2 wk prepartum and 5 wk postpartum, lactating dairy cows lose an average of 21 kg of empty body protein (Komaragiri and Erdman, 1997), indicating that a substantial amount of body protein is catabolized during the periparturient period is likely used to provide amino acids for gluconeogenesis. Thus, many other glucogenic substrates in addition to propionate play critical roles in the ability of the transition cow to meet glucose requirements during late gestation and early lactation.

## **RUMINANT LIVER LIPID METABOLISM**

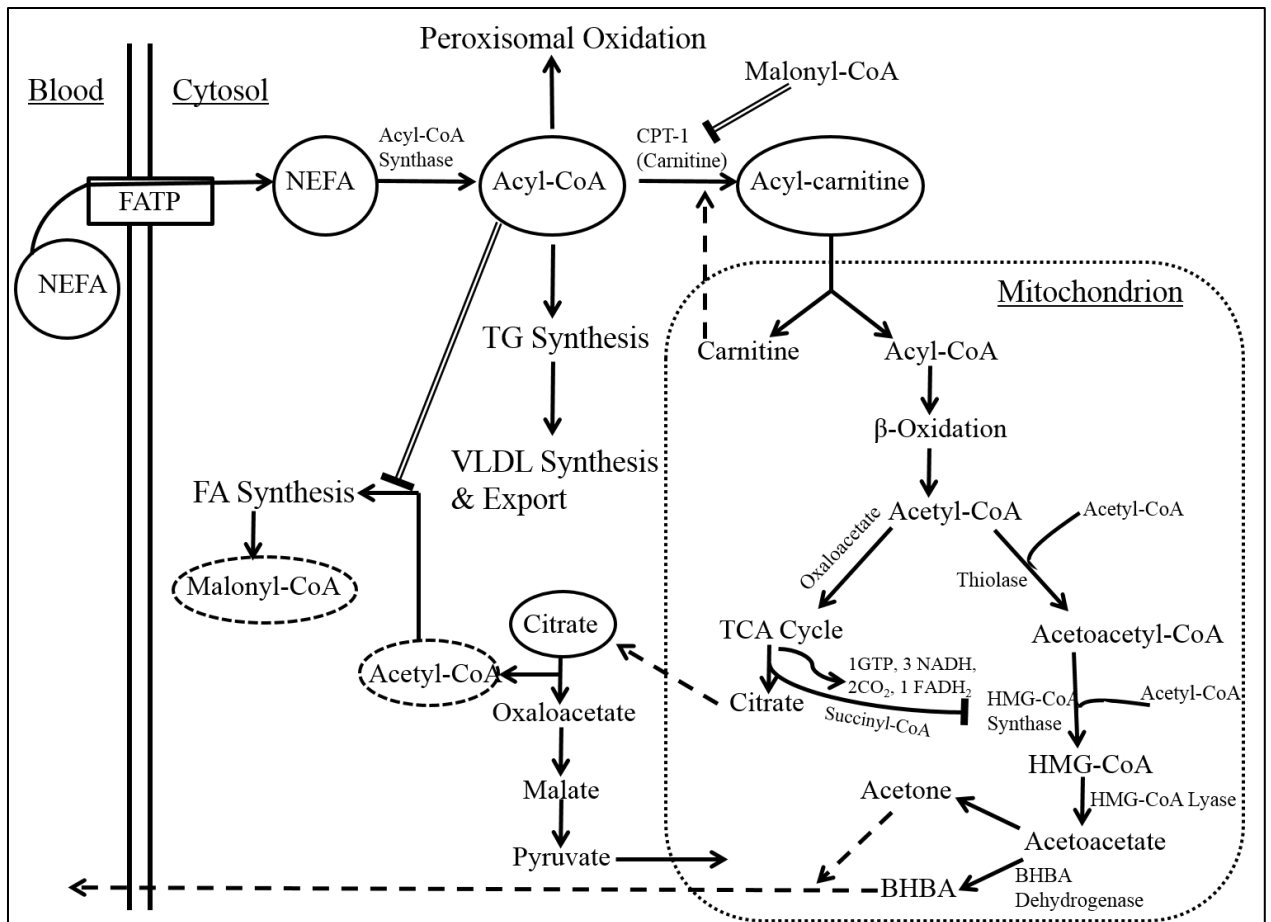
In ruminants, the liver is responsible for supplying glucose to the mammary gland and other extrahepatic tissues, metabolizing nitrogenous compounds and detoxifying ammonia, and processing FA into very low density lipoproteins (VLDL) to supply extrahepatic tissues. The hepatic portal vein supplies the liver with propionate, NEFA, ammonia N, and amino acids that are derived from the diet. Propionate serves as the main glucogenic precursor for early to mid-lactation dairy cows, supplying the liver with over 60% of the substrates used for gluconeogenesis (Reynolds et al., 2003). Non-esterified FA can serve as a direct energy source for the liver through complete oxidation, be partially oxidized to ketone bodies, or be esterified to glycerol to form TGs, which are subsequently incorporated into VLDL and exported to extrahepatic tissues (Grummer, 1993). During the periparturient period, the liver metabolic rate is elevated to increase the rate of

gluconeogenesis and to accommodate the increased supply of ammonia, amino acids, and FA (Drackley et al., 2001).

Liver NEFA uptake is directly proportional to plasma NEFA levels (Bell, 1995; Drackley et al., 2001; Nguyen et al., 2008). Consequently, liver NEFA uptake is enhanced during the periparturient period when plasma NEFA levels are elevated due to adipose mobilization. Drackley et al. (2001) estimated that NEFA uptake by the liver was ~26% of circulating NEFA. Non-esterified FA are delivered to the liver via the portal vein and the hepatic artery, with the latter supplying a greater proportion of NEFA during periods of adipose mobilization. For cows at 11 d postpartum, NEFA flux from the hepatic artery was ~108% greater than the flux of NEFA from the portal drained viscera (Reynolds et al., 2003).

### ***FA Uptake and Activation***

A summary of hepatic NEFA metabolism is illustrated in Figure 2.1. Nonesterified FA are transported into the hepatocyte via FA transport proteins 2 and 5 (FATP2 and FATP5, respectively) and FA translocase (Kawano and Cohen, 2013). Once inside the cytoplasm, NEFA are activated to fatty acyl-CoA (FA-CoA) either by acyl-CoA synthetases (ACS) or by enzymes inherent to FATPs (Nguyen et al., 2008). Once activated, FA-CoA are either oxidized in the mitochondria or peroxisome, or utilized as substrates for TG synthesis (Nguyen et al., 2008; Kawano and Cohen, 2013).



**Figure 2.1.** Schematic of liver lipid metabolism. FATP = fatty acid transport protein; CPT-1A = carnitine palmitoyltransferase 1; HMG-CoA =  $\beta$ -Hydroxy- $\beta$ -methylglutaryl-CoA; BHBA =  $\beta$ -Hydroxy-butyrate.

### *Mitochondrial $\beta$ -Oxidation*

Fatty acyl-CoA cannot freely cross the mitochondrial membrane and a carnitine-dependent shuttle, carnitine palmitoyltransferase 1 (CPT-1), is required to facilitate the transfer (Kawano and Cohen, 2013). This enzyme is associated with the mitochondrial membrane and catalyzes the conversion of acyl-CoA to acyl-carnitine, a process that requires cytosolic carnitine (Nguyen et al., 2008). Acyl-carnitine traverses the mitochondrial membrane via assistance of carnitine acyl-carnitine translocase, and subsequent reaction with the mitochondrial enzyme, CPT-2, yields mitochondrial FA-CoA,

which serves as the primary substrate for  $\beta$ -oxidation, and free carnitine, which can diffuse into the cytosol for future carnitine-dependent translocation reactions (Kawano and Cohen, 2013). Therefore, CPT-1 catalyzes the rate limiting step for mitochondrial  $\beta$ -oxidation because it controls substrate entry into the mitochondrial matrix. Malonyl-CoA, the product of the first step in cytosolic FA synthesis catalyzed by acetyl-CoA carboxylase (ACC), inhibits the action of CPT-1 (Kawano and Cohen, 2013). The first step of mitochondrial  $\beta$ -oxidation is catalyzed by an acyl-CoA dehydrogenase, resulting in production of a trans- $\Delta^2$ -Enoyl-CoA and reduction  $FAD^+$  to  $FADH_2$ , the latter of which enter the electron transport chain (ETC) to produce 1.5 ATP (Nelson and Cox, 2008). The second step of mitochondrial  $\beta$ -oxidation is catalyzed by an enoyl-CoA hydratase and the third step is catalyzed by  $\beta$ -hydroxyacyl-CoA dehydrogenase, producing NADH that can enter the ETC to produce 2 ATP. The final step of  $\beta$ -oxidation, catalyzed by a thiolase, results in a FA-CoA 2 carbons shorter than the initial FA-CoA and one molecule of acetyl-CoA that can enter the TCA cycle to produce ATP (Nelson and Cox, 2008). For even numbered saturated FA, this process repeats until the final step yields two acetyl-CoA molecules from butanoyl-CoA.

Insulin activates FA synthesis by stimulating ACC and results in the production of malonyl-CoA, which inhibits CPT-1 (Kawano and Cohen, 2013). In contrast, glucagon activates a phosphorylation cascade which inhibits ACC and activates malonyl-CoA decarboxylase, effectively reducing the amount of malonyl-CoA and enabling full function of CPT-1 (Kawano and Cohen, 2013). During the periparturient period, plasma insulin levels are low and glucagon levels are relatively constant such that the ratio of glucagon:insulin is increased (Ingvarsten and Andersen, 2000), thereby enabling FA-CoA



transport into the mitochondria for oxidation (Nguyen et al., 2008). Because the rate of NEFA entry into the liver is increased during the periparturient period, large amounts of acetyl-CoA are produced through  $\beta$ -oxidation.

Dann and Drackley (2005) observed an increase in hepatic CPT-1 activity between 30 d prepartum and 1 d postpartum, indicating that the rate of FA oxidation likely also increased during this time to accommodate increased NEFA uptake by the liver. In agreement, results from in vitro incubation studies with liver tissue obtained from transition dairy cows indicate that the hepatic FA oxidation capacity of early lactation cows is significantly greater than mid-lactation cows (Andersen et al., 2002). It is likely that CPT-1 becomes saturated during the periparturient period such that the enzyme simply cannot process all of the FA-CoA in the liver. Activity of CPT-1 plateaus when the concentration of palmitoyl-CoA in pregnant rat liver tissue is  $\sim 0.06$  mEq/L (Saggerson and Carpenter 1982; Gavino and Gavino, 1991). Around the time of calving, plasma NEFA levels can peak as high as 0.9 mEq/L (Grummer, 1993). If the liver takes up 26% of the circulating NEFA (Drackley et al., 2001), liver NEFA concentration would be approximately 0.23 mEq/L at peak adipose tissue mobilization. This concentration of NEFA is more than 280% greater than the concentration of palmitoyl-CoA that maximizes hepatic CPT-1 activity in pregnant rats. Thus, the concentration of FA-CoA in the cytosol during the periparturient period probably exceeds the capacity of CPT-1 to facilitate its transport into the mitochondria for  $\beta$ -oxidation.

## ***Ketogenesis***

Typically, acetyl-CoA produced through  $\beta$ -oxidation will enter the TCA cycle for production of reducing equivalents that are shuttled to the ETC. Alternatively, acetyl-CoA may be diverted to the ketogenic pathway under conditions where propionate supply is limited (Drackley et al., 2001). In the first step of ketogenesis, two acetyl-Co-A molecules condense to form acetoacetyl-CoA (Nelson and Cox, 2008). Acetoacetyl-CoA is converted to  $\beta$ -Hydroxy- $\beta$ -methylglutaryl-CoA (HMG-CoA) which is catalyzed by HMG-CoA synthase (Nelson and Cox, 2008). In the final steps of ketogenesis, HMG-CoA is converted to acetoacetate which is subsequently converted to  $\beta$ -Hydroxybutyrate (BHBA) and acetone (Nelson and Cox, 2008). Acetoacetate and BHBA are the primary ketone bodies that are exported from the hepatocyte and transported to extrahepatic tissues for use as energy sources. The rate limiting step of ketogenesis is catalyzed by of HMG-CoA synthase which is inactivated by the succinylation that occurs in the presence of the TCA cycle intermediate, succinyl-CoA (Nguyen et al., 2008). Drackley et al. (2001) hypothesized that the amount of propionate available to produce succinyl-CoA via the TCA cycle influences the rate of ketogenesis, with, high levels of propionate increasing succinyl-CoA production, thereby reducing the activity of HMG-CoA synthase.

Relative to mid-lactation cows, early lactation cows have a reduced capacity for FA oxidation (Andersen et al., 2002). This is consistent with the classic elevation of plasma BHBA during the early postpartum period (Douglas et al., 2006; Carlson et al., 2007). Incomplete FA oxidation during early lactation (Andersen et al., 2002) probably developed as a mechanism for the ruminant to provide additional energy substrates for extrahepatic tissue during times of limited supply of glucose precursors (Drackley et al., 2001).

However, excessive ketone production leads to ketosis, which has significant negative impacts on milk production potential in early lactation and increases the risk for the development of other diseases during the periparturient period (Duffield et al., 2009). Fatty liver and ketosis are both periparturient metabolic diseases and their occurrence is usually correlated (Grummer, 1993) because both result from increased hepatic NEFA uptake during the periparturient period.

### ***Peroxisomal $\beta$ -Oxidation***

Fatty acyl-CoA can also be oxidized in the peroxisome instead of the mitochondria. Peroxisomal  $\beta$ -oxidation is typically used for very long chain FA; however, during times when FA influx is high, it can apparently serve as an overflow oxidation pathway (Nguyen et al., 2008). In contrast to the first step of  $\beta$ -oxidation in the mitochondria, the first step in peroxisomal  $\beta$ -oxidation is catalyzed by a different enzyme, acyl-CoA oxidase, which results in the production of  $H_2O_2$ . Hydrogen peroxide is subsequently reduced to  $H_2O + 1/2 O_2$  by  $FADH_2$  (Nelson and Cox, 2008; Nguyen et al., 2008). Because the peroxisome does not contain an ETC, NADH produced during the second step must be exported into the cytosol for reoxidation instead of entering the ETC directly to produce ATP (Nelson and Cox, 2008). Thus, fewer ATP are produced through peroxisomal  $\beta$ -oxidation because  $FADH_2$  is used to reduce  $H_2O_2$  in the first step instead of producing ATP through ETC, and because NADH produced during the second oxidative step cannot enter the ETC to produce ATP as in the mitochondria. Similar to mitochondrial  $\beta$ -oxidation, the final step of each round of peroxisomal  $\beta$ -oxidation produces acetyl-CoA, which is exported to the mitochondria for entry into the TCA cycle (Nelson and Cox, 2008).

### ***Triglyceride and VLDL Synthesis***

Activated FA-CoA that are not transported into the mitochondria or peroxisome for  $\beta$ -oxidation are utilized for TG synthesis. In contrast to the rodent and human, the capacity of the ruminant liver to synthesize FA de novo is limited; therefore, most of the FA incorporated into TG in the ruminant liver originate from body tissues or the diet (Grummer, 1993). The first step of TG synthesis involves esterification of FA-CoA to the *sn*-1 position of glycerol-3-phosphate which is catalyzed by glycerol-3-phosphate acyl transferase (Kawano and Cohen, 2013). Acylglycerol-3-phosphate acyl transferase catalyzes esterification at the *sn*-2 position and the resulting diacylglycerol is esterified to TG by diacylglycerol acyl transferase 1 (DGAT1; Kawano and Cohen, 2013).

Triglycerides are incorporated into VLDL and secreted into the blood for transport to extrahepatic tissues. Very-low density lipoproteins are composed of apolipoproteins, phospholipid, free cholesterol, cholesterol esters, and TG. Triglycerides, phospholipids, and proteins make up 45-63%, 12-17%, and 8-16% of this lipoprotein in cattle, respectively (Bauchart, 1993). Apolipoprotein B100 (apoB100) is the major apolipoprotein in VLDL and functions in binding to low-density lipoprotein (LDL) receptors located on target tissues (Bauchart, 1993; Kawano and Cohen, 2013). This lipoprotein is synthesized on the surface of the endoplasmic reticulum (ER) and is transported to the ER lumen for VLDL synthesis by microsomal triglyceride transport protein (MTTP; Tiwari and Siddiqi, 2012). This enzyme is associated with the rough ER membrane and possesses both an apolipoprotein binding region and a lipid binding region (Tiwari and Siddiqi, 2012) and therefore plays an integral role in the assembly of VLDL. Once assembled, the VLDL particles are transported out of the ER to the Golgi apparatus via VLDL-transport vesicles,

where further processing occurs (Tiwari and Siddiqi, 2012). Post-trans-Golgi network VLDL transport vesicles carry mature VLDL particles to the plasma membrane, where fusion occurs and VLDL are released into circulation (Siddiqi, 2015).

## **PERIPARTURIENT FATTY LIVER DISEASE**

During the periparturient period, most (~50%) cows accumulate TG in the liver to some extent (Grummer, 1993; Jorritsma et al., 2001). Excessive accumulation of TG can lead to impaired amino acid and carbohydrate metabolism (Drackley et al., 2001). Bobe et al. (2004) classified hepatic lipidosis, or fatty liver disease, into four categories according to the percentage of TG present in the liver tissue (% of wet weight): normal (<1%), mild (1-5%), moderate (5-10%), and severe (>10%). Jorritsma et al. (2001) reported that 54% of the 218 early lactation cows that were sampled from 9 different grazing herds in The Netherlands suffered from moderate or severe fatty liver disease. Because not all cows experience fatty liver disease, Herdt (2000) suggested that fatty liver occurrence is due to the failure of some cows to adapt to sustained NEB. Fatty liver is categorized as a periparturient metabolic disease because the proportion of TG in the liver increases prepartum, peaks around parturition, and remains elevated throughout early lactation (Grummer, 1993).

### ***Development of Fatty Liver Disease***

Once FA are taken up by the liver and activated to FA-CoA they can either be oxidized completely to CO<sub>2</sub>, oxidized incompletely to ketone bodies, or incorporated into TG for export via VLDL. Ultimately, fatty liver results from the disparity between FA uptake and FA utilization and export. Since the rate of FA export in the form of VLDL-

TG is very slow in ruminants (Grummer, 1993), TG accumulation occurs during the periparturient period because more FA are taken up than can be oxidized or exported. As shown in Figure 2.1, the first regulatory step of mitochondrial FA oxidation is catalyzed by CPT-1, which requires carnitine (Kawano and Cohen, 2013). There is evidence that suggests hepatic carnitine levels are insufficient during the periparturient period. Jesse et al. (1986) observed that oxidation of labeled palmitate increased when liver slices were supplemented with L-carnitine. Abomasal infusion of L-carnitine reduced liver TG accumulation and increased the capacity of the liver to oxidize FA in lactating cows fasted to induce NEB (Carlson et al., 2006). Furthermore, Carlson et al. (2007) observed that carnitine supplementation to cows from -14 to 21 d relative to calving decreased liver TG accumulation, suggesting that carnitine increased in the rate of hepatic FA transport into the mitochondria. The rate of FA export in the form of VLDL-TG likely also plays a key role in the development of periparturient fatty liver disease (NRC, 2001). The supply of choline for phosphatidylcholine (PC) synthesis, the major phospholipid component of VLDL, may be an important determinant of the rate of TG export from the liver (Pinotti et al., 2002).

### ***Impacts on Health, Reproduction, and Profitability***

Fatty liver may develop as a direct result of the NEB induced by DMI depression associated with either parturition or diseases that reduce feed intake (NRC, 2001). Incidence of fatty liver disease correlates strongly with other metabolic diseases, including ketosis (Veenhuizen et al., 1991; Grummer, 1993), displaced abomasum (DA; Wensing et al., 1997; Kalaitzakis et al., 2006), and hypocalcaemia (Goff and Horst, 1997; Katoh, 2002). Ketosis is usually preceded by liver TG accumulation (Veenhuizen et al., 1991)

and cows with fatty liver usually have elevated levels of ketones in blood and urine (Bobe et al., 2004). Severe fatty liver disease can cause a dramatic reduction in DMI (Bobe et al., 2004), which predisposes the animal to a DA (Shaver, 1997) and exacerbates NEB and liver TG accumulation. On the contrary, the depression in DMI associated with a DA (Kalaitzakis et al., 2006), hypocalcaemia (Goff and Horst, 1997), or any other disorder, intensifies NEB which can lead to increased liver TG accumulation. The occurrence of mastitis and metritis during the postpartum period are also positively associated with liver TG content (Bobe et al., 2004) perhaps because excessive lipid mobilization contributes to immune dysfunction and inflammation (Sordillo and Raphael, 2013).

Fatty liver disease is also correlated to infertility in lactating dairy cows, which is likely due to its association with NEB and circulating NEFA levels. The proportion of oocytes obtained from cows with fatty liver that successfully developed into embryos during an in vitro fertilization technique was significantly lower than that of oocytes collected from healthy cows (Wensing et al., 1997). The authors concluded that oocytes that develop during the early postpartum period are probably negatively affected by high NEFA and low glucose concentrations in the blood as well as impaired liver function that is associated with hepatic lipidosis (Wensing et al., 1997). Additionally, liver TG content correlates positively with the number of days open, days to first insemination, days to first estrus, days to first ovulation, and services per cow required to achieve conception (Bobe et al., 2004). Although it is not determined whether the association between fatty liver and fertility is due to the inability of some cows to cope with the severe NEB and the subsequent elevation of plasma NEFA that occur during early lactation (Herdt, 2000) or due to a direct

effect of TG accumulation in the liver, it is clear that cows with fatty liver disease are at a higher risk for reproductive failure than healthy cows.

Severe fatty liver disease likely has a negative impact on whole-lactation milk production and consequently, lifetime profitability. Deluyker et al. (1991) determined that cows that experienced clinical ketosis during the first 3 wk postpartum produced 253 kg less milk throughout the first 119 DIM and had a 2.7 kg lower peak-milk yield than cows that did not develop clinical ketosis during the 3 wk postpartum. Although the study reported production losses coinciding with incidences of clinical ketosis, similar results might also be expected for fatty liver disease because the two diseases are related (Veenhuizen et al., 1991). Bobe et al. (2004) estimated that annual costs associated with fatty liver disease exceed \$60 million in the United States alone.

#### *Detection and Diagnosis of Fatty Liver Disease*

Hepatic lipidosis is difficult to diagnose because outward symptoms of the disease are nonspecific. Liver TG content correlates positively to urinary ketone output and in severe cases of fatty liver (>10% TG), feed intake will be significantly compromised (Bobe et al., 2004). A significant reduction in DMI perpetuates liver TG accumulation by increasing the magnitude of NEB, and enhances the risk for development of other disorders as discussed previously. Currently, the only accurate and reliable method of detection for fatty liver disease is through analysis of TG content of liver tissue obtained via biopsy (Bobe et al., 2004). There are several obvious disadvantages to assessing liver TG content via biopsy. The biopsy procedure is moderately invasive, labor intensive, and requires technical proficiency, making it impractical for producers. Thus, development of a non-



invasive approach to estimate liver TG content using data available on-farm would be beneficial in both industry and research settings.

Jorritsma et al. (2001) attempted to develop a predictive statistical model using data from 9 commercial herds in The Netherlands. Liver and blood samples were collected from 218 cows within 6-17 d postpartum. Additionally, for each cow, a single day milk production record made between wk 4 and 12 wk after calving was obtained. They regressed liver TG as a function of 1) herd (fixed) and blood concentrations of NEFA, glucose, and urea N, and 2) herd (fixed), milk production, and BCS change; the  $R^2$  was 0.33 and 0.22 for the first and second model, respectively. They concluded that their two models were unable to adequately predict liver TG content. However, it is possible that additional blood parameters, such as BHBA, VLDL, or other metabolites, average fat-corrected milk production during the first week postpartum, and measures of prepartum BCS and DMI could have improved the accuracy of the model.

Attempts have also been made to implement ultrasound in the detection of fatty liver disease. Bobe et al. (2008) utilized ultrasound to determine liver TG content in early lactation cows. Using measurements from the ultrasound images, they were able to correctly categorize 82% of the liver samples as normal liver or mild, moderate, or severe fatty liver based on the disease categories described by Bobe et al. (2004). These results suggest that ultrasound may be a useful tool in estimating liver TG content. However, the instrumentation required for and the technical nature of this approach discourages its use in the field.

### *Prevention and Treatment of Fatty Liver Disease*

The high prevalence of fatty liver during the periparturient period (>50% incidence rate; Jorritsma et al., 2001) has motivated the effort to elucidate the best method to prevent or mitigate liver TG accumulation in transition dairy cows. Both management and nutritional strategies have been implicated in the prevention of fatty liver disease. Cows that are fed ad libitum during the prepartum period have lower DMI postpartum and higher levels of liver TG compared with cows that are limit-fed during the prepartum period (Douglas et al., 2006). Douglas et al. (2006) suggested that feed-restricting prepartum cows may have increased the liver's capacity for oxidation of FA, allowing for more efficient NEFA processing than cows fed ad libitum. Management of appropriate BCS is critical for successful transition to lactation. Reid et al. (1986) observed that over conditioned cows at dry-off mobilized more body fat and had higher liver TG content postpartum than their thinner counterparts. Similarly, Roche et al. (2015) observed higher postpartum liver TG levels for cows with a higher BCS than those with a lower BCS at calving. Thus, limit-feeding during the prepartum period and managing late lactation and dry cows might be used to prevent excessive condition gain and may reduce severity of liver TG accumulation.

The effect of the nutrient composition of the prepartum diet on post-partum hepatic TG accumulation has also been examined. Grum et al. (1996) fed cows a high fat diet (6.7% ether extract), a high grain diet (49% concentrate), or a control diet (2.2% ether extract, 30% concentrate) during the dry period until 1 wk prepartum. Cows fed the high fat diet showed significantly lower levels of TG in liver tissue compared with cows fed both the high grain and control diets. In contrast, Bertics and Grummer (1999) concluded

that a diet supplemented with long chain FA to feed-restricted cows did not affect hepatic liver TG accumulation. Grummer (2008) summarized results from multiple studies that examined the effect of increasing non-fiber carbohydrate (NFC) content of the prepartum diet on postpartum liver TG and found that 4 out of 5 studies reported similar liver TG levels across the diets examined. Moreover, in the study by Douglas et al. (2006) where cows were fed either a high fat diet (6.4% fat, 22% NFC) or a low fat diet (2.8% fat, 36% NFC) at either ad libitum or restricted intake levels for 14 d before expected calving, the level of intake prepartum appeared to have a greater impact on postpartum liver TG accumulation than the composition of the prepartum diet. While the composition of the prepartum diet is still important, these studies suggest that the level of intake during the weeks prior to calving as well as the BCS at calving are also important determinants of liver TG accumulation.

Supplementing diets with chromium, niacin, conjugated linoleic acid (CLA), choline, or Met, have also been identified as potential strategies to prevent excessive hepatic TG accumulation during the transition period. Chromium plays a role in augmenting the insulin response through interaction with the insulin receptor (Vincent, 2000); therefore, supplying additional chromium may potentially enhance insulin sensitivity in the early lactation cow and result in depressed adipose tissue lipolysis. However, supplemental chromium (0 - 12 mg chromium per kg BW<sup>0.75</sup> in the form of chromium-Met) delivered orally once daily to periparturient dairy cows did not affect hepatic TG content (Hayirli et al., 2001; Smith et al., 2008). Niacin has been shown to reduce the rate of lipolysis in feed-restricted dry cows (Pires and Grummer, 2007) and may be potentially useful in reducing hepatic TG content. Morey et al. (2011) supplemented

cows with rumen protected niacin at either 0 or 24 g/d from d -21 to d 21 relative to parturition and observed a significant reduction in plasma NEFA at 5 and 10 d postpartum for niacin-treated cows, but similar concentrations of liver TG between control and treated cows. Therefore, although supplemental niacin can apparently reduce lipolysis in periparturient cows, it is still unclear whether or not it will be useful in the prevention of fatty liver. Grummer (2008) suggested that CLA could potentially alleviate periparturient fatty liver disease by reducing the severity of NEB since CLA, specifically *trans*-10, *cis*-12 CLA, has been shown to reduce lipogenesis in the mammary gland but enhance lipogenesis in the adipose tissue of lactating cows (Harvatine et al., 2009). CLA-enriched FA supplements (containing 6 to 29% *trans*-10, *cis*-12 CLA) fed to periparturient cows (21 d pre- through 63 d postpartum; Castañeda-Gutiérrez et al., 2005) and postpartum cows (1 to 40 DIM; Odens et al., 2007), tended to reduce or significantly reduced postpartum plasma NEFA concentrations, respectively. The reduction in plasma NEFA observed in early postpartum cows fed CLA-enriched FA supplements likely decreased liver NEFA uptake which could have potentially limited liver TG production. Choline and Met have been implicated as lipotropic agents that may facilitate the export of TG from the liver by increasing the availability of PC (Grummer, 2008), which is required for VLDL synthesis (Yao and Vance, 1988). Methionine is a precursor for the methyl donor, S-adenosylmethionine (SAM; Martinov et al., 2010), that is required for the de novo synthesis of PC from phosphatidylethanolamine (PE; Zeisell, 1981). Phosphatidylcholine can also be synthesized from a different pathway that utilizes dietary choline (Zeisell, 1981). Thus, increasing the amount of choline absorbed by the cow may reduce the rate of

de novo PC synthesis and spare Met, an already limiting amino acid for cows fed corn-based diets (NRC, 2001), for other needs such as milk protein synthesis.

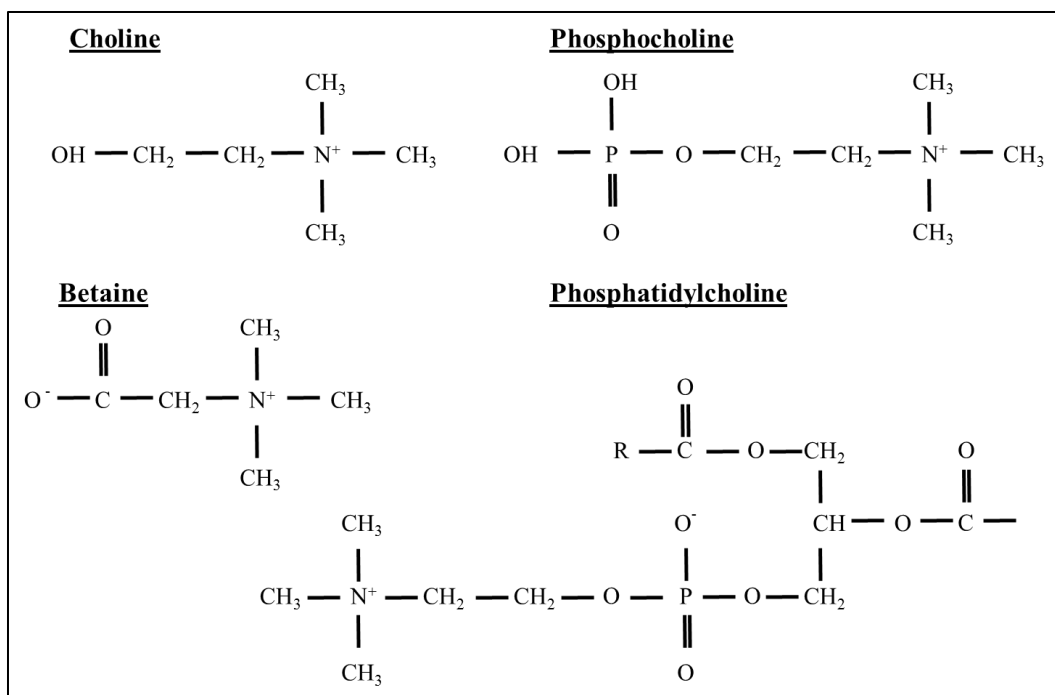
Because ketosis and fatty liver disease are related (Veenhuizen et al., 1991), approaches for the treatment of clinical ketosis could also be effective in the prevention or alleviation of fatty liver disease. Intravenous infusion of glucose precursors and oral delivery of propylene glycol are recognized as treatments for clinical ketosis (Baird, 1982). Propylene glycol reduced plasma NEFA concentrations in non-lactating, feed-restricted cows when administered as part of a concentrate mix or as an oral drench (Christensen et al., 1997), and reduced blood ketone concentrations when administered as a daily oral drench to cows beginning 7 d prepartum (Studer et al., 1993). A once daily oral drench of propylene glycol (500 mL/d) from 1 to 3 DIM resulted in numerically lower liver TG on d 7 postpartum and significantly reduced mean plasma NEFA concentration through 21 DIM (Pickett et al., 2003). Cows that received a 1-L propylene glycol drench daily for 7 d prepartum had significantly reduced liver TG concentrations at 1 and 21 DIM (Studer et al., 1993). Furthermore, Rukkwamsuk et al. (2005) showed that cows receiving a daily 400 mL oral drench of propylene glycol from -7 to 7 d relative to calving had significantly lower plasma NEFA and liver TG concentrations postpartum than control cows.

## **CHOLINE AND METHIONINE METABOLISM**

### ***Choline***

Choline is an essential nutrient for non-ruminant animals, including rats, preruminant calves (NRC, 2001), and humans (Zeisel and da Costa, 2009). It can be consumed in the diet or synthesized de novo in most cell types via the liberation of the

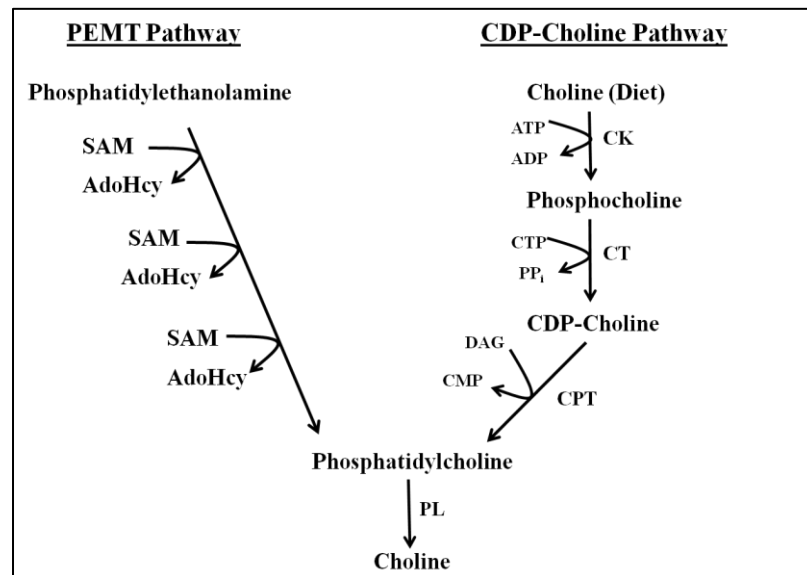
choline moiety from PC produced via the phosphatidylethanolamine *N*-methyltransferase (PEMT) pathway (Li and Vance, 2008). Choline is required for PC synthesis, the major phospholipid comprising cell membranes and lipoproteins, and plays a role in methyl group metabolism as a methyl donor via the betaine-homocysteine methyltransferase (BHMT) pathway (Zeisel and da Costa, 2009). Choline requirements for lactating dairy cows have not been specified (NRC, 2001). However, because choline is rapidly degraded in the rumen (Sharma and Erdman, 1988) requirements are probably met through de novo synthesis. Relative to nonpregnant, nonlactating women, recommended dietary choline intake is 6% and 29% greater for pregnant and lactating women, respectively (Zeisel and da Costa, 2009). Thus it seems that requirements for choline vary depending on physiological state. Perhaps the choline requirement of the periparturient dairy cow exceeds that which can be supplied via de novo synthesis, resulting in choline deficiency. The idea that the transition cow suffers from choline deficiency is supported by the high incidence of fatty liver disease (Jorritsma et al., 2001), which is the primary symptom of choline deficiency in other species (NRC, 2001).



**Figure 2.2.** Choline, phosphocholine, betaine, and phosphatidylcholine structures. Adapted from Nelson and Cox (2008).

Phosphatidylcholine is the primary phospholipid of VLDL and comprises >50% of the phospholipids of cell membranes of mammals (Pinotti et al., 2002; Zeisel and da Costa, 2009). De novo PC synthesis, which occurs in the ER, can occur via the Cytidine Diphosphate-choline (CDP-choline) Pathway or the PEMT Pathway (Figure 2.3; Li and Vance, 2008; Cole et al., 2012). Dietary choline is taken up by the cell via choline transporters and choline kinase (CK) phosphorylates the cellular choline to form phosphocholine (Li and Vance, 2008; Zeisel and da Costa, 2009). Phosphocholine is converted to (CDP-choline) by cytidine triphosphate:phosphocholine cytidylyltransferase (CT) which is the rate-limiting step in the CDP-Choline Pathway (Li and Vance, 2008). Transcription of the gene encoding CT (PCYT1a; Phosphate Cytidylyltransferase 1 Choline,  $\alpha$ ) is associated with cell division and growth instead of the energy status of the cell (Li and Vance, 2008), which is reasonable given that PC is a requirement for cell

membrane development. CDP-choline is then converted to PC via CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CTP), which is bound to the membrane of the ER (Li and Vance, 2008). Phosphatidylcholine synthesis via the PEMT pathway requires a series of 3 methylations of PE by SAM (Cole et al., 2012). The overall contribution of the CDP-choline pathway to the production of PC in the ruminant is probably limited because dietary choline is degraded extensively in the rumen (Sharma and Erdman, 1988). Thus, the PEMT pathway is most likely the main source of de novo PC in the dairy cow.



**Figure 2.3.** Synthesis of phosphatidylcholine via the PEMT Pathway and the CDP-Choline Pathway. SAM (*S*-adenosylmethionine); AdoHcy (*S*-adenosylhomocysteine); CK (choline kinase); CT (cytidine triphosphate:phosphocholine cytidyltransferase); CTP (CDP-choline:1,2-diacylglycerol cholinephosphotransferase); DAG (diacylglycerol); PL (phospholipase). Adapted from Cole et al. (2012).

#### *Choline as a Methyl Donor*

In hepatocytes and probably mammary cells as well, free choline can play a role in methyl metabolism. Free choline is converted to betaine via choline oxidase (Zeisell, 1981; Pinotti et al., 2002; Eklund et al., 2005). Betaine can donate a methyl group to homocysteine to regenerate Met, a process catalyzed by BHMT (Pinotti et al., 2002;



Eklund et al., 2005). Methionine can then enter the amino acid pool for protein synthesis, or serve as a precursor for SAM, a methyl donor required for many metabolic reactions (Selhub, 1999). Thus it seems that supplemental dietary choline could spare Met for protein synthesis by providing additional betaine for the methylation of homocysteine. However, a recent in vitro experiment with cultured calf hepatocytes showed that choline chloride failed to increase the expression of *BHMT*, but instead increased expression of *methionine synthase*, an additional mechanism by which Met can be generated from homocysteine (Chandler et al., 2015).

#### *Supplemental Choline in Dairy Cow Diets*

The study of the effect of choline in dairy cattle rations is complicated by the fact that dietary choline is degraded extensively by rumen microorganisms (Atkins et al., 1988). Early efforts to determine the choline requirement of lactating dairy cattle utilized abomasal infusions of choline chloride to provide a post-ruminal supply of choline (Sharma and Erdman, 1989). Modern encapsulation technology has made it possible to protect choline from degradation in the rumen and these rumen protected choline (RPC) products are now commercially available to livestock producers. Deuchler et al. (1998) estimated that one RPC product achieved 85% protection from ruminal degradation. The availability of RPC products provides a more practical avenue through which the effects of choline supplementation can be investigated in ruminants.

Although RPC products have been developed and protection from rumen degradation is verified (Deuchler et al., 1998), methods to assess the intestinal bioavailability of choline supplied in this form once ingested have not been established.

For some nutrients, this can simply be estimated by measuring total tract digestibility; however, for other nutrients, such as choline and AA, this approach would result in an inaccurate assessment of availability due to microbial fermentation and degradation in the lower gut. In such cases, nutrient bioavailability is better determined indirectly by measuring changes in blood, milk, or body tissue markers in response to increasing nutrient supply. Deuchler et al. (1998) showed that post-ruminal choline chloride, delivered via an abomasal infusion, linearly increased total milk choline secretion. Furthermore, choline chloride fed as RPC (50 g/d choline chloride) increased milk total choline concentration, but not yield in that study (Deuchler et al., 1998). Recent characterization of milk and blood choline metabolite profiles throughout lactation have been reported (Artegoitia et al., 2014). In their study of choline metabolites, Artegoitia et al. (2014) identified several choline metabolites in blood and milk, including betaine, free choline, phosphocholine, glycerophosphocholine, sphingomyelin, as well as several species of PC and lysophosphatidylcholine. These choline metabolites could be useful indicators of post-ruminal choline supply, which could have utility in determining the bioavailability of choline supplied as RPC.

Recently, de Veth and coworkers (2016) suggested that concentrations of specific choline metabolites in blood and milk were more indicative of post-ruminal choline supply than total choline. In their study, de Veth et al. (2016) showed that post-ruminal choline chloride delivered via abomasal infusion, but not RPC, increased plasma betaine, free choline, and phosphocholine. However, modest increases in plasma PC concentration were only apparent for cows supplied post-ruminal choline as RPC (de Veth et al., 2016). Furthermore, milk yields and concentrations of betaine, free choline, phosphocholine, and

acetylcholine were increased by abomasal infusion of choline chloride, but not RPC. These results suggest that betaine, free choline, and phosphocholine could serve as potential markers for estimating post-ruminal choline supply. However, in order to reliably calculate the bioavailability of choline supplied as RPC using the marker approach, it is imperative that such markers are responsive to post-ruminal choline supplied as RPC. It is possible that the short experimental periods (5 days) in the study by de Veth et al. (2016) precluded RPC from eliciting changes in blood and milk choline metabolites similar to those observed when choline chloride was infused into the abomasum. Further research in this area is needed in order to identify markers that will be responsive to post-ruminal choline supplied as RPC.

Several studies have examined the effect of feeding supplemental RPC to periparturient dairy cattle diets with the expectation that RPC would reduce liver TG accumulation and improve postpartum performance. Cooke et al. (2007) induced lipolysis in non-lactating pregnant cows to mimic NEB experienced in the early lactation period by restricting feed intake to 30% of requirements for maintenance and gestation. They observed that cows fed a diet containing 60 g/d RPC had significantly reduced liver TG levels compared with cows that did not receive RPC after 10 d of feed restriction. Recently, Zenobi et al. (2018) demonstrated that increasing levels of RPC (30 to 120 g/d RPC) fed to feed-restricted dry cows reduced liver TG content in a linear fashion. Furthermore, RPC also increased liver glycogen during feed restriction (Zenobi et al., 2018b). Zom et al. (2011) showed that RPC (60 g/d) fed from -21 through 42 d relative to calving reduced liver TG accumulation during wk 1 and wk 4 postpartum compared to control cows. In a companion paper, Goselink et al. (2013) attempted to elucidate the mechanisms by which

choline affects periparturient cow lipid metabolism by assessing changes in the expression of genes related to lipid metabolism in hepatic and adipose tissues. Cows supplemented with 60 g/d RPC had increased *FATP*, *PPAR $\delta$* , *MTP*, and *apoB100* mRNA expression in hepatic tissue. These results suggest that cows supplemented with RPC had an enhanced ability to take up NEFA (as indicated by increased *FATP* expression), perform peroxisomal FA oxidation (as indicated by increased *PPAR $\delta$*  expression), and export TG as VLDL (as indicated by increased *MTTP* and *APOB100* expression). In contrast, Zhou et al. (2016a) showed no effect of choline on liver TG content when fed to cows from -21 through 30 DIM. Although they did not observe changes in liver TG content, Piepenbrink and Overton (2003) showed that liver tissue harvested from periparturient cows (-21 through 63 DIM) fed RPC (45, 60, or 75 g/d) had an elevated capacity to oxidize FA. In addition, RPC increased liver glycogen content for those cows (Piepenbrink and Overton, 2003). Despite mixed reports of the effect of choline on hepatic TG accumulation, results by Goselink et al. (2013) provide support for choline as a lipotropic agent capable of improving hepatic lipid metabolism in periparturient dairy cows.

Production responses to supplemental RPC in lactating cows have been variable. Some studies fail to show a significant effect of feeding RPC on DMI postpartum (Hartwell et al., 2000; Piepenbrink and Overton, 2003; Zhou et al., 2016a; Zenobi et al., 2018a); however, Zom et al., 2011 reported significantly greater postpartum DMI for periparturient cows fed a RPC supplement pre- and postpartum relative to control cows. Both Piepenbrink and Overton (2003), Zom et al. (2011), and Zhou et al. (2016a) observed similar milk yield between control and RPC-fed cows. However, Elek et al. (2008) showed that RPC-supplemented cows (25 g choline/d for 21 d prepartum and 50 g choline/d for 60

d postpartum) had significantly greater milk yield. Furthermore, Zenobi et al. (2018a) provided supplemental choline from -17 through 105 DIM and showed that RPC tended to increase milk yield and 3.5% fat-corrected milk (FCM) yield and this change was sustained throughout lactation, even after RPC supplementation ceased. In the study by Hartwell et al. (2000), RPC was fed at 3 levels (0, 6, 12 g choline/d) across prepartum diets that differed in rumen undegradable protein (RUP; 4 and 6.2%) content. A significant RUP by RPC interaction revealed that the highest level of RPC (12 g/d) coupled with the low RUP prepartum diet (4% RUP) significantly increased milk yield during the first 56 d postpartum; however, the opposite response was observed for the high RUP (6.2%) diet. Positive effects of RPC observed for cows fed the low RUP prepartum diet suggests that choline plays a role in mitigating Met deficiency. There may also be positive effects of feeding RPC during the transition period on milk protein yield (Elek et al., 2008; Zom et al., 2011; Zenobi et al., 2018a), milk fat yield (Elek et al., 2008; Zenobi et al., 2018a), and milk fat and protein concentrations (Leiva et al., 2015). However, Hartwell et al. (2000) observed similar yields of milk fat and protein among RPC and control cows across high and low RUP diets. Furthermore, a significant RUP by RPC interaction for milk protein concentration indicated that the highest level of RPC (12 g choline/d) coupled with the low RUP diet (4% RUP) significantly reduced milk protein concentration while the opposite effect was observed for the high RUP (6.2%) diet. These studies indicate that providing supplemental choline in the diet, in addition to improving liver lipid metabolism, may potentially improve DMI and milk and milk component production which may have important implications for health and profitability. However, results reported to date are variable which likely reflects interactions between choline and other important nutrients,

such as amino acids and B-vitamins, as well as the pre- and postpartum choline status of the cow. More research is needed to determine the conditions under which supplemental choline will have the most beneficial effects.

#### *Effects of Choline on Hepatic Gene Expression*

To further our understanding of the mechanisms by which choline affects periparturient cows, recent research has focused on its effects on the expression of genes associated with lipid metabolism. In their study of periparturient cows, Goselink et al. (2013) showed that RPC increased hepatic expression of *MTTP*, the gene that encodes MTP, and *APOB100*, suggesting that choline increased VLDL assembly. These results corresponded with the choline-induced reduction in liver TG content for those cows (Zom et al., 2011). Furthermore, RPC enhanced expression of *FATP5*, *PPAR $\delta$* , and *CPT1A* (Goselink et al., 2013) indicating that choline could have improved hepatic FA transport and oxidation. Alteration in hepatic expression of genes related to glucose metabolism (*pyruvate carboxylase* and *GLUT2*) also suggest that choline affected glucose production by the liver (Goselink et al., 2013), which supports previous results that showed an increase in hepatic glycogen content in periparturient cows fed RPC (Piepenbrink and Overton, 2003). In contrast to these findings, Morrison et al. (2019) failed to observe any changes in hepatic expression of genes associated with FA oxidation, ketogenesis, VLDL synthesis, or glucose metabolism when periparturient cows were fed a supplement that included a mixture of B-vitamins and choline. The reason for this discrepancy could be related to differences in the supplement fed (choline vs. choline and B-vitamins) as well as variation in the nutrient profile of the diets.

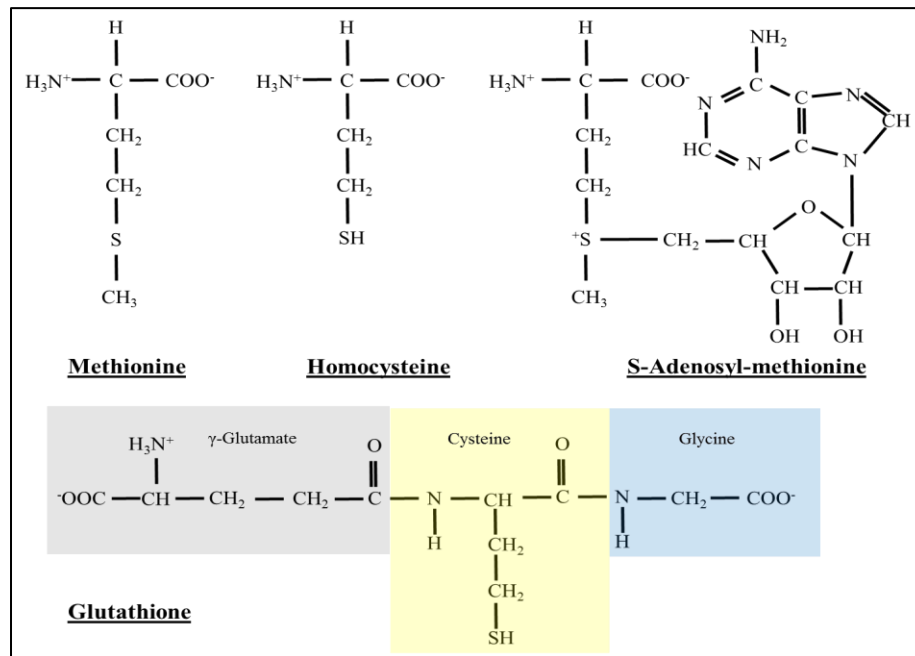
Given that choline and Met metabolism are closely related through one-carbon metabolism, it is of interest whether or not supplementation of choline affects expression of genes associated with these pathways. To date, only one study has examined whether choline supplementation affects expression of genes involved in hepatic one-carbon metabolism in periparturient dairy cows. In their study, Zhou et al. (2017) showed that supplemental choline increased the expression of two genes associated with the conversion of choline to betaine (*choline dehydrogenase* and *betaine aldehyde dehydrogenase*), suggesting that the supplemental choline was being used to synthesize betaine, which is required for Met recycling from Hcy via the BHMT pathway<sup>1</sup>, although BHMT activity was not affected by RPC. Furthermore, RPC reduced the activity of 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR), the enzyme that catalyzes the rate-limiting step of the CH<sub>3</sub>-THF pathway. Together, these results suggest that supplemental choline could have shifted Met recycling toward the BHMT pathway, rather than the CH<sub>3</sub>-THF pathway.

In order to determine whether or not the choline supplied as RPC affects PC synthesis, which could have implications for subsequent VLDL assembly, it is also of interest to examine its effects on the expression of genes associated with the CDP-choline and PEMT pathways. Choline did not alter hepatic expression of *PEMT* (Zhou et al., 2017), suggesting no effect on de novo PC synthesis. However, expression of *PCYT1a*, which encodes the rate-limiting enzyme in the CDP-choline pathway, phosphate cytidyltransferase 1 choline  $\alpha$ , was elevated by RPC, which could be indicative of enhanced PC synthesis via this pathway. Studies in rodents suggest that PC species containing PUFA are likely derived via the PEMT pathway (DeLong et al., 1999). Given the recent characterization of various choline metabolites and PC species in blood and milk

of dairy cows throughout lactation (Artegoitia et al., 2014), assessment of blood or milk PC profile could have utility in determining dietary treatment effects on PC origin. However, specific PC species were not examined in the study by Zhou et al. (2017).

### ***Methionine***

Methionine is the first limiting amino acid in dairy cows fed corn-based diets (NRC, 2001). Because dietary Met is degraded in the rumen, supplemental Met must be fed in a rumen-protected form (Bach and Stern, 2000). Rumen protected Met (RPM) products are commercially available, and many studies have examined the effect of providing additional Met in lactating dairy cow diets. Not only is Met required for protein synthesis, but it also plays a role in methyl donation and the formation of glutathione, an important antioxidant (Martinov et al., 2010), studies also have examined potential effects that Met may have during the periparturient period.



**Figure 2.4.** Structures of methionine, homocysteine, S-adenosyl-methionine, and glutathione. Adapted from Nelson and Cox (2008).



### *Methionine as a Methyl Donor*

Methionine serves as the precursor for the methyl donor, SAM, which is a required participant in many metabolic reactions (Chiang et al., 1996). Methionine is converted to SAM through the addition of an adenosyl group by SAM synthetase, a process that requires 1 ATP (Figures 2.4 and 2.5). *S*-adenosylmethionine is capable of donating a methyl group in a variety of biochemical reactions, such as histone modifications that alter gene expression, protein methylations that change protein function, and phospholipid methylations (Chiang et al., 1996). As shown in Figure 2.3, SAM also is important in the synthesis of PC via the PEMT pathway, which is likely the primary pathway by which ruminants derive PC. After donation of a methyl group, SAM becomes *S*-adenosylhomocysteine (AdoHcy), which is then converted to homocysteine (Hcy; Figure 2.4). Homocysteine can then be converted back to Met via 1) the BHMT pathway, a process which requires betaine, the product of choline oxidation, or 2) the CH<sub>3</sub>-THF pathway which requires vitamin B<sub>12</sub> (Figure 2.5). Thus, it is clear that Met and choline metabolism are intertwined by the requirement of SAM for the synthesis of PC and the requirement of betaine for the regeneration of Met from Hcy via the BHMT pathway.

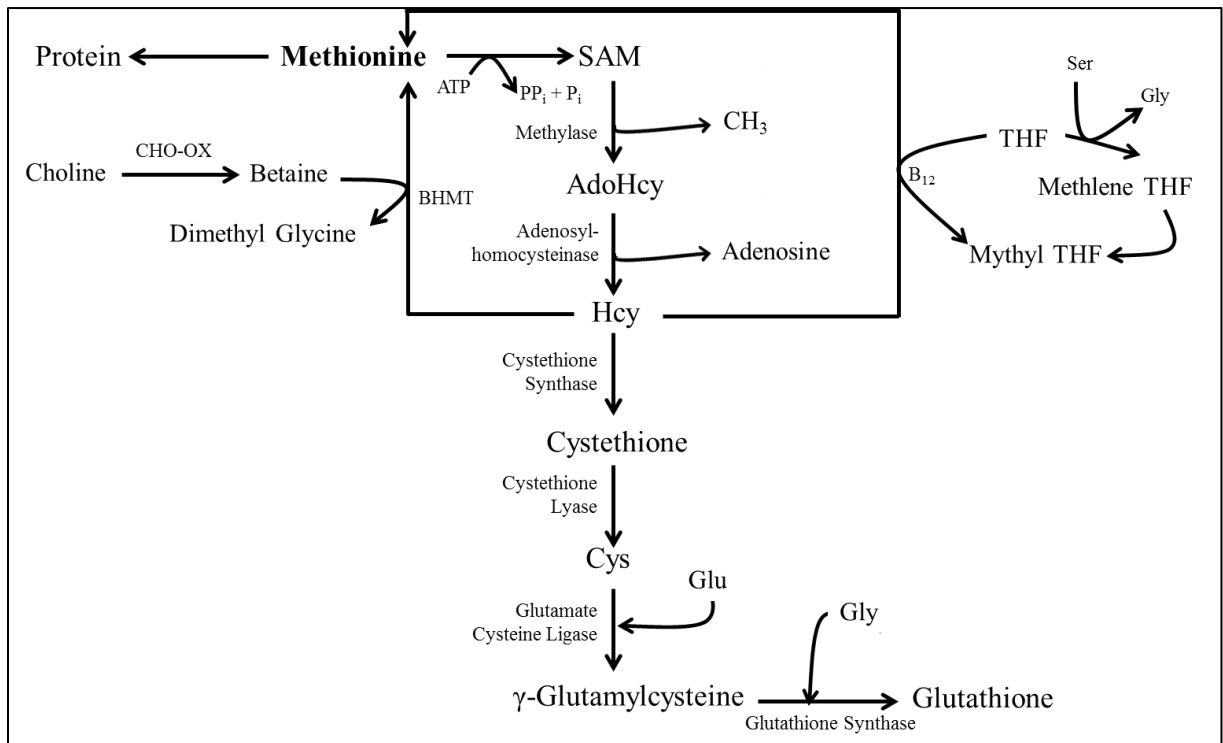
### *Methionine as a Precursor for Glutathione*

Methionine can also play a role in the production of glutathione, a tripeptide formed from  $\gamma$ -glutamine, cysteine, and glycine (Figure 2.4). Glutathione plays several roles in metabolism which includes but is not limited to DNA synthesis, molecule transport, and protection of cells from oxidative damage through neutralization of reactive oxygen species (ROS) and free radicals (Meister and Anderson, 1983). Glutathione is the most abundant

reducing molecule in rat liver cells (Yuan and Kaplowitz, 2009) likely because the liver is the major site for detoxification reactions in the body. Reactive oxygen species, such as  $H_2O_2$  and  $HO^\cdot$ , are produced in abundance during times of inflammation and cellular stress (Yuan and Kaplowitz, 2009). The dairy cow undergoes metabolic stress during the transition period and endures higher than normal levels of oxidative stress due to an imbalance between ROS production and antioxidant function (Sordillo and Raphael, 2013). This oxidative stress can lead to damage and impaired function of other tissues and can be detrimental to immune function (Sordillo and Raphael, 2013) which perpetuates metabolic stress through induction of greater NEB. Strategies to reduce oxidative stress during the periparturient period include feeding antioxidants, such as vitamin E and selenium, and reducing the magnitude of NEB experienced during this time, as high levels of plasma NEFA promote ROS production (Sordillo and Raphael, 2013). As an additional dietary strategy, feeding supplemental Met during the transition period may also help reduce oxidative stress because Hcy can be converted to cysteine, which is a precursor for glutathione (Figure 2.5).

Zhou et al. (2016b) showed that RPM increased hepatic concentration of glutathione in its reduced form during the periparturient period, which is indicative of improved ability to cope with free radicals and ROS. Osorio et al. (2014b) showed that RPM decreased the expression of liver genes associated with glutathione production, but suggested that this finding was related to negative feedback associated with increased glutathione availability. Furthermore, cows fed RPM also had reduced hepatic expression of genes associated with another antioxidant, superoxide dismutase, further suggesting that RPM decreased the need for antioxidants (Osorio et al., 2014b). In a companion analysis

for the same study, the researchers also showed that RPM reduced the apparent oxidative stress postpartum (Osorio et al., 2014a). Therefore, it seems that feeding supplemental Met does have potential to improve the immune status of periparturient cows and this effect is related, in part, to the ability to manage oxidative stress that occurs postpartum.



**Figure 2.5.** Metabolism of methionine. *S*-adenosylmethionine (SAM); AdoHcy (*S*-adenosylhomocysteine); Hcy (homocysteine); THF (tetrahydrofolate); BHMT (betaine-homocysteine *S*-methyltransferase). Adapted from Chiang et al. (1996) and Nelson and Cox (2008).

### *Supplemental Methionine in Dairy Cow Diets*

Several studies have examined production effects associated with feeding RPM throughout lactation. Mixed production responses have been observed when cows in established lactation are supplemented with RPM or post-ruminally infused with Met, which could be explained by differences in the type of Met supplement administered, diet

components, the stage of lactation, or the dietary content of other amino acids such as Lys. Due to the high variability across studies for production responses associated with providing supplemental Met, a meta-analysis can be extremely useful in the detection of potential treatment effects through the use of data obtained from multiple studies. In a recent meta-analysis of production responses for cows in established lactation, Zanton et al. (2014) observed that post-ruminal infusion of Met did not affect DMI, but that feeding a particular RPM product (Smartamine M<sup>®</sup>) resulted in significantly greater DMI. In contrast, feeding a different PRM product (Mepron<sup>®</sup>) resulted in significantly lower DMI compared with control cows. Supplemental Met, whether provided as a protected source or through post-ruminal infusion did not significantly alter milk yield; however, both milk protein and fat yields were significantly increased when compared with non-supplemented control cows (Zanton et al., 2014). The milk protein response was not significantly different between RPM (Mepron<sup>®</sup> and Smartamine M<sup>®</sup>) and post-ruminal infusion of Met, but the milk fat response was significantly lower for Smartamine when compared with post-ruminal infusion of Met (Zanton et al., 2014). Because production results varied with the specific supplemental Met source, Zanton and coworkers (2014) suggest 1) that the mechanism by which these Met sources function differ or 2) the post-ruminal availability of Met supplied varies depending on the source. Other individual studies have consistently shown an increase in milk protein production for cows in established lactation provided supplemental Met (Rogers et al., 1989; Chen et al., 2011).

Because of Met's role as a limiting amino acid, as well as its potential function as a lipotrope and a precursor for glutathione, some research has been conducted to determine if supplemental Met could improve health and performance during the

periparturient period. Some studies have reported increases in postpartum DMI as a result of providing supplemental RPM during the periparturient period (Ordway et al., 2009; Osorio et al., 2013; Zhou et al. 2016a). Ordway et al. (2009) observed a significant improvement in postpartum energy balance for cows supplemented with one of two RPM products throughout the transition period. In contrast, Osorio et al. (2013) and Zhou et al. (2016a) did not observe any effect of RPM supplementation on postpartum energy balance. However, these researchers did observe that RPM increased milk yield, milk protein concentration, as well as yields of both milk fat and protein (Osorio et al., 2013; Zhou et al., 2016a). Piepenbrink et al. (2004) also fed two doses of supplemental RPM to transition dairy cows and observed a significant quadratic effect for milk yield, with the low dose of RPM resulting in improved yield over the control; however, no changes in milk components were observed. Additionally, Davidson et al. (2008) supplemented RPM during the early lactation period and observed a significant increase in milk protein yield but no changes in DMI, milk yield, or milk fat yield. Ordway et al. (2009) showed that RPM increased milk protein concentration, but had no effect on milk or component yields. Thus, production responses to feeding RPM during the periparturient period are variable, but it does seem to increase milk protein yield and/or concentration in most instances.

In addition to observed positive effects on production, several studies have indicated that RPM improves the immune status of periparturient cows (Osorio et al., 2014a; Zhou et al., 2016b; Batistel et al., 2017). Furthermore, Zhou et al. (2016a) also showed a tendency for RPM to reduce incidence of ketosis and retained placenta after calving. In contrast, others failed to see any effect of Met on incidence of metabolic disease (Osorio et al., 2013) and changes in plasma NEFA or BHBA for periparturient cows

(Davidson et al., 2008). Thus, Met seems to be able to consistently elicit immune responses during the periparturient period, but RPM-associated improvements in metabolic disease status appear to be more variable. Much larger studies would be required in order to conclusively determine if RPM reduces incidence of metabolic disease during the periparturient period.

Because Met is a precursor for SAM, which is required for the formation of PC, it may have a role as a lipotropic agent, as hypothesized by Durand et al. (1992). Durand et al. (1992) observed an increase in hepatic VLDL secretion in response to infusion of a mixture of Lys and Met in early postpartum cows and suggested that Met may work to improve TG export from the liver. In contrast, evaluation of liver TG content throughout the periparturient period in studies by Osorio et al. (2013) and Zhou et al. (2016a) showed no effect of RPM on liver TG content. Furthermore, Piepenbrink et al. (2004) failed to observe any change in liver TG content or liver FA oxidation when two different doses of a RPM were fed to periparturient cows. Based on these findings, it seems unlikely that RPM acts as a lipotrope when supplemented during the periparturient period.

#### *Effects of Methionine on Hepatic Gene Expression*

Recently, studies have examined effects of RPM on hepatic expression of genes associated with lipid and one-carbon metabolism in order to further understand the mechanism by which it affects performance of periparturient cows. Osorio et al., (2016) showed that RPM fed during the periparturient period increased expression of *PPAR $\alpha$*  and reduced expression of *HMGCS2* at 21 DIM. These results suggest an increased capacity of peroxisomal FA oxidation and a reduction in partial oxidation through the TCA cycle.

However, *CPT1A* expression was not affected by RPM (Osorio et al., 2016). Reduced expression of *MTTP* and *APOB100* at 21 DIM for cows fed RPM also suggested a decrease in VLDL assembly (Osorio et al., 2016). Although liver TG content was not affected by RPM in that study (Osorio et al., 2013), perhaps this change reflects a reduction in the need for TG export via VLDL and a greater capacity for the liver to cope with the influx of FA during this time. In contrast, Preynat et al. (2010) showed that RPM increased *MTTP* expression in periparturient cows.

Changes in the hepatic expression of genes associated with one-carbon metabolism have also been examined for periparturient cows fed RPM. The RPM-associated increases in methionine adenosyltransferase 1A and S-adenosylhomocysteine hydrolase expression observed by Zhou et al. (2017) and Osorio et al. (2014a) suggest an elevation in SAM production and subsequent increases in SAM-related methyl-donation reactions. Although expression of *BHMT* and *MTR* were not affected in the study by Zhou et al. (2017), RPM reduced MTR activity, suggesting that it reduced the need for Met recycling via the CH<sub>3</sub>-THF pathway. In support of this, Preynat et al. (2010) showed that RPM decreased expression of *5,10 methyl-tetrahydrofolate reductase*, the an enzyme that is important for the recycling of THF intermediates that result from the CH<sub>3</sub>-THF pathway. Similar to results reported by Zhou et al. (2017), expression of *MTR* and *BHMT* were also not affected by RPM (Preynat et al., 2010). In contrast, Osorio et al. (2014a) also showed that RPM increased *MTR* expression, particularly at 10 d prepartum and 7 d postpartum, although *BHMT* expression was not affected. Whether or not RPM has an apparent effect on the CH<sub>3</sub>-THF pathway could be related to the initial methyl status of the cows, their physiological state, as well as the level of Met and other amino acids in the diet. Further

research is needed in order to understand how and under what conditions RPM affects performance and metabolism during the periparturient period.

### ***Relationship between Choline and Methionine Metabolism***

As discussed previously, choline and Met metabolism are connected through their involvement in one-carbon metabolism and PC synthesis. As the precursor for betaine, choline can participate in the regeneration of Met from Hcy via the BHMT pathway (Martinov et al., 2010). Choline is required for PC synthesis via the CDP-choline pathway and Met, as the precursor of SAM, is indirectly required for PC synthesis via the PEMT pathway (Li and Vance, 2008). Because both choline and Met have apparently positive effects on dairy cattle performance, it is of interest to determine if supplementation of both nutrients will have a positive synergistic effect. Furthermore, it is also of interest to determine if providing supplemental choline can enhance Met recycling from Hcy via the BHMT pathway in order to spare Met for other purposes. Only one previous study examined effects of providing both RPC and RPM to periparturient dairy cows, and presence of such a synergistic effect was not substantiated (Zhou et al., 2016a). However, further research in this area is required to improve the understanding of how providing supplemental choline, Met, or both benefit the periparturient dairy cow.



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## **CHAPTER 3: EXPERIMENT 1**

### **Use of Blood and Milk Markers to Assess Post-ruminal Choline Supply<sup>1</sup>**

<sup>1</sup>S.B. Potts, E. Davis, and R.A. Erdman. Use of blood and milk markers to assess post-ruminal choline supply. In preparation for submission to the Journal of Dairy Science.

## ABSTRACT

The objectives of this study were to 1) identify potential blood and milk markers of post-ruminal choline chloride (ChoCl) supply and 2) determine the rate at which rumen-protected choline (RPC) must be fed in order to elicit changes in these metabolites in blood and milk such that apparent bioavailability can be calculated. Study 1 utilized 5 rumen fistulated Holstein cows ( $208 \pm 108$  DIM) in a 5 x 5 Latin square design with 14-d treatment periods. The five treatments included a basal TMR, and the basal TMR plus 15 g/d ChoCl as RPC, 30 g/d ChoCl as RPC, 15g/d ChoCl as a 7-d abomasal infusion, and 30 g/d ChoCl as a 7-d abomasal infusion. Milk and blood were sampled daily during the last 3 days of each period and analyzed for concentrations of choline metabolites by HILC-MS/MS. ChoCl delivered via abomasal infusion, but not RPC, increased concentrations of both betaine and free choline in both blood and milk. Furthermore, abomasal infusion of ChoCl increased milk concentrations and yields of both total choline and phosphocholine. Neither abomasal infusion of ChoCl nor RPC altered the response of blood or milk total phosphatidylcholine, total lysophosphatidylcholine, or individual species of these compounds. Study 2 utilized 20 Holstein cows ( $163 \pm 38$  DIM) in a completely randomized experiment with a 10-d covariate and 21-d treatment period. Dietary treatments included a basal TMR and the basal TMR plus 35, 70, and 105 g/d ChoCl as RPC. Blood and milk were sampled daily during the last 3 days of both the 10-d covariate and the 21-d treatment periods. Treatment had no effect on milk or blood free choline or betaine responses. Collectively, these results suggest that betaine and free choline in milk and blood are the most responsive indicators of post-ruminal ChoCl supplied via abomasal infusion. Perhaps a relatively slower release of choline supplied via RPC into the small intestine, reduces the

efficacy of these metabolites to serve as indicators of post-ruminal choline supplied in this manner.

## INTRODUCTION

Choline is an important micronutrient that plays many crucial roles within the body. The choline ion itself can act as a methyl donor to regenerate the universal methyl donor, *S*-adenosylmethionine, making it an important participant in one-carbon metabolism (Zeisel and da Costa, 2009). Furthermore, choline can serve as a precursor for the neurotransmitter, acetylcholine, as well as the primary phospholipid in cellular membranes, phosphatidylcholine (PC; Zeisel and da Costa, 2009). Phosphatidylcholine is also the predominant membrane lipid in very low density lipoproteins (VLDL), which is required for the export of triglycerides, from the liver (Li and Vance, 2008). Because of this crucial function, in many mammals the primary symptom of choline deficiency is triglyceride accumulation in the liver (NRC, 2001). Although the choline requirement of lactating dairy cattle has not been formally defined (NRC, 2001), the relatively high incidence of liver triglyceride accumulation that occurs during the periparturient period (Jorritsma et al., 2001) suggests that the choline requirement during this period may in fact be higher than during other periods of the lactation cycle.

For most animals, the choline requirement is primarily met through its consumption in the diet (Zeisel and da Costa, 2009). However, in ruminant animals, dietary choline is subject to extensive rumen degradation (Atkins et al., 1988), making it difficult to increase choline availability to the animal by traditional diet supplementation. Because of this, rumen-protected choline (RPC) products have been developed in order to protect choline from microbial degradation in the rumen, while still allowing for digestion and absorption in the small intestine. Some products have been shown to have over 85% protection from

rumen degradation (Deuchler et al., 1998). However, the efficiency with which choline supplied as RPC is absorbed post-ruminally has not been well-established. Traditional methods used for determining nutrient bioavailability, such as digestion studies, cannot be reliably applied for some nutrients such as choline and amino acids due to the ability of microbes of the lower gut to degrade and synthesize these nutrients. Because of this, the bioavailability and intestinal absorption of fermentable nutrients can be estimated indirectly by changes in blood or tissue metabolite concentrations in response to increasing nutrient dose (Rulquin and Kowalczyk, 2003) using the slope-ratio technique (Batterham, 1992; Littell et al., 1997). In order to estimate bioavailability using this approach, a reliable marker of post-ruminal choline supply must be identified.

Previous work in our laboratory demonstrated that milk total choline is responsive to post-ruminal choline supplied via abomasal infusion (Deuchler et al., 1998). In an effort to identify specific marks to assess post-ruminal choline supply, de Veth et al. (2016) showed that specific choline metabolites, namely betaine and free choline, in blood and milk increased in response to post-ruminal choline chloride (12.5 or 25 g/d) supplied via abomasal infusion. These results suggest the utility of betaine and free choline as potential candidates to serve as markers of post-ruminal choline supply. However, when RPC was fed to deliver similar amounts of post-ruminal choline chloride in the same study, no consistent response in blood or milk free choline or betaine was observed. The lack of choline metabolite responses to RPC may have been due to the relatively short feeding period (4 days) prior to sampling. Depending on rate at which RPC mixes with rumen contents and its density relative to particles flowing out of the rumen, there may have been a lag between the initiation of feeding and intestinal appearance of choline. It is also

possible that the bioavailability of choline chloride supplied as RPC is much lower than that of choline chloride supplied via abomasal infusion, such that insufficient levels of RPC were fed during that study to cause significant responses in blood or milk choline metabolites.

The objectives were to 1) identify potential blood and milk markers of post-ruminal choline chloride supply and 2) determine the rate at which RPC must be fed in order to elicit changes in these metabolites in blood and milk such that apparent choline bioavailability can be calculated.

## **MATERIALS AND METHODS**

Both experiments were conducted according to procedures approved by the University of Maryland, College Park Institutional Animal Care and Use Committee.

### ***Study 1***

#### ***Design and Treatments***

Five mid to late-lactation Holstein cows ( $208 \pm 108$  DIM), each fitted with a rumen cannula, were used in a 5x5 Latin square experiment with 14-day experimental periods. The five experimental treatments included: 1) a basal TMR (CON); 2) the basal TMR plus 52 g/d RPC (ReaShure<sup>®</sup>, Balchem Corp., New Hampton, NY) to supply 15 g choline chloride (ChoCl; RPC15); 3) the basal TMR plus 104 g/d RPC to supply 30 g ChoCl (RPC30); 4) basal TMR plus 7-days continuous abomasal infusion of 15g/d ChoCl (ABO15); and 5) basal TMR plus 7-days continuous abomasal infusion of 30g/d ChoCl



(ABO30). The first 7 days of each period served as diet adaptation period for cows fed RPC. Cows were housed in individual tie-stalls fitted with water mattresses and bedded with sawdust to allow for collection of individual feed intake data. Cows were milked twice daily in a milking parlor at 0630 and 1600 h. All cows were fed the same basal TMR (Table 1), balanced to meet requirements of a 624 kg Holstein cow producing 35 kg milk per day at 3.85% fat (NRC, 2001), once daily at 0700 h for ad libitum intake. RPC was top-dressed immediately after feed delivery and hand-mixed into the top 1/3 of the feed offered. Body weight was measured at the start of each experimental period. Body condition was also assessed (1-5 scale; 1=thin, 5=fat) at the beginning of each experimental period by a single trained investigator.

#### *Abomasal Infusion*

Abomasal infusion lines consisted of 2m Tygon<sup>®</sup> tubing attached to a plastisol flange (10 cm) designed to keep the infusion line in the omasal-abomasal orifice. Infusion lines were put in place for all cows at the beginning of the experiment and remained in place throughout the entire study even when an animal was not receiving an infusion treatment. During each period, proper placement of the plastisol flange was assessed every other day during the infusion period.

Continuous abomasal infusions of ChoCl began the morning of d 8 of each period and continued through d 14 for the cows receiving ABO15 and ABO30. Each infusion treatment was prepared by mixing ChoCl (15 g or 30 g; 99% ChoCl, Product # F6522120, Balchem Corp., New Hampton, NY) with 2 L H<sub>2</sub>O. Treatments were continuously infused for 22 h each day via a peristaltic pump (Harvard Apparatus, Holliston, MA) at a rate of

~91 mL/h to allow 2 h per day for cows to be milked in the milking parlor (1 h per milking). During the 7-d infusion period, infusate was changed after the morning milking at approximately 0700 h. Any remaining infusate was weighed to so that total ChoCl infused could be calculated.

### *Sample Collection and Analysis*

Milk yield was electronically recorded for each cow at every milking. The amounts of feed offered and refused for individual animals was recorded daily and BW was recorded at the end of each experimental period. Two samples of milk were collected at each milking during the last 3 days of each 14-d period. One sample was analyzed for concentration of fat, protein, lactose, and somatic cells by Lancaster DHIA (Manheim, PA) and the second sample was stored at -20°C until analysis of choline metabolites. Blood was sampled after the morning milking at 0700 h during the last 3 d of each experimental period. Blood samples were collected by tail venipuncture in 10mL vacutainer tubes containing potassium EDTA. Plasma was separated by centrifugation (2000 x g for 15 min at 4°C) and stored at -20°C within 30 minutes of collection.

Choline metabolite concentrations [Betaine (BET), free choline (Cho), phosphocholine (PCho), total PC, total lysophosphatidylcholine (LPC), and sphingomyelin (SM)] in blood plasma and milk were determined by HILC-MS/MS as described by de Veth et al. (2016). Choline metabolite analysis included measurement of 16 individual FA metabolites of PC and 4 individual FA metabolites of LPC (de Veth et al., 2016). Milk secretion of choline-containing compounds was calculated based on molecular weight.

Secretion of total choline was calculated as the sum of the secretion of Cho, PCho, SM, total PC, and total LPC.

### *Statistical Analysis*

The infusion line for one particularly large cow (> 800 kg BW) with a high feed intake would not remain in the omasal-abomasal orifice. This was in spite of repeated attempts (twice daily) to check and replace if needed the abomasal catheter during her infusion periods. Therefore, data for this cow for both infusion treatments were not included in the analysis. Due to a prolonged recovery from the rumen fistulation surgery, data from period 1 for one cow (CON treatment) was not included in the analysis. Therefore, results presented are based on analysis of 4, 5, 5, 4, and 4 cows for CON, RPC15, RPC30, ABO15, and ABO30, respectively. Due to an infusion pump malfunction on the first sample collection day of period 1, the cow receiving the ABO15 treatment did not receive the entire dose of ChoCl. Therefore, data from this day for that cow were discarded prior to analysis.

All data were summarized by period before statistical analysis. Statistical analyses were performed using SAS (version 9.3, SAS Institute, Inc., Cary, NC). Data were analyzed using a mixed model that included the random effects of cow and period and fixed effect of treatment. When treatment was significant, multiple mean comparisons were performed using Fisher's LSD. Linear contrasts were performed to compare the effects of the infusion treatments with the control and RPC treatments (ABO vs. CON and ABO vs. RPC, respectively) as well as to compare the effects of the RPC treatments with the control (RPC vs. CON). Statistical significance was declared at  $P < 0.05$ .

Principal component analysis was performed using the PrinComp Procedure of SAS to determine which, if any, milk or blood choline metabolites were most related to the amount of ChoCl infused into the abomasum. Thus, data from the CON, ABO15, and ABO30 (corresponding to 0, 15, and 30 g/d ChoCl infused) were used in this analysis. All major metabolite groups (total choline, BET, Cho, PCho, SM, total PC, and total LPC) were kept in the analysis and individual PC or LPC species were added to the analysis one at a time to determine if any clustered with abomasal infusion of ChoCl.

## *Study 2*

### *Design and Treatments*

Twenty Holstein cows (12 primiparous, 8 multiparous;  $163 \pm 38$  DIM) were used in a 31-d experiment with a completely randomized design. Cows were housed and fed in individual tie-stalls as described in Experiment 1. Ten days prior to the start of the 21-d treatment period, cows were moved into tie-stalls to allow for acclimation and collection of covariate data. Throughout the acclimation and treatment periods, all cows were fed the same basal TMR (Table 3.1) once daily at 0730 h and milked twice daily at 0600 and 1600 h in a milking parlor. The basal TMR was formulated to meet the requirements of 635 kg Holstein dairy cow producing 36 kg of milk per day at 3.8% fat.

Experimental treatments were based on results from RPC15 and RPC30 in Experiment 1. Within parity, cows were randomly assigned to one of 4 dietary treatments: the basal TMR only (CON); the basal TMR plus 120 g/d RPC (ReaShure<sup>®</sup> Balchem Corp., New Hampton, NY) to supply 34.5 g ChoCl (RPC35); the basal TMR plus 240 g/d RPC to

supply 69.1 g/d ChoCl (RPC69); or the basal TMR plus 360 g/d RPC to supply 103.7 g/d ChoCl (RPC104). During the 21-d experimental period, treatments were applied daily as a top-dress following feed delivery and mixed with the top ~1/3 of the feed offered as in Experiment 1.

### *Sample Collection and Analysis*

Feed intake and refusals and milk recording was as previously described for Experiment 1. Body weight was measured electronically twice daily upon exit from the milking parlor. During the last 3 days of the 10-d acclimation period and the last 3 days of the 21-d treatment period, blood from each cow was collected by tail venipuncture as previously in Experiment 1. Plasma was retained and stored at -20°C until analysis of Cho and BET as described for Experiment 1.

Two milk samples from each cow were collected from the morning and evening milkings during the last 3 days of both the acclimation and experimental periods. One sample was analyzed for concentration of fat, protein, lactose, and somatic cells by Lancaster DHIA (Manheim, PA) and the second sample was aliquoted into a 15mL conical tube and two 1.5mL microcentrifuge tubes which were subsequently stored at -20°C until analysis of Cho and BET as described for Experiment 1. Additional milk samples were also collected from both morning and evening milkings on d 7 and d 14 of the treatment period for analysis of milk components (Lancaster DHIA, Manheim, PA).

## *Statistical Analysis*

Production data collected daily during the treatment period (feed intake and milk) were averaged by week before statistical analysis. Data obtained during the last 3 days of the acclimation period were averaged by individual cow and used as a covariate for the analysis of production and milk and blood BET and Cho data. Production data (feed intake, milk, milk components) were analyzed using a mixed model (SAS; version 9.3, SAS Institute, Inc., Cary, NC) with repeated measures, which included the fixed effects of treatment (CON, RPC35, RPC69, or RPC104) and wk of the experimental period (1, 2, or 3) as well as the random effect of the covariate measurements taken during the acclimation period. Week was considered as the repeated factor, with cow nested within treatment serving as the subject. The compound symmetry matrix was used for all variables, as it consistently resulted in the lowest Akaike Information Criterion. Milk and blood BET and Cho data were analyzed using a mixed model that included the fixed effect of treatment and random effect of the covariate measurements taken during the acclimation period. Statistical significance was declared at  $P < 0.05$ .

## **RESULTS**

### ***Study 1***

*Production.* Dry matter intake, milk production and milk composition for Study 1 are shown in Table 3.2. Providing supplemental choline, either as RPC or as an abomasal infusion, did not affect production or performance. However, RPC30 and ABO15 cows tended to have reduced DMI relative to other treatments ( $P = 0.06$ ).

*Milk choline metabolites.* Milk choline metabolite concentrations and yields are shown in Tables 3.3 and 3.4, respectively. With the exception of BET, Cho, PCho, and total choline, choline metabolites in milk were not affected by treatment. Concentrations and yields of BET, Cho, PCho, and total choline were greatest for ABO15 and ABO30, and were similar among CON, RPC15, and RPC30.

Relative to CON, post-ruminal infusion of ChoCl increased milk concentrations of total choline, BET, Cho, and PCho by 119, 63, 56, and 68  $\mu\text{M}$ , respectively (ABO vs. CON:  $P = 0.02$ ,  $P < 0.01$ ,  $P = 0.03$ , and  $P < 0.01$ ). Furthermore, abomasal infusion of ChoCl increased milk total choline, BET, Cho, and PCho yields by 4314, 1872, 2049, and 2387  $\mu\text{mol/d}$ , respectively (ABO vs. CON:  $P = 0.02$ ,  $P < 0.01$ ,  $P = 0.03$ , and  $P < 0.01$ ). In contrast, feeding ChoCl as RPC did not alter milk concentrations or yields of these metabolites relative to CON (RPC vs. CON:  $P \geq 0.57$ ). However, there was a tendency for RPC to increase both SM concentration and yield relative to CON (RPC vs. CON:  $P = 0.05$  and  $P = 0.07$ , respectively). Relative to RPC treatments, ABO treatments also had higher milk total choline, BET, Cho, and PCho concentrations by 112, 67, 68, and 66  $\mu\text{M}$ , respectively (ABO vs. RPC: all  $P < 0.01$ ). In accordance, infusion of ChoCl also increased milk total choline, BET, Cho, and PCho yields by 4129, 2128, 2394, and 2444  $\mu\text{mol/d}$ , respectively, relative RPC (ABO vs. RPC: all  $P < 0.01$ ).

*Blood choline metabolites.* Blood choline metabolite concentrations are shown in Table 3.5. Blood BET and Cho concentrations were greatest for ABO15 and ABO30 but similar among CON, RPC15 and RPC30. Concentrations of other choline metabolites were not affected by post-ruminal ChoCl. Abomasal infusion of ChoCl increased BET

concentration by 65  $\mu\text{M}$  relative to CON (ABO vs. CON:  $P < 0.01$ ) and 68  $\mu\text{M}$  relative to RPC (ABO vs. RPC:  $P < 0.01$ ). Similarly, abomasal infusion of ChoCl increased Cho concentration by 2.71  $\mu\text{M}$  relative to CON (ABO vs. CON:  $P = 0.01$ ) and by 2.99  $\mu\text{M}$  relative to RPC (ABO vs. RPC:  $P < 0.01$ ). However, supplying ChoCl as RPC did not affect concentrations of these choline metabolites in blood.

*Principal component analysis.* Loading plots resulting from the principal component analysis of milk and blood choline metabolites in Study 1 are shown in Figures 1.3 and 2.3. For milk choline metabolite yields, Principal Components 1 and 2 collectively accounted for over 96% of variation (Figure 3.1). Milk BET and Cho yields were more closely associated with the amount of ChoCl infused into the abomasum than other metabolites (Figure 3.1). Of the individual PC and LPC species examined (16 total and 4 total, respectively), only the yield of one PC species grouping, PC 18:0/22:6 + 18:1/22:5, showed any association with the amount of ChoCl infused and this association was negative (Figure 3.1). For blood concentration variables, Principal Components 1 and 2 accounted for 99.5% of variation and Components 1 and 3 accounted for 98.9% of variation (Figure 3.2). Blood BET, Cho, and PCho concentrations were more closely associated with the amount of ChoCl infused post-rationally than any other choline metabolite or individual species of PC or LPC (Figure 3.2).

## ***Study 2***

*Production.* Feeding RPC at the levels tested in this study did not have any effect on milk production, milk components, DMI, BW, or feed efficiency (Table 3.6).



*Free choline and betaine.* Milk Cho and BET yields and concentrations were not altered by feeding RPC and were not affected by increasing the amount of RPC fed (Table 3.7). In contrast, feeding RPC tended to reduce blood Cho concentration ( $P = 0.09$ ; Table 3.7), but increasing the amount of RPC supplied did not affect blood Cho or BET concentrations.

## DISCUSSION

Results from Study 1 showed significant changes in milk and blood choline metabolite yields and concentrations in response to abomasal infusion of ChoCl. In contrast, feeding ChoCl as RPC did not affect these parameters. Furthermore, results from Study 2 indicated no response in blood or milk Cho or BET when increasing levels of RPC were fed.

There were no production responses to supplemental ChoCl supplied via abomasal infusion (Study 1) or as RPC (Studies 1 and 2), which is similar to results reported by de Veth et al. (2016) and Deuchler et al. (1998). Neither of these studies, nor those reported by de Veth et al. (2016) and Deuchler et al. (1998), were designed to detect production effects. Sharma and Erdman (1989) showed positive responses for milk, fat percentage, and fat-corrected milk yield when 30 or 50 g/d ChoCl was infused into the abomasum. In contrast, Grummer et al. (1987) failed to observe changes in milk production or feed intake when 22 g/d of choline was infused into the abomasum. Production responses to post-ruminal ChoCl supplied as RPC are also variable, with some reporting improvements in milk yield and milk composition (Elek et al., 2008; Leiva et al., 2015) and others reporting

no effects on production (Hartwell et al., 2000; Guretzky et al., 2006). However, most of these studies examined responses to RPC fed during the periparturient period.

Few experiments have reported individual milk or blood choline metabolites in dairy cows provided with post-ruminal ChoCl. Deuchler et al. (1998) showed that milk total choline concentration and yield increased linearly when ChoCl (0, 25, 50, or 75 g/d) was delivered by post-ruminal infusion. In a follow-up study, they also showed that post-ruminal ChoCl fed as RPC (50 g/d) increased milk total choline concentration and tended to increase milk total choline yield (Deuchler et al., 1998). These results implicated total choline in milk as an indicator of post-ruminal choline supply. It is possible that there are more specific indicators of post-ruminal choline supply than total choline, such as individual choline metabolites. Recently, de Veth et al. (2016) supplied supplemental ChoCl (12.5 or 25 g/d) via abomasal infusion or as RPC and examined individual blood and milk choline metabolite responses. However, their experimental design only allowed 4 days for cows to adapt to post-ruminal ChoCl supplied as RPC, which may have made it difficult to detect changes in choline metabolites in blood and milk for those treatment groups. In study 1, amounts of post-ruminal ChoCl were similar to de Veth et al. (2016), but the diet adaptation period was increased to 11 days and samples were collected over the last 3 days of each 14-d experimental period.

Choline metabolite concentrations and yields in milk, with the exception of total PC and individual PC species, in Study 1 were similar to those reported by Artegoitia et al. (2014) for mid- to late-lactation cows, but slightly lower than those reported by de Veth et al. (2016). Milk PC concentrations and yields in this study were much lower than those

previously reported (Artegoitia et al., 2014; de Veth et al., 2016). Furthermore, blood BET, Cho, and SM concentrations for Study 1 were similar to those reported by de Veth et al. (2016) and Artegoitia et al. (2014) for mid- to late-lactation cows; however, the baseline total PC and total LPC concentrations for this study were much lower. Artegoitia et al. (2014) showed that choline metabolite yield and concentrations in milk and blood, respectively, vary with stage of lactation. However, the discrepancies between PC and LPC data in this study and the study by de Veth et al. (2016) are not likely due to differences in lactation stage as a comparison of these data with those reported by Artegoitia et al. (2014) for mid- to late-lactation cows were also dissimilar. Blood concentrations of several individual PC and LPC species from Study 1 were consistently lower than those reported for mid- to late-lactation cows (Artegoitia et al., 2014). Thus, the reason for these discrepancies remain unclear, but could be related to variation in blood and milk concentrations among individual cows. This idea is supported by observations for total LPC in blood and milk, as cow contributed to ~70% of the error variance. However, cow only accounted for 6 and 27% of the error variance for concentration and yield of total PC in blood and milk, respectively.

Similar to results reported by de Veth et al. (2016), in Study 1 abomasal infusion of ChoCl increased milk total choline, Cho, and BET yields but feeding ChoCl as RPC did not. Furthermore, abomasal infusion of ChoCl increased blood Cho and BET and tended to increase PCho concentrations, which is also similar to the observations of de Veth et al. (2016). However, contrary to results reported by de Veth et al. (2016), who also reported a significant increase in milk PCho yields in response to abomasal infusion of ChoCl and blood PC concentration with both forms of post-ruminal ChoCl, there were no changes in

milk yield or blood concentration of any other major choline metabolite in Study 1. Results reported by de Veth et al. (2016) also showed a significant, albeit minor, increase in milk glycerolphosphocholine yield in response to ChoCl supplied as both abomasal infusion and RPC. However, milk and blood samples were not analyzed for this metabolite in Study 1.

Previous work showed an increase in milk total choline yield in response to post-ruminal ChoCl infusion, with the greatest response (a 41% increase) observed when 25 g/d ChoCl was infused (Deuchler et al., 1998). Observations from Study 1 are in accordance with these results as milk total choline was increased by ~38% when 15 and 30 g/d ChoCl was infused post- ruminally in Study 1. However, Deuchler et al. (1998) only observed a 21% increase in milk total choline yield when 50 g/d ChoCl was supplied as RPC and this result was not statistically significant. Similarly, feeding 15 or 30 g/d ChoCl as RPC during Study 1 in the current study did not affect milk total choline yield.

In an effort to determine if any choline metabolite or specific PC or LPC species is associated with post-ruminal ChoCl supply, a principle component analysis was conducted using data from the abomasal infusion treatments. For both milk metabolite yield and blood metabolite concentrations, both BET and Cho were clustered with the amount of ChoCl infused into the abomasum, suggesting potential utility as markers for post-ruminal ChoCl supply. Of all of the individual PC and LPC species examined (16 and 4 species, respectively), only one PC species in milk (PC 18:0/22:6, 18:1/22:5) was negatively associated with post-ruminal ChoCl. This suggested that this milk metabolite decreases as post-ruminal choline supply increases making it a questionable indicator post-ruminal choline supply.

Based on results for Study 1, and those reported by de Veth et al. (2016), it appears as though blood and milk Cho and BET are the most responsive to post-ruminal ChoCl supply. From Study 1, estimates of the bioavailability of RPC were not possible due to a lack of a response in blood concentration and milk yield of either of these two metabolites. It was hypothesized that insufficient RPC was fed in Study 1 in order to detect a response in BET and Cho in blood and milk. Therefore, Study 2 was conducted in order to determine if feeding higher levels of RPC could elicit such a response. However, differences in milk and blood BET and Cho in response to feeding higher levels of RPC (35, 70, or 105 g/d ChoCl as RPC) were not detected.

Reasons for a lack of response in blood and milk BET and Cho when ChoCl was fed as RPC in both Studies 1 and 2 are unclear. Previous studies using feed-restricted dry cows have shown physiological responses, namely in the form of a reduction in liver triglyceride content (Cooke et al., 2007; Zenobi et al., 2018b), in response to feeding ChoCl as RPC. Responses in lactating cows, although variable, have also been reported (Zom et al., 2011; Zenobi et al., 2018a). It is possible that ChoCl fed as RPC is utilized and metabolized differently than that which is supplied directly to the small intestine via abomasal infusion, making BET and Cho unsuitable markers of post-ruminal ChoCl supply when ChoCl is fed as RPC. Protection of ChoCl supplied as RPC from rumen microbial degradation has been documented (Deuchler et al., 1998; Brusemeister and Sudekum, 2006) and Deuchler et al. (1998) estimated the bioavailability of a former RPC product to be ~36%. However, it is possible that the RPC source fed in Studies 1 and 2, as well as the study reported by de Veth et al. (2016), is so well-protected from rumen degradation that the cow is able to break down and absorb very little ChoCl once it reaches the small

intestine. In several species, dietary choline is primarily absorbed in the jejunum and ileum via a carrier protein at low concentrations and by diffusion at high concentrations (Zeisel, 1981; Sheard and Zeisel, 1986). Thus, any delay in the liberation of ChoCl once RPC reaches the small intestine could thwart the efficiency of choline absorption. Perhaps the break-down of the protective coating of RPC is insufficient by the time it reaches the jejunum and ileum, and ChoCl is not made available until after it passes the primary avenue by which choline is absorbed. Further investigation into this idea is warranted.

### **CONCLUSION**

These findings agree with previous work that investigated indicators of post-ruminal choline supply. It is concluded that free choline and betaine in blood and milk have the greatest potential to be used as markers of post-ruminal choline supply in lactating dairy cows. However, because these metabolites were not responsive to choline chloride supplied in a rumen-protected form, they may not be the best indicators of post-ruminal choline supply when choline chloride is fed as RPC. Further studies should investigate possible over-protection of RPC in an effort to explain this lack of response before the search for other potential biomarkers of post-ruminal choline supply commences.

### **ACKNOWLEDGEMENTS**

The authors would like to acknowledge the staff the Central Maryland Research and Education Center Dairy Unit for their assistance with animal care and management. The authors are also grateful to Claudia Gomez (University of Maryland, Department of

Animal and Avian Sciences) for providing assistance with sample and data collection.

Partial funding for this study was provided by Balchem Corporation (New Hampton, NY).

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**Table 3.1.** Ingredient and nutrient composition of the basal TMR for Studies 1 and 2

Ingredient	Study 1	Study 2
	% of DM	
Corn Silage	47.2	39.0
Alfalfa Silage	--	13.7
Alfalfa Hay	14.4	5.9
Corn, ground	14.4	13.7
Soybean Meal	14.4	15.6
Mineral Mix <sup>1,2</sup>	9.8	10.8
Megalac <sup>®</sup>	--	1.3
Nutrient Composition <sup>3</sup>		
DM	48.9	47.0
NDF	30.8	29.0
CP	16.6	16.1
NFC	44.9	37.9
NE <sub>L</sub> , Mcal/kg	1.66	1.99

<sup>1</sup>Study 1: Mineral mix included 30.7% SoyPlus, 17.4% soybean hulls, 6.1% corn gluten meal, 7.6% wheat middlings, 7.5% limestone, 3.7% Bio-Phos, 1.4% magnesium sulfate, 1.1% magnesium oxide, 4.8% sodium bicarbonate, 1.4% Dyna-mate, 4.8% salt, 2.3% Diamond V. Yeast, 0.27% TM-433, 0.13% 4-PlexC, 0.3% vitamin ADE, 0.19% vitamin E (125000 U), 0.27% Sel-Plex 600, 0.27% selenium, 6.3% Megalac, 2.3% Omigen-AF, 0.25% Mepron, 0.53% Rumensin, 0.4% Bio-Mos.

<sup>2</sup>Study 2: Mineral mix included 30.5% SoyPlus, 16.3% soybean hulls, 6.8% corn gluten meal, 4.1% MetAatein, 5.3% Megalac, 7.24% limestone, 6.4% sodium bicarbonate, 3.6% salt, 4.7% Bio-Phos, 1.6% potassium carbonate, 1.6% molasses, 2.1% Diamond V. Yeast, 2.1% Omigen-AF, 1.3% Dyna-mate, 1.3% magnesium sulfate, 1.1% urea (45%), 1.1% magnesium oxide, 0.5% Rumensin, 0.4% Bio-Mos, 0.3% Clarify Larvicide, 0.3% TM-433, 0.3% vitamin ADE, 0.3% selenium, 0.3% Sel-plex 600, 0.2% Mepron, 0.2% vitamin E (125000 U), 0.2% 4-PlexC.

<sup>3</sup>Nutrient profile of individual diet ingredients was analyzed by wet chemistry at Cumberland Valley Analytical Services (Waynesboro, PA).

**Table 3.2.** Least-square means for feed intake, production, body weight, and body condition score during Study 1

Item	Treatment <sup>1</sup>					SEM	Trt	P-Value <sup>2</sup>		
	CON	RPC15	RPC30	ABO15	ABO30			ABO vs. CON	ABO vs. RPC	RPC vs. CON
DMI, kg/d	25.2	25.6	23.0	22.5	26.1	1.0	0.08	0.47	0.99	0.47
Milk, kg/d	33.5	33.5	33.0	32.7	33.7	2.8	0.33	0.59	0.85	0.65
Milk Fat										
kg/d	1.19	1.21	1.18	1.19	1.22	0.11	0.92	0.79	0.82	0.91
%	3.58	3.60	3.62	3.65	3.60	0.20	0.97	0.64	0.83	0.73
Milk Protein										
kg/d	1.04	1.04	1.03	0.99	1.04	0.09	0.27	0.45	0.36	0.91
%	3.10	3.10	3.13	3.05	3.10	0.11	0.35	0.46	0.17	0.72
BW, kg	711	715	690	716	718	39	0.07	0.50	0.06	0.37
BCS	3.26	3.10	3.05	3.24	3.14	0.25	0.56	0.63	0.30	0.18

<sup>1</sup>Treatments included no supplemental choline (CON), 15 g/d choline chloride supplied as RPC (RPC15), 30 g/d choline chloride supplied as RPC (RPC30), 15 g/d choline chloride supplied as a continuous abomasal infusion (ABO15), and 30 g/d choline chloride supplied as a continuous abomasal infusion (ABO30).

<sup>2</sup>P-value associated with the fixed effect of treatment (trt) or linear contrasts.

**Table 3.3.** Least-square means for milk choline metabolite concentrations ( $\mu\text{M}$ ) during Study 1

Item	Treatment <sup>1</sup>					SEM	Trt	P-value <sup>2</sup>		
	CON	RPC15	RPC30	ABO15	ABO30			ABO vs. CON	ABO vs. RPC	RPC vs. CON
Total Choline	324 <sup>b</sup>	339 <sup>b</sup>	323 <sup>b</sup>	445 <sup>a</sup>	441 <sup>a</sup>	50	0.04	0.02	<0.01	0.86
Betaine	56.5 <sup>b</sup>	43.8 <sup>b</sup>	60.2 <sup>b</sup>	124 <sup>a</sup>	114 <sup>a</sup>	18	<0.01	<0.01	<0.01	0.80
Free Choline	133 <sup>b</sup>	123 <sup>b</sup>	120 <sup>b</sup>	188 <sup>a</sup>	190 <sup>a</sup>	23	0.03	0.03	<0.01	0.57
Sphingomyelin	49.2	52.3	53.5	51.4	51.7	4.02	0.31	0.22	0.34	0.05
Phosphocholine	73.3 <sup>b</sup>	83.9 <sup>b</sup>	66.6 <sup>b</sup>	160 <sup>a</sup>	122 <sup>a</sup>	46	<0.01	<0.01	<0.01	0.90
Total Phosphatidylcholine	84.0	93.2	89.3	89.0	91.8	9.0	0.89	0.49	0.90	0.41
Total Lysophosphatidylcholine	2.30	2.52	2.55	2.36	2.46	0.22	0.75	0.60	0.44	0.24
Phosphatidylcholine Species										
16:0/16:1	7.84	8.60	8.41	8.92	8.87	0.99	0.92	0.39	0.67	0.56
16:0/16:0	36.0	38.7	36.8	40.4	39.6	4.38	0.89	0.40	0.53	0.70
16:0/18:2	0.012	0.011	0.023	0.014	0.016	<0.001	0.42	0.63	0.68	0.41
16:0/18:1	29.8	33.9	32.2	31.7	33.6	3.28	0.75	0.38	0.88	0.29
16:0/20:4	1.41	1.71	1.38	1.34	1.42	0.33	0.20	0.83	0.20	0.40
16:0/20:3	6.61	7.30	6.79	6.15	6.46	0.75	0.37	0.58	0.10	0.41
18:0/18:2,18:1/18:1	10.4	11.4	11.5	10.0	10.3	1.36	0.46	0.81	0.10	0.25
18:0/18:1	3.92	4.48	4.19	4.13	4.04	0.60	0.57	0.64	0.35	0.21
16:0/22:6,18:1/22:5,18:2/20:4	0.14	0.18	0.14	0.15	0.14	0.04	0.43	0.85	0.41	0.38
18:1/20:4,18:0/20:5,16:0/22:5	0.46	0.56	0.46	0.49	0.50	0.13	0.61	0.65	0.83	0.50
18:0/20:4	0.47	0.55	0.45	0.47	0.45	0.07	0.58	0.89	0.47	0.67
18:0/20:3	0.21	0.22	0.28	0.23	0.21	0.06	0.74	0.90	0.47	0.47
18:1/22:6	0.023	0.029	0.027	0.028	0.014	0.007	0.22	0.79	0.18	0.41
18:0/22:6,18:1/22:5	0.002	0.008	0.002	0.0007	0.005	0.004	0.47	0.75	0.47	0.37
18:0/22:5	0.038	0.049	0.054	0.049	0.044	0.017	0.69	0.50	0.53	0.22

Lysophosphatidylcholine Species

16:0	1.91	2.02	2.06	1.88	2.02	0.18	0.66	0.79	0.39	0.33
18:2	0.004	0.015	0.002	0.0034	0.006	0.009	0.71	0.97	0.60	0.65
18:1	0.17	0.18	0.17	0.17	0.15	0.019	0.72	0.80	0.38	0.66
18:0	0.29	0.30	0.32	0.30	0.28	0.049	0.91	0.97	0.47	0.54

<sup>1</sup>Treatments included no supplemental choline (CON), 15 g/d choline chloride supplied as RPC (RPC15), 30 g/d choline chloride supplied as RPC (RPC30), 15 g/d choline chloride supplied as a continuous abomasal infusion (ABO15), and 30 g/d choline chloride supplied as a continuous abomasal infusion (ABO30).

<sup>2</sup>P-value associated with the fixed effect of treatment (trt) or linear contrasts.

**Table 3.4.** Least-square means for milk choline metabolite yields ( $\mu\text{mol/d}$ ) during Study 1

Item	Treatment <sup>1</sup>						Trt	P-Value <sup>2</sup>		
	CON	RPC15	RPC30	ABO15	ABO30	SEM		ABO vs. CON	ABO vs. RPC	RPC vs. CON
Total Choline	10402 <sup>b</sup>	10924 <sup>b</sup>	10251 <sup>b</sup>	14625 <sup>a</sup>	14807 <sup>a</sup>	2102	0.04	0.02	<0.01	0.89
Betaine	1899 <sup>b</sup>	1395 <sup>b</sup>	1891 <sup>b</sup>	3722 <sup>a</sup>	3819 <sup>a</sup>	481	0.01	<0.01	<0.01	0.65
Free Choline	4197 <sup>b</sup>	3934 <sup>b</sup>	3771 <sup>b</sup>	6120 <sup>a</sup>	6372 <sup>a</sup>	821	0.03	0.03	<0.01	0.64
Sphingomyelin	1566	1687	1689	1595	1704	115	0.24	0.23	0.44	0.07
Phosphocholine	2494 <sup>b</sup>	2750 <sup>b</sup>	2124 <sup>b</sup>	5517 <sup>a</sup>	4244 <sup>a</sup>	1685	<0.01	<0.01	<0.01	0.90
Total Phosphatidylcholine	2708	3015	2831	2824	3083	390	0.76	0.43	0.89	0.45
Total Lysophosphatidylcholine	72.2	82.6	81.7	75.0	83.1	9.8	0.51	0.36	0.57	0.16
Phosphatidylcholine Species										
16:0/16:1	244	280	269	279	306	38.7	0.73	0.25	0.56	0.43
16:0/16:0	699	790	743	805	852	105	0.75	0.25	0.46	0.52
16:0/18:2	0.42	0.34	0.72	0.48	0.58	0.22	0.35	0.55	0.99	0.54
16:0/18:1	967	1091	1022	1011	1129	142	0.61	0.34	0.86	0.36
16:0/20:4	45.1	55.7	43.2	46.2	47.8	12.5	0.25	0.75	0.57	0.42
16:0/20:3	214	236	214	197	216	30.5	0.37	0.70	0.19	0.49
18:0/18:2,18:1/18:1	335	366	363	332	355	54.4	0.78	0.81	0.41	0.36
18:0/18:1	128	144	133	138	138	25.6	0.78	0.46	0.98	0.41
16:0/22:6,18:1/22:5,18:2/20:4	4.57	5.81	4.52	5.30	4.63	1.26	0.36	0.61	0.73	0.40
18:1/20:4,18:0/20:5,16:0/22:5	14.8	18.3	14.3	17.0	16.9	4.82	0.40	0.37	0.70	0.49
18:0/20:4	15.1	17.9	14.2	15.2	15.0	2.66	0.57	1.00	0.60	0.67
18:0/20:3	7.04	7.34	8.78	7.21	6.87	1.88	0.87	1.00	0.50	0.58
18:1/22:6	0.75	0.90	0.83	1.01	0.51	0.23	0.30	0.96	0.50	0.55
18:0/22:6,18:1/22:5	0.07	0.21	0.09	0.02	0.17	0.11	0.44	0.77	0.52	0.42
18:0/22:5	1.31	1.53	1.73	1.70	1.45	0.57	0.80	0.50	0.85	0.37

Lysophosphatidylcholine Species

16:0	60.9	66.3	66.1	59.8	68.1	8.12	0.42	0.54	0.54	0.25
18:2	0.16	0.52	0.06	0.12	0.22	0.30	0.72	0.99	0.65	0.70
18:1	5.29	5.88	5.38	5.65	5.10	0.74	0.81	0.90	0.63	0.60
18:0	9.43	9.90	10.17	8.70	9.08	1.71	0.70	0.64	0.19	0.56

<sup>1</sup>Treatments included no supplemental choline (CON), 15 g/d choline chloride supplied as RPC (RPC15), 30 g/d choline chloride supplied as RPC (RPC30), 15 g/d choline chloride supplied as a continuous abomasal infusion (ABO15), and 30 g/d choline chloride supplied as a continuous abomasal infusion (ABO30).

<sup>2</sup>P-value associated with the fixed effect of treatment (trt) or linear contrasts.

**Table 3.5.** Least-square means for blood choline metabolite concentrations (µM) during Study 1

Item	Treatment <sup>1</sup>					SEM	Trt	P-value <sup>2</sup>		
	CON	RPC15	RPC30	ABO15	ABO30			ABO vs. CON	ABO vs. RPC	RPC vs. CON
Total Choline	2837	2697	2934	2682	2940	191	0.44	0.88	0.97	0.89
Betaine	48.3 <sup>b</sup>	39.2 <sup>b</sup>	51.1 <sup>b</sup>	120 <sup>a</sup>	106 <sup>a</sup>	16.7	<0.01	<0.01	<0.02	0.85
Free Choline	4.71 <sup>b</sup>	4.39 <sup>b</sup>	4.48 <sup>b</sup>	7.60 <sup>a</sup>	7.24 <sup>a</sup>	0.73	0.02	0.01	<0.01	0.75
Sphingomyelin	494	472	507	473	504	21.2	0.14	0.72	0.94	0.74
Phosphocholine	0.14	0.31	0.18	0.32	0.44	0.18	0.37	0.12	0.21	0.45
Total Phosphatidylcholine	2237	2138	2340	2135	2357	180	0.58	0.96	0.96	0.99
Total Lysophosphatidylcholine	85.1	82.5	83.6	80.7	85.9	4.76	0.26	0.44	0.87	0.33
Phosphatidylcholine Species										
16:0/16:1	20.5	17.0	22.1	19.0	20.0	2.3	0.48	0.72	0.99	0.71
16:0/16:0	34.5	30.0	34.8	32.8	36.0	3.2	0.58	0.98	0.48	0.56
16:0/18:2	5.25	4.68	5.22	4.85	5.14	0.50	0.45	0.51	0.86	0.39
16:0/18:1	138	122	166	133	139	15.6	0.19	0.93	0.55	0.70
16:0/20:5,16:1/20:4	16.3	12.5	15.1	14.0	15.7	1.95	0.33	0.44	0.45	0.16
16:0/20:4	88.7	76.6	88.0	77.7	87.9	8.57	0.12	0.30	0.91	0.23
16:0/20:3	251	245	254	233	265	20.6	0.17	0.86	0.97	0.88
18:0/18:2,18:1/18:1	973	980	993	952	1052	79.5	0.53	0.60	0.71	0.78
18:0/18:1	173	157	197	163	174	18.2	0.20	0.78	0.51	0.81
16:0/22:6,18:1/22:5,18:2/20:4	7.56	6.46	7.27	6.28	7.40	0.96	0.11	0.19	0.94	0.16
18:1/20:4,18:0/20:5,16:0/22:5	54.5	44.8	53.9	47.0	52.3	6.56	0.39	0.41	0.95	0.35
18:0/20:4	123	112	137	83	96	33.1	0.34	0.21	0.09	0.92
18:0/20:3	321	275	301	298	327	30.5	0.69	0.81	0.40	0.36
18:1/22:6	3.83	3.45	3.68	3.91	3.69	0.40	0.91	0.95	0.55	0.58
18:0/22:6,18:1/22:5	7.10	6.75	7.84	6.50	6.68	1.17	0.86	0.70	0.49	0.88
18:0/22:5	52.8	44.9	52.8	45.5	49.6	7.27	0.76	0.49	0.82	0.59



Lysophosphatidylcholine Species

16:0	24.7	23.9	24.4	23.4	25.4	1.25	0.30	0.77	0.71	0.53
18:2	37.4	36.3	36.6	35.0	38.1	2.89	0.48	0.58	0.94	0.50
18:1	1.99	2.22	2.71	2.85	2.28	0.64	0.84	0.47	0.87	0.54
18:0	20.3	20.1	19.9	19.9	20.8	0.94	0.70	0.99	0.49	0.58

<sup>1</sup>Treatments included no supplemental choline (CON), 15 g/d choline chloride supplied as RPC (RPC15), 30 g/d choline chloride supplied as RPC (RPC30), 15 g/d choline chloride supplied as a continuous abomasal infusion (ABO15), and 30 g/d choline chloride supplied as a continuous abomasal infusion (ABO30).

<sup>2</sup>P-value associated with the fixed effect of treatment (trt) or linear contrasts.

**Table 3.6.** Least-square means for feed intake and production during Study 2

Item	Treatment <sup>1</sup>				SEM	P-value <sup>2</sup>	
	CON	RPC35	RPC69	RPC104		Trt	Week
DMI, kg/d	23.3	23.6	23.3	23.6	0.33	0.93	0.35
Milk, kg/d	33.1	33.5	32.5	33.4	0.47	0.44	<0.01
Milk Fat							
%	3.78	3.89	3.89	3.86	0.09	0.80	0.75
kg/d	1.25	1.28	1.21	1.32	0.04	0.29	<0.01
Milk Protein							
%	2.92	2.91	3.02	2.96	0.03	0.13	<0.01
kg/d	0.98	0.96	0.97	0.99	0.03	0.95	<0.01
BW, kg	644	648	634	625	8.64	0.28	0.27
4% FCM, kg/d	32.0	32.6	31.2	33.2	0.78	0.30	<0.01
FCM/DMI	1.37	1.38	1.34	1.42	0.03	0.42	<0.01

<sup>1</sup>Treatments included no supplemental choline (CON), 34.5 g/d choline chloride supplied as RPC (RPC35), 69.1 g/d choline chloride supplied as RPC (RPC69), or 103.7 g/d choline chloride supplied as RPC (RPC104).

<sup>2</sup>P-value associated with the fixed effect of treatment (Trt) and week of experimental period.

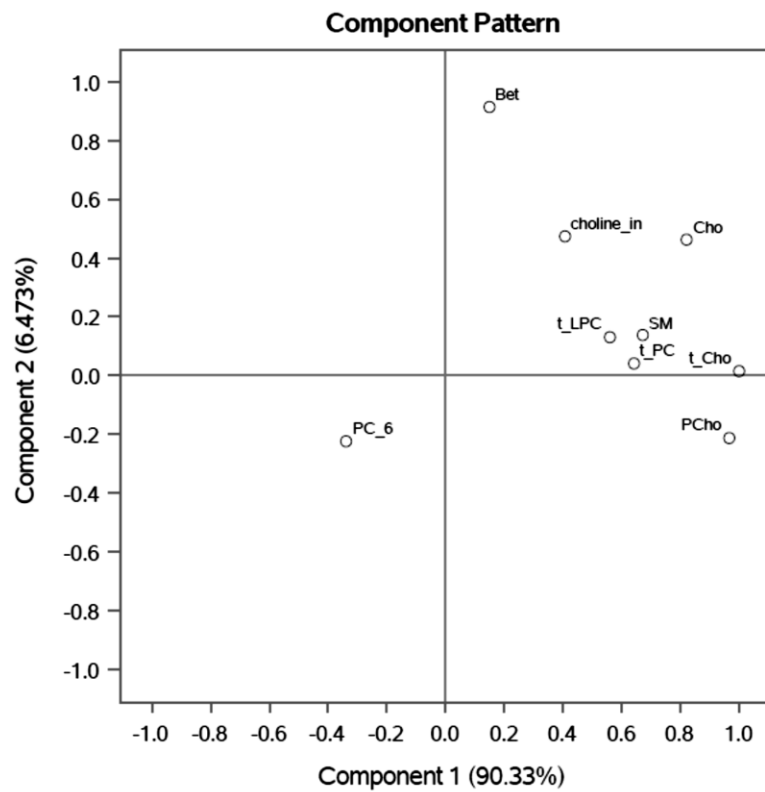
**Table 3.7.** Milk and blood free choline and betaine responses for Study 2

Item	Treatment <sup>1</sup>				SEM	Trt	P-value <sup>2</sup>	
	CON	RPC35	RPC69	RPC104			CON vs. RPC	Linear
Blood								
Free Choline, $\mu\text{M}^2$	7.27	5.56	5.88	5.70	0.74	0.36	0.09	0.23
Betaine, $\mu\text{M}$	20.0	17.4	18.7	20.7	2.15	0.67	0.68	0.68
Milk								
Free Choline, $\mu\text{M}$	208	195	228	205	20.7	0.76	0.95	0.65
Free Choline, $\mu\text{mol/d}$	6192	6166	6615	6339	527	0.96	0.81	0.69
Betaine, $\mu\text{M}$	24.8	24.6	24.7	26.4	2.50	0.94	0.89	0.67
Betaine, $\mu\text{mol/d}$	749	789	737	819	77.0	0.83	0.73	0.71

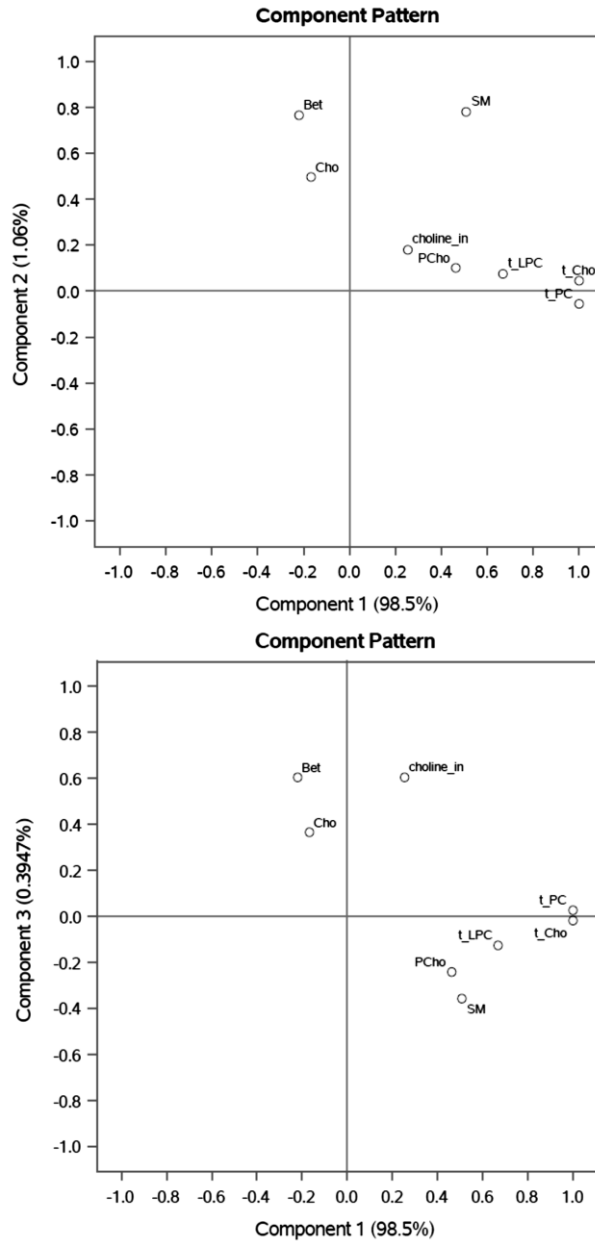
<sup>1</sup>Treatments included no supplemental choline (CON), 34.5 g/d choline chloride supplied as RPC (RPC35), 69.1 g/d choline chloride supplied as RPC (RPC69), or 103.7 g/d choline chloride supplied as RPC (RPC104).

<sup>2</sup>Contrast CON vs. RPC = control vs. average of all three RPC treatments.

**Figure 3.1.** Loading plot of principle components 1 vs 2 derived from principal component analysis describing the relationship among milk choline metabolites and amounts of choline infused into the abomasum. Variable definitions: choline\_in = amount of choline chloride infused (0, 15, or 30 g/d), t\_Cho = total choline ( $\mu\text{mol/d}$ ), Bet = betaine ( $\mu\text{mol/d}$ ), Cho = free choline ( $\mu\text{mol/d}$ ), SM = sphingomyelin ( $\mu\text{mol/d}$ ), PCho = phosphocholine ( $\mu\text{mol/d}$ ), t\_PC = total PC ( $\mu\text{mol/d}$ ), t\_LPC = total LPC ( $\mu\text{mol/d}$ ), PC\_6 = PC 18:0/22:6,18:1/22:5.



**Figure 3.2.** Loading plot of principal components 1 vs 2 and 1 vs 3 derived from principal component analysis describing the relationship among blood choline metabolites and the amounts of choline infused into the abomasum. Variable definitions: choline\_in = amount of choline chloride infused (0, 15, or 30 g/d), t\_Cho = total choline ( $\mu\text{M}$ ), Bet = betaine ( $\mu\text{M}$ ), Cho = free choline ( $\mu\text{M}$ ), SM = sphingomyelin ( $\mu\text{M}$ ), PCho = phosphocholine ( $\mu\text{M}$ ), t\_PC = total PC ( $\mu\text{M}$ ), t\_LPC = total LPC ( $\mu\text{M}$ ), PC\_6 = PC 18:0/22:6,18:1/22:5 ( $\mu\text{M}$ ).



## **CHAPTER 4: EXPERIMENT 2**

### **Production Responses to Rumen-protected Choline and Methionine Supplemented during the Periparturient Period Differ for Primi- and Multiparous Cows<sup>1</sup>**

<sup>1</sup>S.B. Potts, C.M. Scholte, K.M. Moyes, and R.A. Erdman. Production responses to rumen-protected choline and methionine supplemented during the periparturient period differ for primi- and multiparous cows. In preparation for submission to the Journal of Dairy Science.

## ABSTRACT

The objective of this experiment was to examine production performance responses to feeding rumen-protected choline (RPC), methionine (RPM), or both during the periparturient period. Fifty-four Holstein cows (25 primiparous, 29 multiparous) were used in a randomized block design experiment with a 2 x 2 factorial treatment structure. Cows were blocked by expected calving date and parity and assigned to one of 4 treatments: CON (no RPC or RPM); CHO (60 g/d RPC); MET (12 g/d RPM prepartum; 18 g/d RPM postpartum); or CHO + MET. Treatments were applied once daily as a top dress from 3 wk before through 5 wk after calving. Dry matter intake (DMI) and milk production were recorded daily and milk samples were obtained once weekly. Data were analyzed for primi- and multiparous cows separately using a repeated measures mixed model that included random effects of cow and block and fixed effects of CHO, MET, week, and their interactions; week served the repeated effect. Treatment did not affect DMI or calculated energy balance during the pre- or postpartum periods. However, MET increased overall milk fat and protein concentrations by 9 and 7%, respectively, and increased both fat yield and 4% fat-corrected milk (FCM) yield by 0.36 and 5.8 kg/d during wk 3 postpartum for multiparous cows. In contrast, feeding MET had no effect on the production of primiparous cows. For primiparous cows, CHO increased milk yield by 3.5 kg/d and tended to increase FCM and protein yield by 2.6 and 0.10 kg/d, but it did not affect the production of multiparous cows. Although calculated energy balance was not affected, RPC reduced the prepartum body condition score (BCS) of primiparous cows. This, in conjunction with elevated plasma non-esterified fatty acids (NEFA) and  $\beta$ -hydroxybutyrate (BHBA), suggests some effect of CHO on prepartum body tissue mobilization for

primiparous cows fed CHO. During the postpartum period, BCS and plasma NEFA concentrations were not affected by treatment for primiparous cows, although plasma BHBA tended to be increased by CHO. In contrast to the results for primiparous cows, treatment did not affect prepartum BCS or plasma NEFA or BHBA levels for multiparous cows, but postpartum BCS was reduced by CHO. These results suggest that primi- and multiparous cows respond differently to CHO and MET supplemented during the periparturient period. This variation in response could be mediated by differences in choline and methionine requirements of primiparous vs. multiparous cows.



## INTRODUCTION

Choline is an essential nutrient for most animals, including rats, preruminant calves (NRC, 2001), and humans (Zeisel and da Costa, 2009). Choline is required for phosphatidylcholine (PC) synthesis, the major phospholipid comprising cell membranes and lipoproteins, and plays a role in methyl group metabolism as a methyl donor (Zeisel and da Costa, 2009). Because choline is a precursor for PC, which is required for very low density lipoprotein (VLDL) synthesis (Li and Vance, 2008), choline deficiency impedes triglyceride (TG) export from the liver and can result in fatty liver disease (Zeisel, 1981). Choline requirements for lactating dairy cows have not been specified (NRC, 2001); however, increased prevalence of fatty liver disease during early lactation (Jorritsma et al., 2001) suggest that choline supply may be insufficient during this time. In humans, relative to nonpregnant, nonlactating women, recommended dietary choline intake is 6% and 29% greater for pregnant and lactating women, respectively (Zeisel and da Costa, 2009). Thus, it seems logical that the choline requirement of the dairy cow might increase during the periparturient period.

Feeding rumen-protected choline (RPC) to feed-restricted dry cows consistently reduces liver TG content (Cooke et al., 2007; Zenobi et al., 2018b). In contrast, supplementing periparturient cows with RPC has decreased (Zom et al., 2011) or has had no effect (Zhou et al., 2016a; Zenobi et al., 2018a) on liver TG content. Furthermore, production responses of periparturient cows fed RPC are also variable (Hartwell et al., 2000; Piepenbrink and Overton, 2003; Elek et al., 2008; Zom et al., 2011; Zhou et al., 2016a). This variation is likely due to many factors, such as different length of RPC

supplementation prepartum, the amount of RPC fed, the choline status of the cows before supplementation, and the availability of other methyl donors.

Methionine (Met) and lysine are the two most limiting amino acids in dairy cattle diets (NRC, 2001). Methionine is not only required for protein synthesis, but also serves as the precursor for *S*-adenosylmethionine (SAM), the most predominant methyl donor in the body that is required for many metabolic reactions, including the *de novo* synthesis of PC from phosphatidylethanolamine in the phosphatidylethanolamine methyl-transferase (PEMT) pathway (Li and Vance, 2008). Therefore, methionine may indirectly improve liver TG export via VLDL through its participation in PC synthesis. Furthermore, Met can also be used as a precursor of glutathione, an important antioxidant (Yuan and Kaplowitz, 2009; Meister and Anderson, 1983), which might help the dairy cow cope with the increased metabolic stress that is characteristic of the periparturient period (Sordillo and Raphael, 2013).

Supplementing rumen-protected Met (RPM) during the periparturient period generally increases milk protein production (Osorio et al., 2013; Ordway et al., 2009; Zhou et al., 2016a) and improves immune status (Zhou et al., 2016b). However, feeding RPM during this time has not been shown to have an effect on liver TG content (Osorio et al., 2013; Zhou et al., 2016a; Batistel et al., 2017). It is possible that the availability of Met as well as the other major limiting amino acid, Lysine (NRC, 2001), plays a role in determining the type of response observed when RPM is supplemented to periparturient cows. It is also possible that additional Met supplied in the diet is diverted to protein

synthesis, instead of methyl group metabolism, which could explain the lack of a liver TG response.

Because both choline and Met have the apparent potential to improve hepatic TG export from the liver, it could be expected that supplementing either or both would promote production performance during the periparturient period. Zhou et al. (2016a) fed both RPC and RPM to periparturient cows and observed increased milk yield and DMI for cows supplemented with RPM but not RPC, and there was no effect of either supplement on liver TG content. Furthermore, no synergistic effect of both RPC and RPM fed simultaneously was observed in that study.

Additional research is warranted to further the understanding of potential roles that choline and Met may play during the transition period and how they may affect cow performance during this time. Therefore, the objective of this experiment was to compare the effects of feeding RPC, RPM, or both during the periparturient period on production and liver TG accumulation.

## **MATERIALS AND METHODS**

All experimental procedures that utilized animals were approved by the University of Maryland, College Park Institutional Animal Care and Use Committee.

### ***Animals and Study Design***

Between March and December 2017, 25 primiparous and 29 multiparous Holstein cows from the Central Maryland Research and Education Center were selected for use in

a randomized block design experiment with a 2 x 2 factorial treatment structure. The two factors were 0 or 60 g/d RPC (28.8% choline chloride; ReaShure<sup>®</sup>; Balchem Corp., New Hampton, NY) and 0 or 12 g/d RPM (75% DL-Methionine; Smartamine<sup>®</sup> M; Adisseo USA, Inc., Alpharetta, GA) prepartum and 18 g/d RPM postpartum. Thus, the four resulting treatments included: 1) no supplemental choline or methionine (control; CON); 2) 60 g/d RPC (CHO); 3) 12 g/d RPM prepartum and 18 g/d RPM postpartum (MET); and 4) a combination of CHO and MET treatments (CHO + MET; 60 g/d RPC + 12 g/d RPM prepartum and 18 g/d RPM postpartum).

Prior to the start of the experiment, animals were blocked by age (primiparous vs. multiparous) and expected calving date and randomly assigned to treatment. Treatments were applied daily as a top dress from 21 d before expected calving through 35 d postpartum. Throughout the experiment, cows were housed in a free stall-barn equipped with electronic feeding gates (American Calan, Inc., Northwood, NH) to allow for individual cow feed intakes to be recorded. Cows were trained to use the electronic feeding gates approximately 31 d before expected calving. Once trained to a specific gate, cows retained their assigned feeding gate for the duration of the experiment. Free-stalls were equipped with rubber mattresses and bedded with sawdust. Due to facility limitations, pre- and postpartum animals were housed in the same group throughout the study except during calving. When calving appeared imminent, animals were moved to individual box-stalls until after parturition. Stocking density of the group pen never exceeded 80% throughout the study.

Prepartum and postpartum cows were fed their respective total mixed rations (TMR) throughout the study. All cows received the same basal prepartum diet (Table 4.1) starting 21 d before expected calving. After calving, cows received the same basal lactation diet (Table 4.1) until 35 days postpartum. Cows were fed once daily at 0800 h for ad-libitum intake (~5-10% orts). Feed refusals were removed each morning before the delivery of fresh feed. After calving, cows were milked twice daily in a milking parlor at 0600 and 1600 h. All animals were visually observed twice daily for signs of illness. Upon signs of illness, cows were treated as directed by the herd veterinarian.

### ***Data Collection and Sample Analysis***

*Feed intake and diet composition.* Individual feed intake was recorded daily as feed offered minus feed refused. Samples of individual feed ingredients were collected once weekly throughout the study and composited before analysis of nutrient composition by wet chemistry methods (Cumberland Valley Analytical Services; Waynesboro, PA). Additional samples of silage were analyzed monthly by NIR (Cumberland Valley Analytical Services) so that diet adjustments could be made to maintain consistency throughout the study. Dry matter (DM) of haylage and corn silage was determined twice weekly in order to maintain a constant mixture of feed ingredients on a DM basis.

*Body weight, body condition, rumination and calving data.* Postpartum body weight (BW) was recorded electronically twice daily upon exit from the milking parlor; prepartum BW was recorded three times per week using the same scale system. Body condition scores (BCS; 5-point scale; 1 = thin, 5 = fat; Wildman et al., 1982) were determined independently by two trained investigators each week. Daily rumination time

(min/d) was collected electronically using the SCR system (Allflex<sup>®</sup> USA, Inc., Airport, TX). Calving difficulty scores were assigned on a 1-5 scale (1 = no assistance, 5 = extreme difficulty; Djemali et al., 1987), and calf sex and BW were recorded at calving.

*Milk.* After calving, milk yield was recorded electronically at each milking. At approximately d 7, 14, 21, 28, and 35 postpartum, samples of milk were collected for analysis of fat, true protein, and somatic cell count by Lancaster DHIA (Lancaster, PA).

*Blood.* Blood was collected via coccygeal venipuncture in the morning before feeding on approximately -21, -14, -7, 0, 4, 7, 11, 14, 21, 28, and 35 d relative to calving. Because some cows calved early or late, prepartum samples were actually collected ( $\pm 2$  d) on -19, -12, and -6 d relative to calving. Samples were collected into two 10mL evacuated tubes containing potassium EDTA, one 6mL evacuated tube containing sodium fluoride as a glycolytic inhibitor, and one 10mL evacuated tube containing sodium heparin. Tubes were immediately placed on ice and then centrifuged at 2000 x g for 15 min at 4°C. Plasma aliquots were stored at -20°C until analysis. Concentrations of non-esterified fatty acids (NEFA) were determined using a colorimetric assay (HR Series NEFA HR(2), FUJIFILM Wako Diagnostics U.S.A, Mountain View, CA). Glucose and  $\beta$ -hydroxybutyrate (BHBA) concentrations were also determined using commercial colorimetric assays (Autokit Glucose, FUJIFILM Wako Diagnostics U.S.A, Mountain View, CA;  $\beta$ -hydroxybutyrate LiquiColor<sup>®</sup>, #2440, Stanbio Laboratory, Boeme, TX).

*Liver.* Approximately 120 mg of liver tissue was collected from each animal via percutaneous liver biopsies on approximately d -21 (actual prepartum samples were obtained on  $-19 \pm 2$  d) and d 7 relative to calving. The biopsy incision site was identified as

the location where an imaginary line from the tuber coxae to the olecranon intersected the 11<sup>th</sup> intercostal space. Cows were restrained in a chute and local anesthesia (2% lidocaine) was provided before a small incision was made in the skin and the biopsy needle (14G x 15 cm; Tru-Cut<sup>®</sup>, Merit Medical, Jordan, UT) was inserted. Approximately 10 passes of the biopsy needle were required in order to obtain 120 mg of tissue. Tissue was immediately snap frozen in liquid nitrogen and stored at -80°C until TG analysis. Liver lipids and triglycerides were determined similar to procedures outlined by Zenobi et al. (2018a) but with slight modifications to accommodate the analysis of 50 mg of tissue instead of 100 mg. Approximately 50 mg of liver tissue was homogenized in 1 mL of saline, followed by addition of 0.5 mL of saline:methanol (1:1). Total lipids were extracted according to Folch et al. (1957) using 1 mL of homogenate; the remaining 0.5 mL of homogenate was used to determine DM content by drying at 60°C for 24 h. Lipid extracts were analyzed for TG content using a colorimetric procedure (Foster and Dunn, 1973).

### *Statistical Analysis*

Four cows were removed from the study early due to various reasons. One multiparous cow from the CHO + MET treatment was removed during wk 3 postpartum due to a severe coliform mastitis infection. Another multiparous cow that calved with twins on the CHO + MET treatment was removed due to a retained placenta and severely depressed DMI that eventually led to a right displaced abomasum during wk 2 postpartum. Two cows (one multiparous on the CON treatment, one primiparous on the CHO + MET treatment) were removed due to injuries. Data for these cows were utilized in the statistical analyses up until the day before they were removed from the study. Data from other cows

that required health-related interventions for various reasons throughout the study (Table 4.2) were not excluded from the analysis. Instead, potential outliers were detected using Cook's Distance for each variable, using a cutoff of  $n/4$ . If a particular week for a cow was identified as a potential outlier for more than 4 response variables, it was excluded from the statistical analysis for all variables.

Initial statistical analysis of data from both primi- and multiparous cows revealed that primi- and multiparous cows seemed to respond to RPC and RPM differently. Unfortunately, the experimental design precluded proper investigation of parity by treatment interactions because parity was confounded with block. Therefore, all data were analyzed for primi- and multiparous cows separately. Production and plasma NEFA, BHBA, and glucose measurements were reduced to weekly means before statistical analysis. Data were analyzed using a repeated-measures mixed model using SAS (version 9.4; SAS Institute, Cary, NC). The model included the random effect of cow nested within block and fixed effects of week relative to calving (-3, -2, -1, 1, 2, 3, 4, or 5), the main effects of CHO and MET, and all two- and three-way interactions. Week relative to calving served as the repeated factor and the auto-regressive (1) covariance structure was chosen because it resulted in the lowest Akaike Information Criterion values for most variables. Postpartum liver TG data (d 7 postpartum) were analyzed using a mixed model (SAS, version 9.4) that included the fixed main effects of CHO and MET and their interaction. Prepartum liver TG content, determined from samples collected in the morning before first treatment application (~d -21), was also included in the model as a random covariate effect.



All data are presented as least-square means unless otherwise noted. Comparison of least-square means was carried out using Fisher's LSD only if a significant  $P$ -value was observed. Significance was declared at  $P \leq 0.05$  and tendencies were declared at  $P \leq 0.10$ .

## RESULTS

### *Days on Treatment, Calving Details, and Health*

Details regarding the number of days on treatment prepartum, calving, and incidence of health disorders are shown in Table 4.2. On average for all treatments, multiparous and primiparous cows received their respective treatments for ~20 and 18 d before calving, respectively (Table 4.2). Although statistics were not performed on health data, there were numerically more cases of mastitis for both primi- and multiparous cows on the CON treatment compared to all other treatments (Table 4.2).

### *Prepartum Performance*

Prepartum DMI, BCS, energy balance, and daily rumination results for primi- and multiparous cows are shown in Table 4.3. With the exception of BCS for primiparous cows and BW for multiparous cows, prepartum performance was not affected by treatment. Feeding CHO reduced prepartum BCS ( $P = 0.02$ ) for primiparous cows but not multiparous cows. Multiparous cows that received MET had a lower BW prepartum ( $P = 0.02$ ), but prepartum BW for primiparous cows was not affected by treatment. For both primi- and multiparous cows, prepartum DMI and energy balance decreased as calving approached ( $P < 0.01$ ; Figure 4.1). For multiparous cows, daily rumination time also decreased as calving approached ( $P = 0.02$ ; Figure 4.1).

### ***Postpartum Production: Multiparous Cows***

Production responses for multiparous cows are shown in Table 4.4. A tendency for a CHO x MET interaction for milk yield ( $P = 0.10$ ) indicated that cows fed CHO + MET had numerically lower milk yield relative to all other treatments. MET increased milk fat percentage ( $P = 0.03$ ), and this difference tended to be more pronounced during wk 3 postpartum (MET x Week:  $P = 0.06$ ; Figure 4.2 A). By contrast, CHO reduced milk fat percentage during wk 4 postpartum (Figure 4.2 B). Milk fat yield was not affected by CHO, but MET increased milk fat yield during wk 3 postpartum (Figure 4.2 C). Furthermore, cows fed MET had greater 4% fat-corrected milk (FCM) yield during wk 3 postpartum (MET x Week:  $P = 0.02$ ; Figure 4.3 A). Cows that received MET had greater overall milk protein percentage ( $P < 0.01$ ), but there tended to be a positive synergistic effect of feeding both CHO and MET for milk protein concentration (CHO x MET:  $P = 0.07$ ; Table 4.4). Protein yield was not affected by MET, but a CHO x Week interaction ( $P = 0.04$ ; Figure 4.4) indicated that CHO reduced it during the first week after calving. A tendency for a MET x Week interaction ( $P = 0.08$ ; Table 4.4) indicated that cows that were fed MET had numerically lower MUN during wk 1 and wk 3 postpartum. Furthermore, cows fed CHO had numerically greater MUN during wk 1 and wk 3 postpartum (CHO x Week:  $P = 0.06$ ; Table 4.4). Milk somatic cell count was not affected by treatment (Table 4.4).

Postpartum energy balance, DMI, daily rumination, and BW of multiparous cows were not affected by treatment (Table 4.4). However, feeding CHO reduced the average BCS during wk 3 and 4 postpartum (CHO x Week:  $P = 0.05$ ).

### ***Postpartum Production: Primiparous Cows***

Production responses for primiparous cows are shown in Table 4.5. Feeding CHO increased overall milk yield by 3.5 kg/d ( $P = 0.04$ ). A MET x Week interaction ( $P < 0.01$ ) indicated that cows fed MET had numerically greater milk yield during wk 1 postpartum. Furthermore, a significant CHO x MET x Week interaction ( $P < 0.01$ ) indicated that cows that received CHO + MET had greater 4% FCM yield during wk 3 postpartum (Figure 4.3 B).

A significant CHO x MET x Week interaction for milk fat percentage ( $P < 0.01$ ) indicated that cows on the CHO treatment had a lower milk fat percentage than cows on the MET treatment during wk 2, 3, and 5 (Figure 4.5). Furthermore, cows on the CHO treatment had a lower milk fat percentage than cows on the CON and CHO + MET treatments during wk 3 postpartum (Figure 4.5). A significant CHO x MET x Week interaction for milk fat yield ( $P = 0.01$ ) indicated an increase in milk fat yield for the CHO + MET treatment over the MET treatment during wk 3 postpartum, although fat yield for CHO + MET cows was not statistically different from CON or CHO cows. In contrast to milk fat, milk protein percentage was not affected by treatment; however, CHO tended to increase overall milk protein yield ( $P = 0.06$ ; Table 4.5). There was no effect of CHO or MET on MUN or SCC (Table 4.5).

Postpartum energy balance, DMI, daily rumination, and BCS of primiparous cows were not affected by treatment. However, cows fed MET had greater BW during the first week postpartum compared with cows that did not receive MET (MET x Week:  $P = 0.03$ ).

### ***Blood Metabolites***

Prepartum plasma BHBA, glucose, and NEFA concentrations for primi- and multiparous cows are shown in Table 4.6. For multiparous cows, prepartum plasma metabolites were not affected by treatment. In contrast, CHO increased NEFA concentration ( $P = 0.05$ ) and tended to increase plasma BHBA concentration ( $P = 0.08$ ) in primiparous cows during the prepartum period.

During the postpartum period, treatment did not affect plasma BHBA, glucose, or NEFA concentrations in multiparous cows (Table 4.6), although there was a tendency for MET to increase BHBA concentration ( $P = 0.10$ ). Similarly, plasma NEFA concentration were not affected by treatment for primiparous cows during the postpartum period; however, CHO significantly increased plasma BHBA concentrations during this time ( $P = 0.05$ ). Additionally, a tendency for a MET x CHO x Week interaction ( $P = 0.06$ ) for plasma glucose indicated that cows fed CHO or MET alone had numerically lower concentrations than CON cows during wk 1 postpartum.

### ***Liver Triglyceride***

Liver TG concentrations on d 7 postpartum are shown in Figure 4.6. Neither CHO nor MET affected postpartum liver TG content even after the prepartum TG measurement (taken before treatment initiation at day  $-19 \pm 2$  relative to calving) was used as a covariate in the statistical model.

## DISCUSSION

These results indicate that feeding CHO and MET during the periparturient period can have positive, but different effects on the production of both primi- vs multiparous cows. Feeding MET had positive effects on milk fat and protein percentages for multiparous cows, whereas feeding CHO had positive effects on milk yield for primiparous cows. These differences in responses by parity can likely be attributed to a difference in choline and Met requirements for these two groups of cows. This experiment was not designed to investigate treatment by parity interactions. However, the lack of a uniform response among primi- and multiparous cows is not surprising since the two groups of cows likely differ in their choline and methionine requirements during this period. Because the average number of lactations for dairy cows in the U.S. is 3, primiparous cows make up a large portion of the U.S. dairy herd. Therefore, future experiments should be designed in order to further investigate these differences so that strategies can be implemented to better meet the needs of primi- vs multiparous cows.

Results from previous studies indicate variable milk and milk component responses to RPC and RPM during the periparturient period. Similar to results reported by Ordway et al. (2009), MET did not elicit an increase in milk yield for primi- or multiparous cows in the current study. This is in contrast to other studies (Zhou et al., 2016a; Batistel et al., 2017) that showed an increase in milk yield when RPM was fed to periparturient cows. However, MET increased milk fat percentage and yield for multiparous cows, especially during wk 3 postpartum in the current study. Others (Zhou et al., 2016a; Batistel et al., 2017) have also shown that RPM fed during the periparturient period increases milk fat

yield, but this increase was associated with an increase in overall milk yield, since fat percentage remained unchanged in those studies. Because no increase in milk yield was observed, the increase in milk fat and FCM yield during wk 3 postpartum for the multiparous cows supplemented with MET in this study can be mostly attributed to the increase in milk fat percentage that also occurred at this time. In contrast to results for multiparous cows, but similar to those reported by Ordway et al. (2009) for primi- and multiparous periparturient cows, MET did not affect milk fat yield or content for primiparous cows.

Milk yield and FCM yield of multiparous cows were not affected by CHO, which is similar to results reported in other studies (Zom et al., 2011; Leiva et al., 2015; Zhou et al., 2016a). In contrast, CHO increased milk yield by ~3.4 kg/d and tended to increase FCM yield for primiparous cows, which is similar to results by Elek et al. (2008), who observed a 4.4 kg/d increase in milk yield and 2.5 kg/d increase in FCM yield when RPC was fed to multiparous cows before and after calving. Furthermore, feeding RPC during the transition period has also been shown to increase milk yield of multiparous cows in early lactation and through wk 40 postpartum even after supplementation ceased (Zenobi et al., 2018a). In contrast, Davidson et al. (2008) showed that RPC did not alter milk yield for primiparous cows when fed during early lactation. Consistent with previous work (Hartwell et al., 2000; Piepenbrink and Overton, 2003; Elek et al., 2008; Zhou et al., 2016a; Zenobi et al., 2018a), overall milk fat percentage was not affected by RPC. However, a CHO x Week interaction for multiparous cows indicated that CHO reduced milk fat percentage during wk 4 postpartum. Milk yield was not altered by CHO for multiparous cows, so this change in milk fat concentration during wk 4 was not an artifact of dilution.

Furthermore, a significant CHO x MET x Week interaction for primiparous cows indicated that cows on the CHO treatment had lower milk fat percentage during wk 3 postpartum, which is likely due to the numerically greater milk yield for those cows at that time. In the current study, CHO also did not alter milk fat yield, although primiparous cows fed CHO + MET had greater milk fat yield than those fed CHO or MET alone due, in part, to the numerically greater milk yield and fat percentage for those cows. These observations are similar to previously reported results (Zom et al., 2011; Zhou et al., 2016a), where RPC did not affect milk fat yield when fed to periparturient cows. Reported increases in milk fat yield in response to RPC in the studies by Zenobi et al. (2018a) and Elek et al. (2008) were likely due to the concurrent increase in overall milk yield that was observed.

Similar to results reported by Ordway et al. (2009), MET increased milk protein percentage, but not yield, for multiparous cows in the current study. However, MET did not alter milk protein percentage or yield for primiparous cows. In contrast to these findings, others (Osorio et al., 2013; Zhou et al., 2016a; Batistel et al., 2017) reported increased milk protein concentration and yield in response to feeding RPM during the periparturient period. However, the increased protein yield observed in these studies was partially due to the simultaneous increase in milk yield.

Milk protein percentage was not affected by CHO for both primi- and multiparous cows, which is similar to results reported by others (Piepenbrink and Overton, 2003; Zom et al., 2011; Zhou et al., 2016a; Zenobi et al., 2018a). Because CHO increased milk yield for primiparous cows, there was a tendency for CHO to also increase milk protein yield. Previous work by Zenobi et al. (2018a) also showed an increase in milk protein yield in

response to RPC fed during transition. However, similar to results of the current study, this response was also likely due to the increase in milk yield also observed for cows fed RPC since milk protein concentration was not affected by treatment (Zenobi et al., 2018a). In the current study, CHO reduced milk protein yield for multiparous cows during the first wk after calving. As milk protein percentage was not affected by CHO, this change was likely due to the numerically lower milk yield of multiparous cows fed CHO during this time.

There were no changes in DMI in response to MET or CHO. In contrast to this observation, Osorio et al. (2013) showed that RPM increased postpartum DMI when fed to periparturient cows. Similarly, Zhou et al. (2016a) and Batistel et al. (2017) also showed an increase in pre- and postpartum DMI in response to RPM supplemented to during the periparturient period. Ordway et al. (2009) reported a significant treatment x parity interaction, whereby RPM supplementation during the periparturient period reduced DMI for multiparous cows, but not for primiparous cows. However, DMI as a percentage of BW was not affected by feeding RPM, which is in accordance with the observations of the current study. Similar to the findings of the current study, previous studies have also shown that RPC supplementation to periparturient cows does not impact pre- or postpartum DMI (Hartwell et al., 2000; Piepenbrink and Overton, 2003; Zhou et al., 2016a; Zenobi et al., 2018a).

Feeding MET reduced prepartum BW for multiparous cows, but this was due to a lower initial BW for these cows, and this difference was not significant during the postpartum period. Prepartum BW of primiparous cows was not affected by MET;



however, a MET x Week interaction postpartum indicated a higher BW for MET-treated cows during week 1 postpartum. Feeding MET did not affect BCS or energy balance during the pre- or postpartum period; however, a MET x Week interaction indicated that primiparous cows fed MET had a lower energy balance at the beginning of the experiment, but energy balance was similar between MET-treated cows and those that did not receive MET by week 2 prepartum. These results suggest that MET had minimal impact on apparent tissue mobilization. This conclusion is further supported by a lack of plasma NEFA and BHBA responses to MET for both multiparous and primiparous cows. The results of the current study are concordant with other studies (Zhou et al., 2016a; Batistel et al., 2017) that reported no effect of RPM on postpartum BW, BCS, or energy balance. In contrast, Osorio et al. (2013) observed that RPM reduced postpartum BCS and energy balance and increased plasma NEFA during the first 5 weeks postpartum, which suggested an increase in tissue mobilization.

Similar to previous results for multiparous cows (Piepenbrink and Overton, 2003; Leiva et al., 2015; Zenobi et al., 2018ba), CHO did not affect pre- or postpartum BW for cows in the current study. Furthermore, CHO did not affect energy balance before or after calving. However, CHO reduced prepartum BCS for primiparous cows, suggesting potential modification of body tissue mobilization before calving. This idea is further supported by the increase in prepartum plasma NEFA and BHBA that was observed for primiparous cows fed CHO. These prepartum responses were not apparent for multiparous cows fed CHO. During the postpartum period, CHO reduced BCS for multiparous cows but did not affect BCS for primiparous cows. This may suggest that CHO increased tissue mobilization for multiparous cows in the postpartum period; however, the lack of change

in plasma NEFA and BHBA for multiparous cows in response to CHO does not support this. Although BCS, BW, and energy balance of primiparous cows were not affected by CHO during the postpartum period, elevated BHBA levels suggest that CHO may have increased hepatic ketogenesis. Overall, these results suggest that choline may modify body tissue mobilization. Contrary to the results of the current study, previous studies have not indicated alterations in body tissue mobilization in response to RPC (Peipenbrink and Overton, 2003; Zhou et al., 2016a; Zenobi et al., 2018a). Furthermore, Goselink et al. (2013) failed to observe changes in the expression of genes associated with lipid mobilization in adipose tissue harvested from cows fed RPC during the periparturient period. Nevertheless, Hartwell et al. (2000) observed an increase in BW loss for cows fed 12 g/d choline as RPC during the postpartum period, suggesting that RPC can have effects that manifest as changes in body tissue mobilization.

Liver TG content was not affected by MET for multiparous or primiparous cows in the current study, although the liver TG levels were relatively low for all treatments, which may have minimized responses to CHO or MET. However, this lack an effect of CHO or MET on liver TG is similar to results presented elsewhere (Osorio et al., 2013; Zhou et al., 2016b; Batistel et al., 2017). These observations suggest that additional Met supplied as RPM during the periparturient period may not be used to support PC synthesis via SAM. Responses to MET by multiparous cows suggest that the additional dietary Met was diverted toward milk component production rather than the methyl-metabolism cycle to support PC and, subsequently, VLDL synthesis in the liver.

Studies that used feed restriction to induce negative energy balance in dry cows have shown that RPC can reduce liver TG accumulation (Cooke et al., 2007; Zenobi et al., 2018b). It has been hypothesized (Pinotti et al., 2002) that choline increases the rate of TG export from the liver by increasing the availability of PC and subsequently increasing the production of VLDL. This seems to be the case for dry cows under conditions of negative energy balance (Cook et al., 2007; Zenobi et al., 2018b). However, results obtained from periparturient cows have not supported this possibility. Similar to the results of the current study, other experiments failed to demonstrate reductions in liver TG by feeding RPC during the periparturient period (Hartwell et al., 2000; Zenobi et al., 2018a; Zhou et al., 2016a). In contrast, Zom et al. (2011) showed a reduction liver TG content during the first week postpartum when RPC was fed. As follow-up to that study, Goselink et al. (2013) showed that hepatic expression of genes associated with liver lipid metabolism were altered by RPC. Among the genes affected, expression of those involved in VLDL synthesis (*apolipoprotein B100* and *microsomal triglyceride transfer protein*) was increased with RPC. While it is apparent from restricted feeding experiments in dairy cows that feeding RPC will reduce liver TG, the specific conditions under which such responses would occur outside of restricted feeding experiments is unclear.

Feeding CHO and MET together was expected to have a synergistic effect on performance. Such an effect was not observed in the current study, which is similar to results reported by Zhou et al. (2016a). Both choline and Met have the ability to participate in the methyl-group metabolism pathway, and transition cows may in fact be deficient in both nutrients. The primary symptom of choline deficiency is fatty liver (Zeisel et al., 1981), and the relatively high incidence of fatty liver disease in early lactation dairy cows

(Jorritsma et al., 2001) suggests insufficient choline availability during this time. It might be expected that feeding additional choline could function to spare Met for other uses. However, the lack of milk protein responses to choline in the current study do not appear to support this concept. Production and immune responses to Met supplied during the transition period (Zhou et al., 2016a; Zhou et al., 2016b) also suggest that cows require additional Met during this time.

Because no increase in apparent tissue mobilization or a change in liver TG content was observed in response to MET, it seems as though the additional Met supplied to the cows in this study was used for other purposes. In the case of multiparous cows, MET could have been used to support milk production, as evidenced by changes in milk composition. For primiparous cows, the additional Met supplied as RPM may have been used to support continued skeletal and muscle growth and development, since MET did not affect milk production responses. In addition, Met can also be used to support the immune system through its role in the synthesis of glutathione (Martinov et al., 2010). Although the current study was not designed to analyze the effects of MET on animal health, results shown in Table 4.2 do not suggest any trends for improved health for cows fed MET. Previous work in dairy cattle has shown positive effects on immune function when RPM was fed during the transition period (Osorio et al., 2013; Zhou et al., 2016b).

Variation in responses to RPM supplemented during the periparturient period could be due to a variety of factors, including the Met requirements of the cow, the Lys:Met ratio of the diet, and the length and timing of RPM supplementation. The NRC (2001) recommends a Lys:Met ratio of 3:1, or a Lys content of 7.2% of MP and Met content of

2.4% of MP. The Lys:Met ratios for the base prepartum and postpartum diets in the current study, as predicted by NRC (2001), were 3.79:1 and 3.70:1, respectively. Thus, Lys should not have been limiting in the base diet. The Lys:Met ratios for the prepartum and postpartum base diets in the current study are similar to those reported by Zhou et al. (2016a), Osorio et al. (2013), and Ordway et al. (2009). For the postpartum diets supplemented with MET (MET and CHO + MET), the predicted (NRC, 2001) Lys:Met ratio was reduced to 2.94:1, which is similar to the ratios of the MET-supplemented postpartum diets fed by others (Zhou et al., 2016a; Osorio et al., 2013; Ordway et al., 2009). The amino acid profile of the individual ingredients and diets from this study as well as previous studies are estimated by book values and model predictions, respectively, and not via chemical analysis, which represents an inherent source of error of ration formulation. It is possible that the amino acid profile of the diets fed in the current study as well as previous studies could have differed such that the supply of Lys and Met, and other amino acids, was not accurately described. This makes it difficult to ascertain reasons for the variation in responses of cows to RPM across studies.

Many factors likely also play a role in determining the responses observed when choline is supplemented during the periparturient period. Dietary factors, such as the Lys:Met ratio and the amount and type of protein supplied prepartum, postpartum, or both, may affect responses. Hartwell et al. (2000) fed three levels of RPC to transition cows that were fed either a high or low RUP diet (4.0 or 6.2% RUP, respectively) during the prepartum period. A significant diet x RPC interaction for milk production indicated that the increase in milk yield in response to RPC was dependent upon the level of RUP supplied in the prepartum diet. It is also possible that the prepartum metabolic status of

the cow as well as her BCS and adiposity may affect her response to RPC. Zahra et al. (2006) showed that over-conditioned cows (BCS > 4) had increased milk production in response to RPC fed during the transition period. Feeding supplemental choline should only improve production in situations when the choline requirement of the animal is not met; however, the choline requirement of dairy cattle has not been established (NRC, 2001) and likely fluctuates depending on physiological state, as is apparent with other mammals (Ziesel and da Costa, 2009). Furthermore, accurate methods for predicting the choline status of a cow are lacking, so it is difficult to determine when and if choline supply is insufficient.

## **CONCLUSION**

Feeding Met as RPM during the periparturient period had positive effects on milk production for multiparous cows, while feeding choline as RPC had positive effects on milk production for primiparous cows. Neither nutrient had any apparent impact on postpartum liver TG accumulation. Variation in responses to these nutrients between primi- and multiparous cows are likely due to differences in Met and choline requirements. Further investigation into potential interactions between parity and choline or Met supplementation to periparturient cows is warranted.

## **ACKNOWLEDGMENTS**

The author would like to acknowledge the staff the Central Maryland Research and Education Center Dairy Unit for their assistance with animal care and management. The authors are also grateful to Claudia Gomez and Emily Davis (University of Maryland,

Department of Animal and Avian Sciences) for providing assistance with sample and data collection. Funding for this study was provided by Balchem Corporation (New Hampton, NY).

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**Table 4.1.** Ingredient and nutrient composition (% of DM) of the basal prepartum and postpartum diets<sup>1</sup>

<b>Item</b>	<b>Prepartum</b>	<b>Postpartum</b>
<b>Ingredient</b>		
Corn silage	44.0	29.2
Legume silage	--	11.6
Timothy hay	30.0	--
Alfalfa hay	--	7.9
Ground corn	2.0	19.5
Soybean meal	6.8	6.5
Fresh meal <sup>2</sup>	--	25.3
Close-up meal <sup>3</sup>	17.2	--
<b>Nutrient</b>		
DM, % of as-fed	48.4	51.9
NDF	45.7	32.9
ADF	27.7	21.4
Lignin	3.9	3.3
CP	14.3	16.7
MP <sup>4</sup>	9.32	10.40
Lys, % of MP <sup>4</sup>	6.71	6.74
Met, % of MP <sup>4</sup>	1.77	1.82
Lys:Met	3.79	3.70
Starch	16.0	24.0
TDN	66.7	67.0
Fat	2.4	2.6
NE <sub>L</sub> , Mcal/kg	1.51	1.62
Ash	6.7	7.6
Calcium	1.1	1.1
Phosphorus	0.38	0.40
Magnesium	0.44	0.30
Potassium	1.5	1.9
Sulfur	0.42	0.30
Sodium	0.08	0.50
Chloride	0.95	0.60
DCAD, mEq/kg <sup>5</sup>	-103	369

<sup>1</sup>Nutrient composition was calculated from wet chemistry analysis of individual feed ingredients sampled weekly throughout the study. Cows on all treatments received the same base diet throughout the study, with treatments applied daily as a top-dress.

<sup>2</sup>Fresh meal included: 35.5% soybean hulls, 26.1% SoyPlus<sup>®</sup> (Dairy Nutrition Plus<sup>™</sup>, Ames, IA), 11.2% dried molasses, 6.5% ProvAAI<sup>2</sup> MetAAtein<sup>®</sup> (Perdue Agribusiness LLC<sup>®</sup>, Binghamton, NY), 5.6% MegaLac<sup>®</sup> (Church & Dwight Co., Inc, Ewing, NJ), 3.1% limestone, 2.8% sodium bicarbonate, 1.87% salt, 1.16% OmniGen-AF<sup>®</sup> (Phibro Animal Health Corporation<sup>™</sup>, Teaneck, NJ), 1.16% Diamond V XP<sup>™</sup> (Diamond V<sup>™</sup>, Cedar Rapids, IA), 0.93% potassium carbonate, 0.93% cane molasses, 0.84% Dynamate<sup>®</sup> (The Mosaic Company, Plymouth, MN), 0.58% urea, 0.49% magnesium oxide, 0.37% Bio-fos<sup>®</sup> (The Mosaic Company, Plymouth, MN), 0.28% Rumensin<sup>®</sup> (Elanco, Greenfield, IN), 0.14% vitamin A,D, E mix, 0.13%

Selenium, 0.13% Sel-Plex 600<sup>®</sup> (Alltech<sup>®</sup>, Lexington, KY), 0.13% trace mineral salt, and 0.08% vitamin E (125,000 U).

<sup>3</sup>Close-up meal included: 30.9% soybean hulls, 22.2% Animate<sup>®</sup> (Phibro Animal Health Corporation<sup>™</sup>, Teaneck, NJ), 15.8% SoyPlus<sup>®</sup> (Dairy Nutrition Plus<sup>™</sup>, Ames, IA), 11.6% limestone, 6.33% ProvAAI<sup>2</sup> MetAAtein<sup>®</sup> (Perdue Agribusiness LLC<sup>®</sup>, Binghamton, NY), 2.64% Diamond V XP<sup>™</sup> (Diamond V<sup>™</sup>, Cedar Rapids, IA), 2.64% OmniGen-AF<sup>®</sup> (Phibro Animal Health Corporation<sup>™</sup>, Teaneck, NJ), 2.11% cane molasses, 1.48% Bio-fos<sup>®</sup> (The Mosaic Company, Plymouth, MN), 1.27% urea, 0.63% salt, 0.63% Rumensin<sup>®</sup> (Elanco, Greenfield, IN), 0.63% magnesium oxide, 0.27% vitamin A,D, E mix, 0.23% vitamin E (125,000 U), 0.23% Sel-Plex 600<sup>®</sup> (Alltech<sup>®</sup>, Lexington, KY), 0.23% selenium, and 0.21% trace mineral salt.

<sup>4</sup>MP = metabolizable protein; MP, Lys as a % of MP, and Met as a % of MP were predicted from NRC (2001) using the average prepartum or postpartum DMI for all cows.

<sup>5</sup>DCAD = dietary cation anion difference; predicted from NRC (2001) using average prepartum or postpartum DMI for all cows.

**Table 4.2.** Number of days on treatment prepartum, calving details, and health event counts for primiparous and multiparous cows fed the control (CON) diet or the control diet plus RPC (CHO), RPM (MET), or both (CHO + MET)<sup>1</sup>

Item	Treatment <sup>2</sup>			
	CON	CHO	MET	CHO + MET
<b>Multiparous</b>				
<i>n</i>	8	7	7	7
Prepartum treatment days	21 ± 5	22 ± 5	19 ± 2	20 ± 5
Incidence of twins	1	0	0	1
% heifer calves <sup>3</sup>	67	43	57	50
Calf birth weight, kg <sup>4</sup>	43 ± 2	48 ± 2	50 ± 4	45 ± 4
Calving Difficulty Score <sup>5</sup>	1.50	1.57	1.14	1.14
No. Cases				
Clinical Mastitis <sup>6</sup>	4	0	2	1
Displaced Abomasum	0	1	0	1
Lameness	0	1	0	0
Retained Placenta <sup>7</sup>	0	2	1	1
<b>Primiparous</b>				
<i>n</i>	7	6	6	6
Prepartum treatment days	17 ± 6	17 ± 2	20 ± 3	18 ± 4
Incidence of twins	0	0	0	0
% heifer calves	43	83	83	33
Calf birth weight, kg	42 ± 4	42 ± 4	42 ± 4	43 ± 4
Calving Difficulty Score	1.86	1.50	1.17	1.17
No. Cases				
Clinical Mastitis	3	0	1	0
Displaced Abomasum	0	0	1	0
Lameness	0	0	0	0
Retained Placenta	0	1	0	1

<sup>1</sup>Prepartum, cows were supplemented with 60 g/d RPC (ReaShure<sup>®</sup>, Balchem Corp., New Hampton, NY; CHO), 12 g/d RPM (Smartamine<sup>®</sup> M; Adisseo USA, Inc., Alpharetta, GA; MET), or both (CHO + MET). Postpartum, cows were supplemented with 60 g/d RPC, 18 g/d RPM, or both. Cows were monitored and treatments were applied from 21 days before expected calving through 35 DIM.

<sup>2</sup>Results are reported as treatment means ± standard deviation.

<sup>3</sup>Percentage of female calves born from cows on each treatment; calculations included one set of female twins born on the CON treatment and one set of male twins born on the CHO + MET treatment for multiparous cows.

<sup>4</sup>Calf birth weight was determined using a weight tape within 24 h of birth.

<sup>5</sup>Calving Difficulty Score: 1 = no problem or unobserved; 2 = slight problem, but no assistance; 3 = needed assistance; 4 = considerable force required; 5 extreme difficulty.

<sup>6</sup>Clinical symptoms of mastitis (milk/quarter appearance) for which treatment was administered.

<sup>7</sup>Defined as failure to expel fetal membranes within 24 h of calving.

**Table 4.3.** Prepartum performance of primi- and multiparous cows (n=25 and 29, respectively) fed the control (CON) diet or the control diet plus RPC (CHO), RPM (MET), or both (CHO + MET)<sup>1</sup>

Item	Treatment					P-value						
	CON	CHO	MET	CHO + MET	SEM	CHO	MET	MET x CHO	Week	CHO x Week	MET x Week	MET x CHO x Week
<b>Multiparous</b>												
DMI, kg/d	11.8	11.2	11.4	11.3	0.7	0.60	0.80	0.74	<0.01	0.18	0.72	1.00
Rumination, min/d	477	473	480	469	24	0.77	1.00	0.87	0.02	0.38	0.38	0.50
BW, kg	749	780	703	692	25	0.69	0.02	0.41	0.33	0.88	0.09	0.49
BCS	3.54	3.54	3.52	3.49	0.06	0.82	0.66	0.81	0.64	0.60	0.73	0.90
Energy Balance, Mcal/d <sup>2</sup>	2.64	1.99	2.53	2.30	1.40	0.76	0.94	0.89	<0.01	0.26	0.90	0.80
<b>Primiparous</b>												
DMI, kg/d	9.18	9.15	8.72	8.50	0.63	0.85	0.39	0.88	<0.01	0.88	0.18	0.42
Rumination, min/d	470	499	433	447	29	0.47	0.15	0.82	0.11	0.32	0.98	0.86
BW, kg	616	624	631	634	18	0.76	0.50	0.89	0.47	0.50	0.65	0.65
BCS	3.65	3.60	3.81	3.60	0.05	0.02	0.13	0.10	0.57	0.43	0.92	0.56
Energy Balance, Mcal/d <sup>2</sup>	1.00	0.77	0.30	-0.75	1.19	0.59	0.36	0.73	<0.01	0.96	0.05	0.51

<sup>1</sup>Primi- and multiparous cows were supplemented with 60 g/d RPC (ReaShure<sup>®</sup>, Balchem Corp., New Hampton, NY; CHO), 12 g/d RPM (Smartamine<sup>®</sup> M; Adisseo USA, Inc., Alpharetta, GA; MET), or both (CHO + MET) prepartum. Cows were monitored and treatments were applied from 21 days before expected calving through 35 DIM.

<sup>2</sup>Energy balance was calculated according to NRC (2001) equation 2-19.

**Table 4.4.** Postpartum performance of multiparous cows ( $n = 29$ ) fed the control (CON) diet or the control diet plus RPC (CHO), RPM (MET), or both (CHO + MET)<sup>1</sup>

Item	Treatment				SEM	P-value						
	CON	CHO	MET	CHO + MET		CHO	MET	MET x CHO	Week	CHO x Week	MET x Week	MET x CHO x Week
DMI	19.2	18.1	20.7	19.2	1.1	0.26	0.26	0.84	<0.01	0.49	0.95	0.90
Milk, kg/d	35.0	37.0	37.6	32.9	1.9	0.49	0.69	0.10	<0.01	0.42	0.70	0.86
Milk Fat												
%	3.77	3.58	3.95	4.06	0.14	0.75	0.03	0.30	<0.01	0.05	0.06	0.96
Yield, kg/d	1.37	1.36	1.56	1.43	0.08	0.36	0.11	0.44	0.03	0.18	0.02	0.52
Milk Protein												
%	2.86	2.76	2.96	3.07	0.05	0.87	<0.01	0.07	<0.01	0.45	0.18	0.42
Yield, kg/d	1.05	1.07	1.15	1.08	0.05	0.59	0.28	0.34	0.11	0.04	0.06	0.41
4% FCM, kg/d	33.6	34.1	37.7	35.2	1.7	0.57	0.14	0.39	<0.01	0.07	0.02	0.75
Rumination, min/d	508	510	520	503	17	0.68	0.89	0.60	<0.01	0.37	0.85	0.18
BW, kg	651	627	626	606	22	0.33	0.31	0.92	<0.01	0.39	0.41	0.68
BCS	3.26	3.11	3.23	3.05	0.06	0.01	0.44	0.84	<0.01	0.05	0.50	0.53
Energy Balance, Mcal/d	-3.04	-5.57	-3.37	-2.87	1.35	0.46	0.39	0.28	<0.01	0.61	0.58	0.71
MUN, mg/dL	10.4	10.6	9.2	10.5	0.6	0.29	0.31	0.39	0.73	0.06	0.08	0.46
SCC, 1000 cells/mL	853.0	416.0	79.2	72.0	392.0	0.58	0.17	0.59	0.27	0.11	0.57	0.44

<sup>1</sup>Cows were supplemented with 60 g/d RPC (ReaShure<sup>®</sup>, Balchem Corp., New Hampton, NY; CHO), 18 g/d RPM (Smartamine<sup>®</sup> M; Adisseo USA, Inc., Alpharetta, GA; MET), or both (CHO + MET) postpartum. Cows were monitored and treatments were applied from 21 days before expected calving through 35 DIM.

<sup>2</sup>Energy balance was calculated according to NRC (2001) equation 2-16.

**Table 4.5.** Postpartum performance of primiparous cows ( $n = 25$ ) fed the control (CON) diet or the control diet plus RPC (CHO), RPM (MET), or both (CHO + MET)<sup>1</sup>

Item	Treatment					P-value						
	CON	CHO	MET	CHO + MET	SEM	CHO	MET	MET x CHO	Week	CHO x Week	MET x Week	MET x CHO x Week
DMI	14.5	15.1	13.8	15.3	0.8	0.21	0.81	0.55	<0.01	0.74	0.59	0.98
Milk, kg/d	23.2	26.7	23.0	26.5	1.6	0.04	0.90	0.99	<0.01	0.45	<0.01	0.15
Milk Fat												
%	3.98	3.68	4.18	3.97	0.16	0.13	0.15	0.78	<0.01	0.51	0.69	<0.01
kg/d	0.97	1.05	1.00	1.11	0.07	0.18	0.49	0.88	0.10	0.55	0.89	0.01
Milk Protein												
%	3.03	2.91	3.06	2.96	0.11	0.33	0.71	0.95	<0.01	0.76	0.88	0.18
kg/d	0.72	0.82	0.75	0.82	0.04	0.06	0.80	0.78	0.01	0.56	0.94	0.94
4% FCM, kg/d	23.0	25.3	23.4	26.2	1.5	0.10	0.63	0.86	<0.01	0.82	0.15	<0.01
Rumination, min/d	511	504	493	477	23	0.62	0.34	0.83	<0.01	0.43	0.30	0.80
BW, kg	533	537	545	560	15	0.54	0.26	0.69	<0.01	0.26	0.03	0.72
BCS	3.32	3.34	3.46	3.37	0.05	0.52	0.13	0.25	0.07	0.51	0.85	0.82
Energy Balance, Mcal/d	-2.09	-2.81	-3.65	-3.35	0.80	0.79	0.21	0.54	<0.01	0.45	0.80	0.41
MUN, mg/dL	11.2	11.0	11.6	12.7	0.5	0.46	0.08	0.22	0.72	0.83	0.42	0.29
SCC, 1000 cells/mL	504.0	148.0	65.4	139.0	191.0	0.47	0.26	0.28	0.63	0.72	0.65	0.62

<sup>1</sup>Cows were supplemented with 60 g/d RPC (ReaShure<sup>®</sup>, Balchem Corp., New Hampton, NY; CHO), 18 g/d RPM (Smartamine<sup>®</sup> M; Adisseo USA, Inc., Alpharetta, GA; MET), or both (CHO + MET) postpartum. Cows were monitored and treatments were applied from 21 days before expected calving through 35 DIM.

<sup>2</sup>Energy balance was calculated according to NRC (2001) equation 2-16.

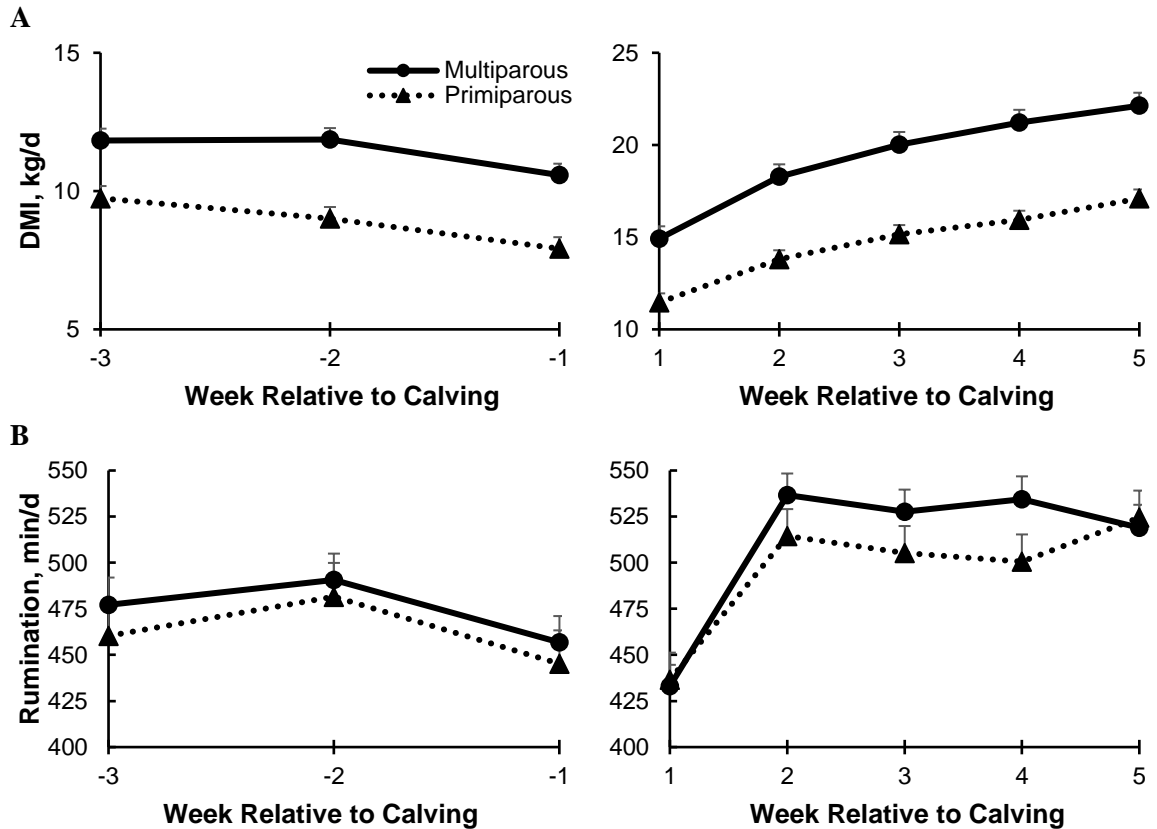


**Table 4.6.** Pre- and postpartum blood metabolite responses of primi- and multiparous cows ( $n = 25$  and  $29$ , respectively) fed the control (CON) diet or the control diet plus RPC (CHO), RPM (MET), or both (CHO + MET)<sup>1</sup>

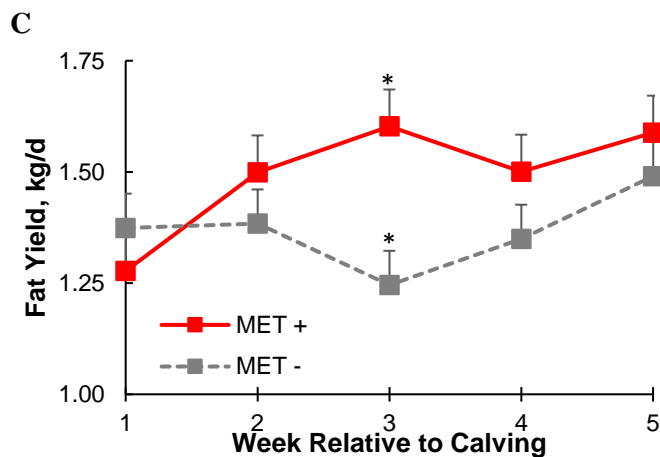
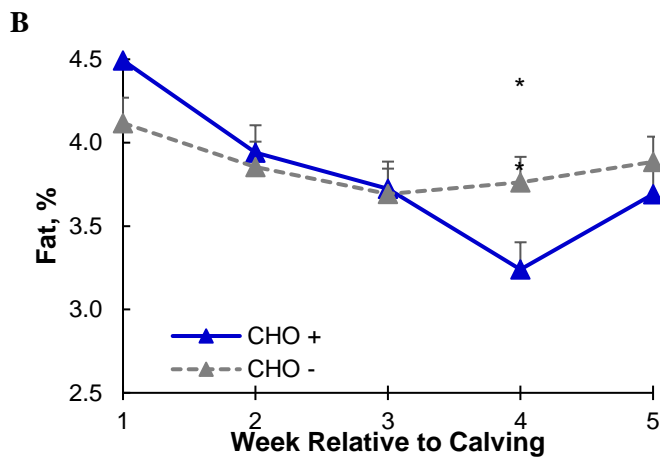
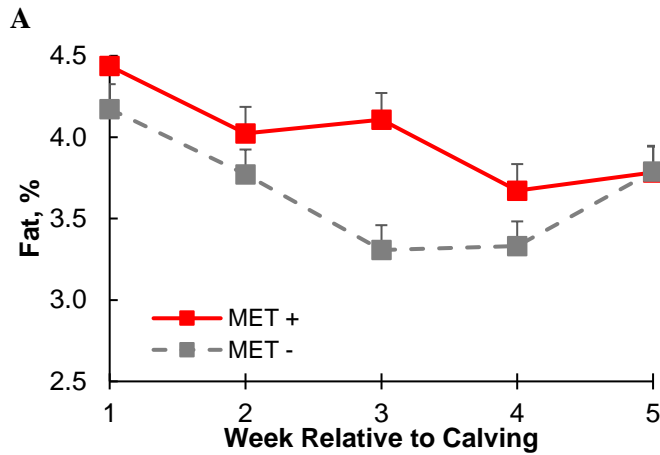
Item	Treatment					P-value						
	CON	CHO	MET	CHO + MET	SEM	CHO	MET	MET x CHO	Week	CHO x Week	MET x Week	MET x CHO x Week
<b>Prepartum</b>												
Multiparous												
BHBA, mM	0.25	0.30	0.29	0.33	0.04	0.30	0.42	0.86	<0.01	0.21	0.14	0.07
NEFA, mEq/L	0.36	0.43	0.40	0.40	0.07	0.65	1.00	0.64	0.27	0.91	0.75	0.32
Glucose, mg/dL	56.2	54.8	55.4	55.5	2.0	0.74	0.99	0.72	0.72	0.76	0.27	0.52
Primiparous												
BHBA, mM	0.31	0.37	0.29	0.36	0.04	0.08	0.65	0.85	0.07	0.37	0.45	0.67
NEFA, mEq/L	0.44	0.47	0.37	0.55	0.05	0.05	0.99	0.14	0.79	0.54	0.14	0.30
Glucose, mg/dL	61.3	59.6	60.1	58.8	2.6	0.57	0.69	0.93	0.74	0.78	0.34	0.43
<b>Postpartum</b>												
Multiparous												
BHBA, mM	0.47	0.61	0.57	0.52	0.09	0.64	0.99	0.32	0.09	0.28	0.18	0.96
NEFA, mEq/L	0.71	0.67	0.81	0.66	0.06	0.15	0.46	0.38	<0.01	0.50	0.33	0.62
Glucose, mg/dL	53.6	49.5	54.1	54.8	1.7	0.31	0.10	0.17	0.32	0.58	0.82	0.73
Primiparous												
BHBA, mM	0.40	0.46	0.45	0.49	0.02	0.05	0.14	0.75	0.13	0.96	0.29	0.44
NEFA, mEq/L	0.52	0.58	0.63	0.60	0.06	0.77	0.29	0.42	0.02	0.68	0.40	0.28
Glucose, mg/dL	62.4	59.0	58.3	59.2	2.0	0.54	0.35	0.30	0.01	0.54	0.76	0.06

<sup>1</sup>Prepartum, cows were supplemented with 60 g/d RPC (ReaShure<sup>®</sup>, Balchem Corp., New Hampton, NY; CHO), 12 g/d RPM (Smartamine<sup>®</sup> M; Adisseo USA, Inc., Alpharetta, GA; MET), or both (CHO + MET). Postpartum, cows were supplemented with 60 g/d RPC, 18 g/d RPM, or both. Cows were monitored and treatments were applied from 21 days before expected calving through 35 DIM.

**Figure 4.1.** Mean DMI (A) and daily rumination (B) for primi- and multiparous cows. For primi- and multiparous cows, DMI decreased as calving approached ( $P < 0.01$ ) and subsequently increased after calving ( $P < 0.01$ ). For primiparous cows, daily rumination tended to decrease as calving approached ( $P = 0.11$ ) and increased after calving ( $P < 0.01$ ). For multiparous cows, daily rumination decreased as calving approached ( $P = 0.02$ ) and increased after calving ( $P < 0.01$ ).

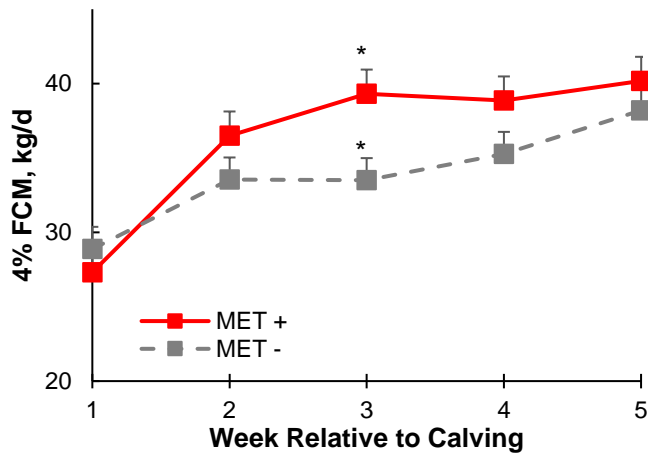


**Figure 4.2.** Milk fat percentage (A and B) and yield (C) for multiparous cows fed the control diet (CON), 60 g/d RPC (CHO), 12 g/d RPM prepartum and 18 g/d RPM postpartum (MET), or both (CHO + MET) from 21 days before expected calving through 35 DIM. Milk fat percentage: CHO x Week:  $P = 0.05$ ; MET x Week:  $P = 0.06$ ; Milk fat yield: MET x Week:  $P = 0.02$ . Asterisks indicate that individual means are significantly different ( $P < 0.05$ ).

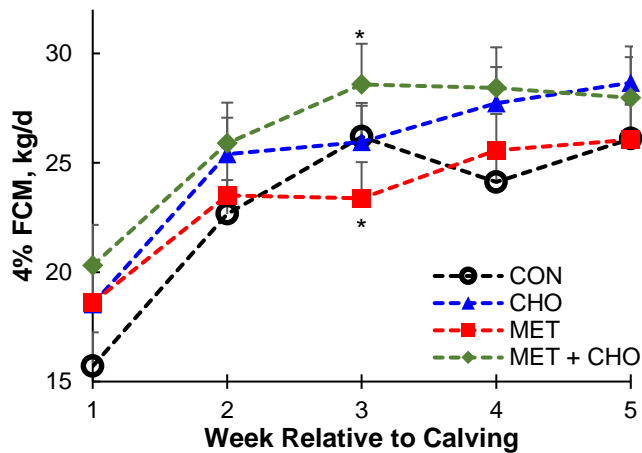


**Figure 4.3.** Fat-corrected milk for multiparous (A) and primiparous cows (B) fed the control diet (CON), 60 g/d RPC (CHO), 12 g/d RPM prepartum and 18 g/d RPM postpartum (MET), or both (CHO + MET) from 21 days before expected calving through 35 DIM. For multiparous cows, MET x Week: ( $P = 0.02$ ). For primiparous cows, CHO x MET x Week ( $P < 0.01$ ). Asterisks indicate that individual means are significantly different ( $P < 0.05$ ).

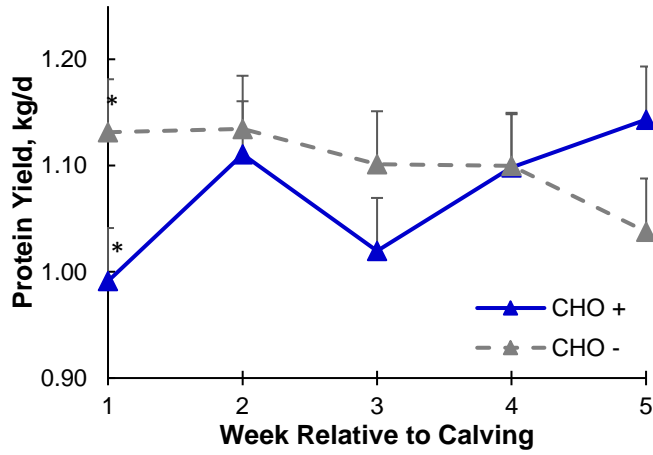
A



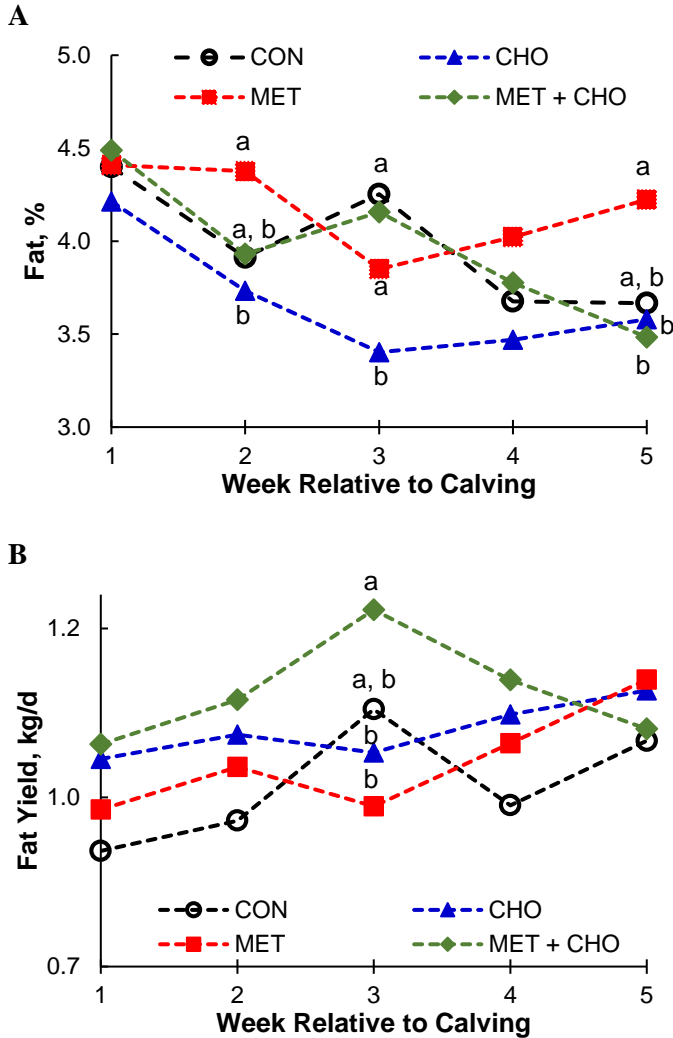
B



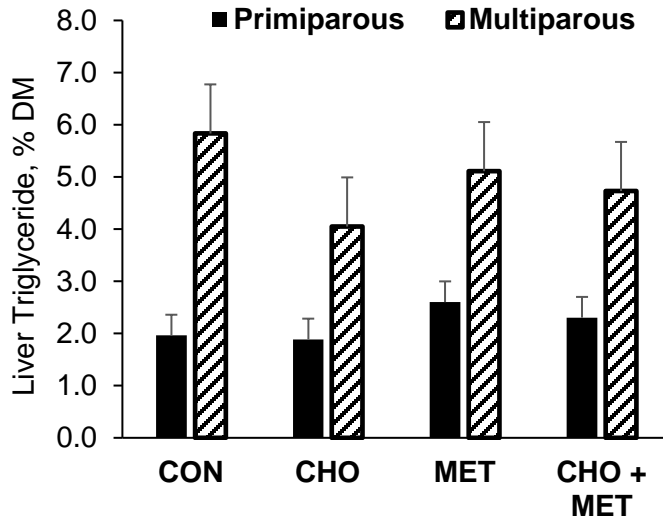
**Figure 4.4.** Milk protein yield for multiparous cows fed the control diet (CON), 60 g/d RPC (CHO), 12 g/d RPM prepartum and 18 g/d RPM postpartum (MET), or both (CHO + MET) from 21 days before expected calving through 35 DIM. CHO x Week: ( $P = 0.04$ ). Asterisks indicate means that are significantly different ( $P < 0.05$ ).



**Figure 4.5.** Milk fat percentage (A) and yield (B) for primiparous cows fed the control diet (CON), 60 g/d RPC (CHO), 12 g/d RPM prepartum and 18 g/d RPM postpartum (MET), or both (CHO + MET) from 21 days before expected calving through 35 DIM. Milk fat concentration: CHO x MET x Week:  $P < 0.01$ ; Milk fat yield: CHO x MET x Week:  $P = 0.01$ . Means that lack common superscripts at each week relative to calving are significantly different ( $P < 0.05$ ).



**Figure 4.6.** Liver TG concentration (% of DM) for primi and multiparous cows ( $n = 23$  and  $n = 29$ , respectively) fed the control diet (CON), 60 g/d RPC (CHO), 12 g/d RPM prepartum and 18 g/d RPM postpartum (MET), or both (CHO + MET) from 21 days before expected calving through 35 DIM. Data show liver TG concentrations from samples obtained at ~7 d postpartum. Triglyceride concentrations of liver samples obtained on the day of study enrollment (prepartum) were used as covariates in the statistical analysis. CHO and MET did not affect liver TG content for primiparous ( $P = 0.65$ ,  $P = 0.20$ , respectively) or multiparous cows ( $P = 0.26$ ,  $P = 0.98$ , respectively).



## **CHAPTER 5: EXPERIMENT 3**

### **Feeding Rumen-protected Choline or Methionine during the Periparturient Period affects Plasma and Milk Choline Metabolites, Plasma Amino Acids, and Hepatic Expression of Genes Associated with Choline and Lipid Metabolism<sup>1</sup>**

<sup>1</sup>S.B. Potts, K.M. Brady, C.M. Scholte, K.M. Moyes, N.E. Sunny, and R.A. Erdman. Feeding rumen-protected choline or methionine during the periparturient period affects plasma and milk choline metabolites, plasma amino acids, and hepatic expression of genes associated with choline and lipid metabolism. In preparation for submission to the Journal of Dairy Science.



## ABSTRACT

Feeding supplemental choline and Met during the periparturient period can have positive effects on cow performance; however, the mechanisms by which these nutrients affect performance and metabolism are unclear. The objective of this experiment was to determine if providing rumen-protected choline (RPC), rumen-protected Met (RPM), or both during the periparturient period modifies plasma AA, the choline metabolite profile of plasma and milk, and hepatic mRNA expression of genes associated with choline, Met, and lipid metabolism. Cows (25 primiparous, 29 multiparous) were blocked by expected calving date and parity and assigned to one of 4 treatments: CON (no RPC or RPM); CHO (60 g/d RPC); MET (12 g/d RPM prepartum; 18 g/d RPM postpartum); or CHO + MET. Treatments were applied daily as a top dress from ~21 d prepartum through 35 days in milk (DIM). On the day of treatment enrollment (d -19±2 relative to calving), liver and blood samples were collected for covariate measurements. At ~7 and 14 DIM, samples of blood and milk were collected for analysis of choline metabolites, including 16 species of phosphatidylcholine (PC) and 4 species of lysophosphatidylcholine (LPC). Blood was also analyzed for AA and select organic acid concentrations. Liver samples were collected from multiparous cows at ~7 DIM for gene expression analysis. Choline reduced milk betaine yield for multiparous cows but tended to increase it for primiparous cows. Choline increased and Met tended to increase yields of 4 PC species as well as LPC 18:0 in multiparous cows. However, only LPC 18:1 yield was increased by choline for primiparous cows. Met reduced blood free choline concentrations and choline tended to increase glycerolphosphocholine but decrease sphingomyelin concentrations in multiparous cows. For primiparous cows, Met decreased concentrations of total PC and

several species of PC during the second week of lactation, but choline only affected concentrations of 2 PC species. For both groups of cows, Met increased blood Met concentrations, but choline did not. Choline significantly reduced lactate and pyruvate concentrations in the blood of multiparous cows, but only numeric differences were observed for primiparous cows. When fed without Met, choline increased hepatic mRNA expression of *betaine-homocysteine methyltransferase* and *phosphate cytidyltransferase 1 choline,  $\alpha$* . In addition, choline also tended to decrease mRNA expression of *3-hydroxy-3-methylglutaryl-CoA synthase 2* and *peroxisome proliferator activated receptor  $\alpha$*  when fed with or without Met. These results indicate that both Met and choline can have effects on choline metabolism when supplemented during the periparturient period and that these effects differ between primi- and multiparous cows.

## INTRODUCTION

The choline requirement for dairy cattle has not been defined (NRC, 2001), although it is considered to be an essential nutrient for other mammals, including humans (Zeisel and da Costa, 2009). Because lactation increases the choline requirement in humans (Zeisel and da Costa, 2009), the same is probably also true for cows. Choline deficiency can limit an animal's ability to export lipids from the liver because it is required for synthesis of phosphatidylcholine (PC), the major phospholipid that comprises very low-density lipoproteins (Yao and Vance, 1988). Phosphatidylcholine is synthesized via one of two pathways: 1) the cytidine diphosphate (CDP)-choline pathway using choline derived from the diet; and 2) the phosphatidylethanolamine methyl-transferase (PEMT) pathway whereby a series of three methylation reactions occur to convert phosphatidylethanolamine to PC (Caudill, 2010). Because dietary choline is rapidly degraded in the rumen (Atkins et al., 1988), it is likely that in ruminants, the majority of PC is synthesized via the PEMT pathway. Feeding rumen-protected choline (RPC) could potentially lead to more PC synthesis via the CDP-choline pathway. Delong et al. (1998) suggested that PC species that contained poly-unsaturated FA (PUFA) were derived from PEMT origin in rats. Thus, the profile of individual PC species in blood or milk that result when RPC is supplemented in periparturient cows could be indicative of a change in PC source.

Methionine is considered to be the most limiting AA in dairy cattle diets (NRC, 2001) and it is used not only for protein synthesis, but also for synthesis the methyl donor *S*-adenosylmethionine (SAM), one of the most important methyl donors in the body (Chiang et al., 1996). *S*-adenosylmethionine is required for de novo synthesis of PC via

the PEMT pathway (Li and Vance, 2008) and for this reason, studies in dairy cattle have investigated the lipotropic potential of Met, although liver triglyceride (TG) responses to Met have not been reported (Bertics and Grummer, 1999; Zhou et al., 2016b). Furthermore, SAM is also implicated in regulation of gene expression via histone methylation reactions (Mentch and Locasale, 2016). After donating its methyl group, SAM is converted to homocysteine (Hcy), which can be used to regenerate Met or synthesize glutathione, an important antioxidant in the body (Martinov et al., 2010). Thus, through these roles, Met has the potential to have significant impacts on immune function, as well as lipid and protein metabolism.

Choline and Met metabolism are integrated through their participation in one-carbon metabolism. Choline indirectly serves as a methyl donor in the one-carbon metabolic pathway via betaine, the product of choline oxidation. Betaine is required for the regeneration of Met from Hcy via the betaine-homocysteine methyltransferase (BHMT) pathway (Martinov et al., 2010). Alternatively, Hcy can also be converted to Met via the methyl-tetrahydrofolate (CH<sub>3</sub>-THF) pathway by methionine synthase (MS), an enzyme that requires vitamin B<sub>12</sub> to function (Preynat et al., 2009). Methionine also indirectly influences PC synthesis through the PEMT pathway via the availability of SAM. Because of these connections, as well as down-stream effects of both PC, SAM, and Hcy, feeding supplemental choline or Met has the potential to affect the metabolism of each other as well as several other metabolic processes within the body.

During the periparturient period, the availability of many nutrients can be limited due the increase in nutritional demands associated with the onset of lactation coupled with

the slow increase in feed intake after parturition (Grummer, 1995). In an effort to derive nutritional strategies that improve cow health and performance during this time, recent attention has been given to nutrients involved in one-carbon metabolism, such as choline, Met, and B-vitamins (Zhou et al., 2016b; Duplessis et al., 2017; Zenobi et al., 2018; Zang et al., 2019) due to the aforementioned functions associated with lipid transport and metabolism, immune function and gene expression. Previous investigations have shown that supplementing many of these nutrients around the time of calving can modify hepatic expression of genes associated with choline, Met, and lipid metabolism (Preynat et al., 2010; Goselink et al., 2013; Zhou et al., 2017a) as well as the plasma AA profile (Zhou et al., 2017b; Zang et al., 2019). However, only the study by Zhou et al. (2017a,b) investigated specific effects associated with supplementation of choline, Met, or both during the periparturient period. Furthermore, none of these studies investigated possible effects of dietary Met or choline on the milk and blood choline metabolite profile of periparturient cows. Such changes could help further our understanding of how these nutrients affect the metabolism and production performance of postpartum cows.

The objective of this study was to determine if providing supplemental choline, Met, or both during the periparturient period modifies plasma AA concentrations, the choline metabolite profile of plasma and milk, as well as hepatic expression of genes associated with choline, Met, and lipid metabolism. It was also of interest to determine if such responses were different between primiparous and multiparous cows, as was the case for production responses.

## MATERIALS AND METHODS

All procedures that utilized animals were approved by the University of Maryland, College Park, Institutional Animal Care and Use Committee.

### *Animals and Study Design*

Between March and December of 2017, 25 primiparous and 29 multiparous Holstein cows from the Central Maryland Research and Education Center were selected for use in a randomized block design experiment with a 2 x 2 factorial treatment structure. The two factors were 0 or 60 g/d RPC (ReaShure<sup>®</sup>; Balchem Corp., New Hampton, NY) and 0 or 12 g/d RPM (Smartamine<sup>®</sup> M; Adisseo USA, Inc., Alpharetta, GA) prepartum and 18 g/d RPM postpartum. Thus, the four resulting treatments included: 1) no supplemental choline or Met (control; CON); 2) 60 g/d RPC (CHO); 3) 12 g/d RPM prepartum and 18 g/d RPM postpartum (MET); and 4) a combination of CHO and MET treatments (CHO + MET; 60 g/d RPC + 12 g/d RPM prepartum and 18 g/d RPM postpartum). Prior to the start of the experiment, cows were blocked by age (primiparous vs. multiparous) and expected calving date and randomly assigned to treatment. Treatments were applied daily as a top dress from 21 d before expected calving through 35 DIM. Additional details regarding basal pre- and postpartum diets and animal care and housing were as described in Chapter 4.

### *Sample Collection and Analysis*

On approximately d 7 and 14 postpartum, samples of milk were collected and stored at -80°C until analysis of choline metabolites by HILC-MS/MS (Artegoitia et al., 2014).

Metabolites examined included betaine, free choline, glycerophosphocholine (GPC), sphingomyelin (SM), 16 species of PC, and 4 species of lysophosphatidylcholine (LPC).

Blood was collected via coccygeal venipuncture in the morning before feeding on approximately -21, 7, and 14 d relative to calving. Because some cows calved early or late, prepartum samples were actually collected on  $-19 \pm 2$  d relative to calving. Samples were collected into one 10mL evacuated tube containing potassium EDTA and one 10mL evacuated tube containing sodium heparin. Tubes were immediately placed on ice and then centrifuged at  $2000 \times g$  for 15 min at  $4^{\circ}\text{C}$ . Plasma aliquots were stored at  $-80^{\circ}\text{C}$  until analysis. Amino acid and organic acid (citrate, fumarate, lactate, malate, oxaloacetate, pyruvate, succinate, and  $\alpha$ -ketoglutarate) concentrations of plasma were determined using the methods described by Sunny and Bequette (2010). Briefly, plasma samples (50  $\mu\text{L}$ ) were deproteinized with 500  $\mu\text{L}$  of cold acetonitrile and evaporated to dryness under  $\text{N}_2$ . Amino acids and organic acids were then converted to their respective t-butyltrimethylsilyl derivatives by heating at  $90^{\circ}\text{C}$  for 1 h. Metabolites were separated by gas chromatography (HP-5ms, 30 m  $\times$  0.25 mm  $\times$  0.25  $\mu\text{m}$ , Agilent Technologies, Santa Clara, CA) before selected ion monitoring of specific ion fragments with mass spectrometry under electron ionization. Choline metabolite concentrations of plasma were determined as described previously for milk (Artegoitia et al., 2014).

Approximately 150 mg of liver tissue was collected from each animal via percutaneous liver biopsies on approximately -21 d (actual prepartum samples were obtained on  $-19 \pm 2$  d) and 7 d relative to calving using a 14G biopsy needle (14G x 15 cm; Tru-Cut<sup>®</sup>, Merit Medical, Jordan, UT). Further details regarding the liver biopsy procedure

are described in Chapter 4. Tissue was immediately snap frozen in liquid nitrogen and stored at -80°C. Liver samples obtained from 5 of the 7 blocks of multiparous cows ( $n = 20$ , 5 cows per treatment) were used for gene expression analysis as described below. These 5 blocks were selected because all of the cows within each of them completed the experiment.

Hepatic expression of genes related to choline and methionine metabolism (betaine-homocysteine S-methyltransferase, *BHMT*; phosphatidylethanolamine N-methyltransferase, *PEMT*; phosphate cytidyltransferase 1, *PCYT1A*; glutathione synthesis, *GSS*; 5-methyltetrahydrofolate-homocysteine methyltransferase, *MTR*) and lipid metabolism (carnitine palmitoyltransferase 1A, *CPT1A*; diacylglycerol O-acyltransferase 1, *DGAT1*; 3-hydroxy-3-methylglutaryl-CoA synthase 2, *HMGCS2*; microsomal triglyceride transfer protein, *MTTP*; peroxisome proliferator activated receptor alpha, *PPAR $\alpha$* ) was determined via RT-PCR. Expression of phosphoglycerate kinase 1 (*PGK1*) was also determined for use as the housekeeping gene. Primers were designed (Table 5.1) using the National Center for Biotechnology Information (NCBI) primer BLAST Software (NCBI, Bethesda, MD). Primers needed to: 1) span an intron; 2) target a region as close to the 3' end of the sequence as possible; 3) amplify all splice variants; 4) have a melting temperature of 58-60°C; 5) have a G/C content of 40-60%; 6) be between 18-30 nucleotides in length; and 6) generate a PCR product that was 100-250 nucleotides in length. Primers (25 nmol DNA oligos; standard desalting purification) were obtained from Integrated DNA Technologies (Coralville, IA) and reconstituted in ultrapure water. Amplification efficiencies were determined for each primer pair by performing RT-qPCR (QuantiTect SYBR<sup>®</sup> Green PCR Kit, Qiagen Inc., USA, Germantown, MD) using 2-fold serial dilutions



of pooled cDNA (1µg). Efficiency was calculated using the equation  $[(10^{(-1/\text{slope})})-1]$ , where slope is equal to the slope of the regression of the  $C_T$  value on the  $\log_{10}(\text{copy number})$ . Efficiencies ranged from 0.96 to 1.08 for all primers tested. Primer specificity was verified by dissociation curve analysis, agarose gel electrophoresis, and sequencing of PCR products.

Liver total RNA was extracted using the RNeasy Lipid Tissue Mini Kit with on-column DNase digestion (Qiagen Inc.). Approximately 20-30 mg of liver tissue was weighed and kept frozen in liquid nitrogen until homogenization in 0.5 mL of QIAzol Lysis Reagent (Qiagen Inc.). The remainder of the RNA extraction protocol was carried out according to manufacturer instructions. After extraction, RNA was stored at  $-80^{\circ}\text{C}$ . The concentration of RNA in each sample was determined using a commercially available kit (Quant-iT™ RiboGreen™ RNA Assay Kit, Catalog #R11490, ThermoFisher Scientific, Waltham, MA) and 1 µg of RNA was used for cDNA synthesis (QuantiTect Reverse Transcription Kit, Qiagen Inc.). For the reverse transcription reactions, a reaction of a pool of total RNA without reverse transcriptase was conducted as a control for genomic DNA contamination. Complementary DNA was not diluted prior to PCR analysis and was stored at  $-20^{\circ}\text{C}$ .

The PCR reactions were carried out using a commercially available kit according to the manufacturer instructions (QuantiTect SYBR® Green PCR Kit, Qiagen Inc.) in a CFX-Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA). The PCR reactions were performed in 96-well plates (VWR International, LLC, Radnor, PA) in a CFX-Connect™ Real-Time PCR Detection System (Bio-Rad

Laboratories, Inc.) for 40 cycles using the following program: 95°C for 15 min (activation), 94°C for 15s (denaturation), 55°C for 30s (annealing), and 72°C for 30s (extension). Dissociation curve analysis ensured amplification of a single PCR product and absence from the no RT controls. Data were normalized to PGK1 and analyzed by the  $2^{-\Delta\Delta C_t}$  method.

### ***Statistical Analysis***

Similar to production data (presented in Chapter 4), AA, organic acid, and choline metabolite data were analyzed for primi- and multiparous cows separately. Postpartum blood AA, organic acid, and choline metabolite concentrations were analyzed using a repeated-measures mixed model (SAS, version 9.4) that included the random effect of cow nested within block and fixed effects of week relative to calving (1 or 2), the main effects of CHO and MET, and all two- and three-way interactions. Prepartum metabolite concentration, determined from samples collected in the morning before first treatment application ( $-19 \pm 2$  d relative to calving), was also included in the model as a covariate. Week relative to calving served as the repeated factor and the compound symmetry covariance structure was used. Milk choline metabolite concentrations and corresponding yields were analyzed using a similar model that did not include a prepartum covariate measurement.

Gene expression fold-changes (relative to postpartum CON) were analyzed in a mixed model that included the main effects of CHO and MET, their interaction, and the random effect of cow. Prepartum gene expression fold-change (relative to postpartum CON) was included in the model as a covariate. Thus, postpartum gene expression results

are expressed as covariate-adjusted fold-changes relative to CON. Cook's Distance was used to detect potential outliers using a threshold of  $4/n$ . If a cow was identified as a potential outlier for more than 4 of the 10 genes of interest, she was removed from the analysis. Based on this method, results from two cows (one from CON and one from CHO) were considered outliers and removed from the final analysis. Statistical significance was declared at  $P \leq 0.05$  and tendencies were declared at  $P \leq 0.10$ .

## **RESULTS AND DISCUSSION**

There were several changes in plasma AA, organic acid, and choline metabolite concentrations as well as alterations in milk choline metabolite yields in response to providing supplemental choline and Met. This is the first study in dairy cattle that has examined effects of providing these nutrients during the periparturient period on several species of choline metabolites in blood and milk. These results demonstrate that both choline and Met can modify both AA and choline metabolism when supplemented during the periparturient period and that these responses vary between primi- and multiparous cows.

### ***Plasma Amino Acids***

Plasma AA concentrations for multiparous and primiparous cows are shown in Tables 5.2 and 5.3, respectively. All changes in plasma AA concentrations due to treatment occurred without any changes in protein intake, as DMI was not affected by treatment (presented in Chapter 4). With the exception of Ser, there were no main effects ( $P > 0.05$ ) for any of the non-essential AA. This is in contrast to previous work by Zhou et al. (2017b),

who showed that feeding MET fed during the periparturient period increased Ala, Asp, Asn, and Pro concentrations but did not affect Ser. For the multiparous cows in this study, a MET x Week interaction ( $P = 0.05$ ; Table 5.2) indicated that MET reduced Ser concentrations during week 2 but not week 1 postpartum. Similarly, for primiparous cows, MET reduced Ser concentrations regardless of time ( $P < 0.01$ ; Table 5.3), although there tended to be a MET x CHO interaction ( $P = 0.08$ ), with a more pronounced reduction in Ser occurring when both CHO and MET were fed. Ser is required for the first step toward glutathione production from Hcy as well as for regeneration of 5, 10-methyl-tetrahydrofolate from tetrahydrofolate (THF) in the CH<sub>3</sub>-THF pathway (Selhub, 1999). Thus, the reduction in Ser observed for cows fed MET could be indicative of enhanced Met recycling via the CH<sub>3</sub>-THF pathway or elevated glutathione production. However, as discussed later, hepatic mRNA expression of *MTR*, the gene that encodes the rate-limiting enzyme in the CH<sub>3</sub>-THF pathway, and *GSS*, the gene that encodes glutathione synthetase, were not affected by CHO or MET.

Feeding CHO tended to increase plasma Gly concentrations during the first week of lactation for primiparous cows (CHO x Week:  $P = 0.08$ ; Table 5.3) but not multiparous cows. Dimethylglycine, a byproduct of the BHMT pathway, can be converted to sarcosine and, subsequently, glycine (Eklund et al., 2005). Thus, the observed increase in Gly for primiparous cows fed choline in the current study could indicate enhanced flux of Hcy through the BHMT pathway. Glycine is also produced as a byproduct from the conversion of THF to 5, 10-methyl-tetrahydrofolate, so this increase in plasma Gly could also be indicative of enhanced Met recycling through the CH<sub>3</sub>-THF pathway. However, plasma

Gly levels were not altered by CHO for multiparous cows, which is similar to results reported by Zhou et al. (2017b).

Changes in plasma concentrations of non-essential AA during the early postpartum period are not surprising, given that cows are generally in negative energy and protein balance even as DMI gradually increases (Grummer, 2008). Concentrations of Pro and Tyr were greater during the second week of lactation as compared to the first for both groups of cows. However, there tended to be a MET x Week interaction for Tyr concentration for primiparous cows ( $P = 0.08$ ; Table 5.3), which indicated that MET dampened this increase during the second week of lactation. The increase in Pro and Tyr was not surprising, given that Pro is glucogenic and Tyr is both glucogenic and ketogenic. These results are similar to previous reports by Doepel et al. (2002) and Zhou et al. (2017b). Consistent with results reported by Zhou et al. (2016a), plasma Ala concentrations were greater during week 2 postpartum for both groups of cows, but only results for multiparous cows showed statistical significance ( $P < 0.01$ ; Table 5.2). This finding likely indicates an increase in muscle tissue break-down to support gluconeogenesis via the alanine cycle.

Feeding CHO tended to reduce essential AA (EAA) concentrations ( $P = 0.10$ ; Table 5.2) for multiparous but not primiparous cows. This is in contrast to the observations of Zhou et al. (2017b), who showed that feeding choline to multiparous periparturient cows did not affect overall EAA concentrations. Zhou et al. (2016a) used a liver functionality index, an index that utilizes blood concentrations of albumin, cholesterol, and bilirubin to indicate immune and metabolic status, to rank cows according to how well they adapted during the periparturient period. They observed that a low index (poor immune and

metabolic status) was associated with a reduction in circulating EAA during the postpartum period (Zhou et al., 2016a). The authors suggested that this change could be the result of enhanced uptake of these AA for synthesis of acute phase proteins, which are associated with inflammation. Thus, the CHO-induced decrease in EAA for multiparous cows in the current study could reflect a poor transition to lactation; however, immune function of cows in the current study was not assessed, so further inferences related to this hypothesis cannot be made.

For multiparous cows, CHO numerically reduced Leu, Lys, and Val concentrations, although only the decrease in Leu concentration was statistically significant ( $P = 0.04$ ; Table 5.2). However, these EAA were not affected by CHO for primiparous cows. The reduction in Leu and Val is consistent with results reported by Zang et al. (2019) when a supplement containing several methyl donors (choline, Met, betaine, riboflavin, and vitamin B<sub>12</sub>) was provided to cows during the periparturient period. In agreement with previous observations (Zhou et al., 2017b), CHO did not affect plasma Met concentrations in the current study.

Consistent with previous work (Blum et al., 1999; Zhou et al., 2017b), MET increased plasma Met ( $P < 0.01$ ; Tables 5.2 and 5.3) for both groups of cows, indicating that the Met fed as RPM was absorbed. Feeding MET without CHO numerically increased concentrations of all EAA examined for both groups of cows relative to CON, suggesting that providing MET improved the AA profile (Zhou et al., 2017a) and increased availability of these AA to allow for greater protein synthesis. This is consistent with previous findings (Zhou et al., 2017b) as well as the production responses that were observed for these cows

(presented in Chapter 4), where milk protein percentage was enhanced for multiparous cows fed MET. Although milk protein production was not altered by MET for primiparous cows (presented in Chapter 4), it is possible that stimulation of protein synthesis by MET was directed instead toward skeletal growth or maintenance. There was a tendency for a MET x Week interaction for BCAA ( $P = 0.07$ ; Table 5.2) for multiparous cows which indicated that MET numerically increased these AA during the second but not the first week of lactation. This could indicate that MET stimulated gluconeogenesis and ketogenesis during this time. There appeared to be a negative synergistic effect of feeding CHO + MET for plasma Phe concentration for multiparous cows (MET x CHO:  $P = 0.03$ ; Table 5.2) because cows fed CHO + MET had lower Phe concentrations than cows fed CHO or MET alone. A similar trend was observed for primiparous cows, but the MET x CHO interaction was not statistically significant ( $P = 0.12$ ; Table 5.3).

For both primi- and multiparous cows, plasma concentrations of Thr increased from the first week to the second week of lactation ( $P < 0.01$  and  $P = 0.04$ , respectively; Tables 5.2 and 5.3). All other EAA, with the exception of Met and Phe, also increased from week 1 to 2, but these changes were only statistically significant for Lys and Val concentrations in primiparous cows ( $P = 0.04$  and  $P < 0.01$ , respectively; Table 5.3). This finding is congruent with previous findings (Doepel et al., 2002; Zhou et al., 2016a) and likely coincides with an increase in the demand for AA postpartum.

### ***Plasma Organic Acids***

Plasma organic acid concentrations for multiparous and primiparous cows are shown in Table 5.4. For multiparous cows, CHO drastically decreased plasma lactate

concentration ( $P < 0.01$ ) which coincided with a decline in pyruvate concentrations ( $P < 0.01$ ), although pyruvate concentrations were only reduced when CHO was fed without MET (MET x CHO,  $P = 0.04$ ). Furthermore, CHO reduced plasma oxaloacetate concentration ( $P = 0.01$ ) in multiparous cows. This observation, in conjunction with the reduction in plasma pyruvate concentration for cows fed CHO, could be indicative of elevated TCA cycle activity. In support of this, work by Goeslink et al. (2013) showed that periparturient cows fed RPC had decreased hepatic expression of *pyruvate carboxykinase* mRNA, which suggested a decrease in the need to replenish oxaloacetate. However, because the results from the current study reflect metabolite concentrations in venous plasma, it is difficult to make extrapolations to TCA function. Alternatively, these findings could be indicative of an alteration in muscle glycolysis or the Cori cycle.

In contrast to results for multiparous cows, plasma lactate, pyruvate, and oxaloacetate were not affected by CHO for primiparous cows (Table 5.4). However, MET tended to increase oxaloacetate concentrations during week 1, but not week 2 postpartum (MET x Week,  $P = 0.07$ ; Table 5.4), which might indicate reduced TCA cycle activity during week 1. Furthermore, a MET x CHO interaction for plasma pyruvate concentrations ( $P = 0.05$ ; Table 5.4) indicated that primiparous cows fed CHO or MET alone had numerically lower concentrations compared to CON or CHO + MET. However, because the changes in plasma pyruvate concentrations occurred in the absence of altered plasma lactate concentrations, treatment effects were probably not due to changes in the Cori cycle, as was postulated for multiparous cows.



Feeding CHO increased plasma fumarate and malate concentrations for primiparous cows ( $P = 0.02$  and  $P = 0.05$ , respectively; Table 5.4), but not multiparous cows. Accumulation of fumarate or malate could indicate insufficient oxaloacetate to maintain TCA cycle activity, but a lack of a CHO effect on oxaloacetate for primiparous cows does not support this. There was a tendency for CHO x Week interaction for succinate concentrations in primiparous cows ( $P = 0.07$ ; Table 5.4), where CHO increased succinate concentrations during week 1 but reduced them during week 2 postpartum. The increase in succinate during week 1 occurred parallel to an increase in Gln for cows fed CHO during this time (CHO x Week:  $P = 0.04$ ; Table 5.3), which might suggest an increase in glutaminolysis (Klein et al., 2013).

In contrast to primiparous cows, there were no main effects of CHO or MET on fumarate or malate for multiparous cows. A MET x CHO x Week interaction ( $P = 0.03$ ; Table 5.4) for multiparous cows indicated that CHO, MET, and CHO + MET had numerically greater fumarate concentrations during week 1 postpartum than CON (12.4 vs. 10.6  $\mu\text{M}$ ). However, during week 2 postpartum, CON had numerically greater fumarate concentrations than each of the three experimental treatment groups (12.9 vs. 11.8  $\mu\text{M}$ ). Furthermore, a tendency for a MET x CHO x Week interaction for malate ( $P = 0.09$ ; Table 5.4) indicated a similar pattern for malate concentrations during week 2 postpartum (4.0  $\mu\text{M}$  for CON vs. 3.7  $\mu\text{M}$  for CHO, MET, and CHO + MET), which could have been a downstream effect of the changes in fumarate concentrations that observed at this time. These results could indicate that MET and CHO altered TCA cycle activity during week 2 postpartum for multiparous cows.

For both primi- and multiparous cows, plasma citrate concentrations increased for primiparous (Week:  $P = 0.04$ ; Table 5.4) and tended to increase for multiparous cows (Week:  $P = 0.10$ ; Table 5.4) from week 1 to week 2. Plasma citrate concentrations observed in this study were similar to those reported by Klein et al. (2013) for Brown Swiss cows in early lactation. Enhanced FA oxidation during the early postpartum period increases TCA cycle activity (Roche et al., 2013); however, due to a sudden influx of FA, there could be a lag until the TCA cycle is fully equipped to oxidize these FA, which could explain the delayed increase in citrate concentrations observed in the current study.

For both primi- and multiparous cows, plasma concentrations of  $\alpha$ -ketoglutarate were greater during week 1 than week 2 postpartum ( $P < 0.01$  and  $P = 0.03$ , respectively; Table 5.4). It has been suggested that  $\alpha$ -ketoglutarate dehydrogenase, a rate-limiting enzyme of the TCA cycle that converts  $\alpha$ -ketoglutarate to succinyl-CoA, is inhibited by oxidative stress (McLain et al., 2011). This theory, in conjunction with the idea that cows are under increased oxidative stress during the early postpartum period (Sordillo and Raphael, 2013), could explain the increase in  $\alpha$ -ketoglutarate during week 1 postpartum. Greater  $\alpha$ -ketoglutarate concentrations after calving could also be reflective of enhanced activities of the alanine and urea cycles at this time.

### ***Plasma Choline Metabolites***

Postpartum plasma choline metabolite concentrations are shown in Table 5.5. Treatment did not affect concentrations of betaine for primi- or multiparous cows. Furthermore, free choline concentrations were not affected by CHO for either group of cows, but MET reduced it ( $P = 0.03$ ; Table 5.5) in multiparous cows. The lack of a CHO

effect on betaine and free choline concentrations was somewhat surprising, given that recent work (de Veth et al., 2016; results presented in Chapter 3) has shown that plasma concentrations of betaine and free choline are most responsive to post-ruminal choline supplied by abomasal infusion. However, in both of those studies, plasma betaine and free choline concentrations were minimally affected by post-ruminal choline supplied in the diet as RPC, which is in agreement with the results described here. It is possible that post-ruminal choline supplied as RPC is absorbed and utilized differently than that supplied by abomasal infusion. Because the reduction in free choline concentrations for multiparous cows fed MET did not coincide with a reduction in betaine concentration, these results do not suggest any MET-induced changes in the BHMT pathway. This idea is further supported by the fact that mRNA expression of *BHMT* was not altered by MET in this study, as discussed later. Alternatively, the MET-induced reduction in free choline concentration could indicate an increase in PC synthesis, although MET did not affect total PC concentrations.

For multiparous cows, CHO tended to increase GPC but decrease SM concentrations ( $P = 0.07$  and  $P = 0.10$ , respectively; Table 5.5). In contrast, these metabolites were not affected by CHO in primiparous cows. De Veth et al. (2016) showed that supplying post-ruminal choline via abomasal infusion or as RPC increased milk GPC concentration and yield, which is consistent with the observations for blood plasma in multiparous cows. Glycerophosphocholine, a choline carrier in milk, is synthesized from PC (Gallazzini and Burg, 2009; Bernhard et al., 2018). Thus, its increase in plasma when CHO was fed could indicate an increase in PC availability to support its synthesis, although total PC concentrations were not affected by CHO. Similar to results for primiparous cows,

previous studies have not shown any changes in SM concentrations when choline is supplied as RPC (de Veth et al., 2016; results presented in Chapter 3).

Total LPC concentrations were not affected by treatment for multiparous cows (Table 5.5), which agrees with results observed for cows supplied with post-ruminal choline via RPC or abomasal infusion (presented in Chapter 3). However, CHO tended to increase LPC 18:1 concentration for multiparous cows ( $P = 0.07$ ; Supplemental Table 5.S1). In contrast, CHO numerically increased total LPC concentrations during week 2 postpartum (CHO x Week interaction:  $P = 0.07$ ; Table 5.5) for primiparous cows. Furthermore, CHO x Week interactions for LPC 16:0, 18:0, 18:1, and 18:2 ( $P = 0.05$ ,  $P = 0.04$ ,  $P = 0.06$ , and  $P = 0.05$ , respectively; Supplemental Table 5.S2) indicated that CHO numerically increased concentrations of each of these species for primiparous cows during week 2 postpartum as well. These results are contrary to previous observations where post-ruminal Cho did not affect concentrations of these LPC species in the blood (presented in Chapter 3). However, that study was conducted using mid- to late-lactation cows, not periparturient cows, and previous work has shown that LPC concentrations vary throughout lactation (Artegoitia et al., 2014). Lysophosphatidylcholines play several roles in the body and can modify gene expression, function in metabolic signaling pathways, and affect cellular mechanisms associated with survival (D'Arrigo and Servi, 2010). Heimerl et al. (2014) showed that in humans, plasma concentrations of certain LPC species, such as LPC 18:0, 18:1, 18:2, 18:3, and 16:0, are lower in obese individuals and that these differences persisted even after weight-loss intervention, suggesting that these metabolites play a role in energy metabolism. Elevation of these compounds by CHO in the current study could have been a direct result of the increased supply of dietary choline. It is also

possible that these changes reflect a mechanism by which dietary choline could modulate changes in body reserves and energy metabolism. In support of this, CHO reduced BCS for multiparous cows during the postpartum period and primiparous cows during the prepartum period (presented in Chapter 4).

Other than PC 16:0/18:2, plasma concentrations of total PC and individual PC species were not affected by treatment for multiparous cows (Supplemental Table 5.S1). A tendency for a MET x CHO x Week interaction ( $P = 0.09$ ) indicated that CHO and MET numerically increased concentrations of PC 16:0/18:2 relative to CON during the second week of lactation (820 vs. 648  $\mu\text{M}$ ), but CHO + MET was similar to CON during this time. This result is contrary to previous observations (presented in Chapter 3), where PC 16:0/18:2 was not affected in mid-to late-lactation cows supplied with post-ruminal choline. DeLong et al. (1999) classified PC origin in rats and suggested that PC species with a high proportion of long chain FA or long chain poly-unsaturated FA (PUFA) are produced via the PEMT pathway and PC species with a high proportion of short or medium chain saturated FA are derived from the CDP-choline pathway. Based on this classification, PC 16:0/18:2 is probably produced via the CDP-choline pathway, and the changes observed in the current study could be indicative of alterations in this pathway.

Concentrations of PC 18:0/18:1 and PC 18:1/22:6 tended to be reduced by CHO for primiparous cows ( $P = 0.07$  and  $P = 0.09$ , respectively; Supplemental Table 5.S2), but total PC concentrations were unaffected. Dietary choline is used for the synthesis of PC via the CDP-choline pathway (Li and Vance, 2008). Thus, it was hypothesized that CHO would enhance PC synthesis via this pathway. The reduction in PC 18:1/22:6

concentration by CHO substantiates this, as this PC species is considered to be derived from the PEMT pathway (Delong et al., 1999). However, a lack of change in other PC species by CHO, specifically those of CDP-pathway origin that contain mostly saturated FA (Delong et al., 1999), do not support this hypothesis.

Feeding MET reduced total PC during week 2 but not week 1 for primiparous cows (MET x Week:  $P = 0.02$ ). In line with this observation, concentrations of several PC species, including PC 16:0/16:0, PC 16:0/16:1, PC 16:0/18:2, PC 16:0/20:3, PC 18:0/18:1, PC 18:0/20:3, and PC 18:0/18:2 + 18:1/18:1, were reduced or tended to be reduced for primiparous cows fed MET during week 2 postpartum (MET x Week interactions: all  $P \leq 0.05$ ; Table 5.S2). These results observed for MET suggest a reduction in the CDP-choline pathway for PC synthesis because most of these species would be considered to be of CDP-choline-pathway origin (Delong et al., 1999). However, MET also reduced or tended to reduce concentrations of several PC species that contained  $\geq 5$  double bonds (PC 18:1/20:4 + 18:0/20:5 + 16:0/22:5, PC 18:1/22:6, PC 18:0/22:5, and PC 18:0/22:6, 18:1/22:5; Table 5.S2) for primiparous cows. These PC species are considered to be of PEMT origin (Delong et al., 1999). Previous research in rats also suggested that dietary Met alters plasma concentrations of various PC species, specifically by reducing those that contain linoleic acid and PC 16:0/20:4 (Sugiyama et al., 1997). For ruminants, it is likely that the majority of PC is synthesized via PEMT because most dietary choline is degraded in the rumen (Atkins et al., 1988), which would conceivably reduce the functionality of the CDP-pathway. The observations of the current study suggest that either the classification system proposed by Delong et al. (1999) is not applicable to ruminants, or that MET was not being used to enhance the PEMT pathway.

For multiparous cows, concentrations of most of the choline metabolites measured were greater during week 2 postpartum than week 1 ( $P \leq 0.05$ ; Table 5.5), although free choline and betaine were reduced over time ( $P = 0.03$  and  $P < 0.01$ , respectively; Table 5.5). Similarly, concentrations of nearly all metabolites were greater or tended to be greater in week 2 ( $P \leq 0.11$ ; Table 5.5) for primiparous cows, with the exception of free choline and GPC, which did not change. These results are consistent with those reported by Artegoitia et al. (2014), who showed increases in plasma concentrations of SM, total LPC, total PC, and GPC, as well as most species of LPC and PC during the first 3 weeks of lactation. Furthermore, Artegoitia et al. (2014) also reported a decrease in plasma betaine concentration from week 1 to week 3 of lactation. Although concentrations of free choline were similar to those reported in the current study, results from Artegoitia et al. (2014) did not indicate any temporal effects for plasma free choline concentrations over the course of lactation, which is contrary to findings for multiparous cows.

### ***Milk Choline Metabolites***

Milk choline metabolite yields are shown in Table 5.6. Results for concentrations of milk choline metabolite and individual PC and LPC species can be found in Supplemental Tables 5.S5 and 5.S6 for multi- and primiparous cows, respectively. Free choline yield and concentration were not affected by treatment for either group of cows. For multiparous cows, CHO reduced milk betaine yield during week 1 postpartum (3629 vs. 4912  $\mu\text{mol/d}$ ), but not week 2 (CHO x Week:  $P = 0.03$ ; Table 5.5). In contrast, CHO tended to increase milk betaine yield ( $P = 0.09$ ; Table 5.5) but not concentration (Table 5.S.6) for primiparous cows. Feeding MET increased betaine concentrations during week

1 (154 vs. 131  $\mu\text{M}$ ), but not week 2 for multiparous cows (MET x Week:  $P = 0.02$ ; Table 5.S5), but MET did not affect milk betaine concentration for primiparous cows (Table 5.S6). Results for milk free choline concentration are similar to results observed for lactating sows fed supplemental choline during the perinatal period (Mudd et al., 2016). In contrast, previous work in mid- to late lactation dairy cattle showed that milk betaine and free choline yields were increased by post-ruminal choline supplied via abomasal infusion (de Veth et al., 2016; results presented in Chapter 3) and RPC (de Veth et al., 2016). However, results reported by de Veth et al. (2016) indicated that milk betaine and free choline responses were much greater when post-ruminal choline was supplied as an abomasal infusion rather than RPC.

During week 2 postpartum, MET tended to increase milk GPC yield for multiparous cows (86,833 vs. 76,599  $\mu\text{mol/d}$ ; MET x Week:  $P = 0.08$ ; Table 5.6), but CHO had no effect. In contrast, milk GPC yield for primiparous cows was not impacted by treatment (Table 5.6). However, a synergistic effect of CHO and MET for milk GPC concentration was observed for primi- and multiparous cows (MET x CHO:  $P = 0.01$  and  $P = 0.02$ , respectively; Tables 5.S5 and 5.S6), whereby CHO + MET had greater GPC concentrations than either CHO or MET. The lack of a CHO effect on milk GPC yield and concentration is inconsistent with research in lactating women, where choline supplementation has been shown to increase milk GPC content (Davenport et al., 2015), and dairy cows, in which post-ruminal choline tended to increase milk GPC yield (de Veth et al., 2016). However, similar to results of the current study, Mudd et al. (2016) did not observe changes in milk GPC content when supplemental choline was provided to lactating sows fed a choline-deficient diet. Because GPC is synthesized from PC, it is logical that its concentration



could be affected by choline supply and that an increase in its concentration might reflect ample availability of PC.

Yields of SM and total LPC were not affected by treatment for multiparous cows. However, feeding CHO increased SM concentration during week 1 but not week 2 postpartum (52.1 vs. 42.1  $\mu\text{M}$ ; CHO x Week,  $P = 0.03$ ; Table 5.S5) which could have been the result of the lower milk yield (~2.8 kg) for multiparous cows fed CHO during this time (presented in Chapter 4). Furthermore, CHO + MET tended to increase overall total LPC concentrations of multiparous cows (MET x CHO,  $P = 0.08$ ; Table 5.S5) which could be attributed to the lower milk yield for these cows (presented in Chapter 4). For primiparous cows, CHO tended to increase SM and total LPC yields during week 2 (964 vs. 844  $\mu\text{mol/d}$  and 100 vs. 82  $\mu\text{mol/d}$ , respectively) but not week 1 (CHO x Week,  $P = 0.06$  and  $P = 0.07$ , respectively; Table 5.S6). This observation for primiparous cows was likely due to the increased milk yield observed for cows fed CHO during week 2 postpartum (presented in Chapter 4), as milk SM and total LPC concentrations were not affected. Similar to these results, choline supplementation in swine during the perinatal period did not affect milk concentrations of SM or LPC during the 3-week lactation period (Mudd et al., 2016). Furthermore, de Veth et al. (2016) and results presented in Chapter 3 also showed no change in milk SM or total LPC yields when post-ruminal choline was provided to lactating cows.

For multiparous cows, CHO increased yield and concentration of LPC 18:0 ( $P = 0.03$  and  $P < 0.01$ , respectively; Tables 5.S3 and 5.S5). Additionally, MET increased concentration and tended to increase yield of LPC 18:0 ( $P < 0.01$  and  $P = 0.08$ ,

respectively; Tables 5.S3 and 5.S5). Although yield was not affected by treatment, there tended to be a positive synergistic effect of CHO + MET on LPC 16:0 concentration (MET x CHO,  $P = 0.09$ ). For primiparous cows, CHO increased yield of LPC 18:1 ( $P = 0.05$ ; Table 5.S4) and tended to increase yield of LPC 16:0 during week 2 postpartum (CHO x Week,  $P = 0.07$ ; Table 5.S4). Because concentrations of these metabolites were not affected by CHO, this result was likely due to increased milk yield by primiparous cows fed CHO (presented in Chapter 4). A significant MET x CHO x Week interaction ( $P = 0.03$ ; Table 5.S4) indicated that CHO + MET increased yield of LPC 18:2 relative to CON during week 2 (6.7 vs. 5.6  $\mu\text{mol/d}$ ) but not week 1. Previous findings did not show any change in milk content or yield of LPC species when post-ruminal choline was supplied to lactating cows (presented in Chapter 3). However, that study examined responses for cows in established lactation, which could explain the discrepancy between observations.

For both primi- and multiparous cows, total PC yield was not affected by treatment. However, CHO tended to increase total PC concentration for multiparous cows ( $P = 0.07$ ; Table 5.S5) but decrease it for primiparous cows ( $P = 0.07$ ; Table 5.S6). This discrepancy is likely explained by the different milk yield responses observed between primi- and multiparous cows fed CHO, as it had a positive effect on milk yield for primiparous cows but no effect for multiparous cows (presented in Chapter 4). The results of the current study are similar to those reported by de Veth et al. (2016) for dairy cows as well as those reported for sows (Mudd et al., 2016) and lactating women (Davenport et al., 2015).

Yields of individual PC species were not affected by treatment for primiparous cows (Table 5.S4). However, for multiparous cows, CHO increased the yield of several

PC species with FA that contained  $\geq 4$  double bonds, including PC 16:0/20:5 + 16:1/20:4, PC 18:1/22:6, PC 18:0/22:6 + PC 18:1/22:5, PC 18:0/22:5 (all  $P \leq 0.02$ ; Table 5.S3). In addition, CHO tended to increase yield of PC 18:0/18:1 ( $P = 0.07$ ; Table 5.S3). These changes all corresponded with increases in concentration (all  $P \leq 0.03$ ; Table 5.S5). Feeding MET also increased the yield of several PUFA-containing PC species, including PC 18:1/22:6, PC 18:0/22:6 + PC 18:1/22:5, PC 18:0/22:5 (all  $P \leq 0.03$ ; Table 5.S4), which also coincided with MET-induced increases in concentration ( $P \leq 0.02$ ; Table 5.S5). Because the majority of these changes involved PC species that contained PUFA, these results suggest, similar to those for plasma discussed previously, that CHO and MET could have altered the balance between of the two PC synthesis pathways. These results are contrary to previous observations for mid- to late lactation cows that received post-ruminal choline (Chapter 3). However, the results of the current study demonstrate that both CHO and MET modify choline metabolism in periparturient dairy cattle, although the significance of these changes remains to be determined.

### ***Liver Gene Expression***

Relative expression of hepatic genes associated with choline, Met, and FA metabolism are shown in Table 5.7. Gene expression was assessed for a subset ( $n = 20$ ) of multiparous cows only. Feeding CHO tended to increase, while MET decreased expression of *PCYT1A* ( $P = 0.10$  and  $P < 0.01$ , respectively). However, a tendency for a CHO x MET interaction ( $P = 0.10$ ) indicated that mRNA expression was only increased when CHO was fed separate from MET (Figure 1). Because the *PCYT1A* gene encodes phosphate cytidylyltransferase 1 choline  $\alpha$ , the enzyme catalyzes the rate-limiting step of the CDP-

choline pathway (Li and Vance, 2008), these results lend support to the hypothesis that supplemental choline increases PC synthesis via the CDP-choline pathway. That providing MET reduced expression of this gene suggests that availability of methyl donors (e.g., SAM or CH<sub>3</sub>-THF) was sufficient to support PC synthesis from phosphatidylethanolamine via the PEMT pathway. Zhou et al., (2017a) also observed an increase in *PCYT1A* mRNA expression for periparturient cows fed choline. However, in contrast to findings of the current study, they showed that supplemental Met also increased *PCYT1A* mRNA expression.

For multiparous cows fed MET, an elevation in *PEMT* mRNA expression would have coincided with some of the increases observed for the milk yield of several PC species that are presumably synthesized via the PEMT pathway. However, there were no treatment effects on mRNA expression of *PEMT* in the current study. Contrary to these results, previous research that examined hepatic mRNA expression of *PEMT* in periparturient dairy cows showed an increase in *PEMT* expression when supplemental Met was fed (Preynat et al., 2010; Osorio et al., 2014; Zhou et al., 2017a).

Similar to previous results (Preynat et al., 2010; Osorio et al., 2014; Zhou et al., 2017a), MET did not affect mRNA expression of *BHMT*. However, expression of *BHMT* tended to be elevated when choline was fed alone (Figure 1; CHO x MET,  $P = 0.09$ ). This result could indicate an increase in Met recycling from Hcy via the BHMT pathway for cows fed CHO without MET. The fact that the elevation in *BHMT* mRNA expression was absent for MET + CHO suggests that when Met supply is sufficient, there is less need to resynthesize Met from Hcy via this pathway. In support of this hypothesis, recent in vitro

work by Zhang et al. (2016) showed a reduction in *BHMT* mRNA expression with increasing concentrations of Met, indicating that when Met is in ample supply, less Met is regenerated from Hcy through the BHMT pathway. However, the observations of the current study are contrary to those reported by Zhou et al. (2017a), who did not observe changes in *BHMT* mRNA expression or activity when choline was supplemented to periparturient cows.

Treatment did not affect the mRNA expression of *MTR* or *GSS*. These results suggest that neither CHO nor MET affected the CH<sub>3</sub>-THF pathway or the production of the antioxidant, glutathione. Contrary to these results, Osorio et al. (2014) showed that Met supplemented during the periparturient period increased mRNA expression of both *MTR* and *GSS* during the first week of lactation. However, similar to findings of the current study, other studies have also shown no effect of choline or Met supplementation during the periparturient period on mRNA expression of *MTR* or *GSS* (Preynat et al., 2010; Zhou et al., 2017a). However, in the study by Zhou et al. (2017a), activity of MTR was reduced by both choline and Met supplementation, which suggests that alterations in MTR function by choline and Met may not be at the transcriptional level. Contrary to this hypothesis, Zhang et al. (2016) showed a reduction in *MTR* mRNA expression for calf hepatocytes cultured with increasing levels of Met. The reduction in MTR activity for cows fed choline or Met observed by Zhou et al. (2017a) corresponds with the hypothesis that when Met is fed, there is less need for Met regeneration from Hcy via the CH<sub>3</sub>-THF pathway and that when choline is fed, there is likely more reliance on Met recycling via the BHMT pathway.

The mRNA expression of *CPT1A*, the protein that is responsible for transporting FA into the mitochondria for oxidation, was also not affected by feeding CHO or MET. These results are similar to those reported by Goselink et al. (2013) and Morrison et al. (2018) for periparturient cows fed choline. Furthermore, Osorio et al. (2016) also showed no change in *CPT1A* expression when Met was supplemented during the periparturient period. Together, these results suggest that neither choline nor Met affect the rate at which FA become available for mitochondrial oxidation. However, enzyme activity or concentration was not measured, so this is only speculative.

The mRNA expression of *HMGCS2* and *PPAR $\alpha$*  tended to be reduced by CHO ( $P = 0.10$  and  $P = 0.08$ ). Similar to results reported by Osorio et al. (2016), MET did not affect mRNA expression of *HMGCS2* or *PPAR $\alpha$*  in the current study. These results would suggest reductions in ketogenesis and hepatic FA oxidation by cows fed choline, although CHO did not affect postpartum plasma BHBA or NEFA concentrations for multiparous cows (presented in Chapter 4). Results from the current study are contrary to previous research that did not show changes in *HMGCS2* (Morrison et al., 2018) or *PPAR $\alpha$*  (Goselink et al., 2013; Morrison et al., 2018) mRNA expression when choline was fed to periparturient cows.

Feeding MET tended to reduce mRNA expression of *DGATI* ( $P = 0.08$ ), suggesting a reduction in liver TG synthesis, although there was no effect of MET on liver TG content (presented in Chapter 4). In contrast, CHO did not alter the expression of *DGATI*. In agreement, Goselink et al. (2013) did not observe changes in the mRNA expression of hepatic *glycerol-3-phosphate O-acetyltransferase 1*, the enzyme that catalyzes the first step

in TG synthesis, when choline was fed to periparturient cows. Taken together, these results suggest no change in the rate of liver TG synthesis when choline is fed before and after parturition.

Contrary to results reported by Goselink et al. (2013), but similar to those reported by Morrison et al. (2018), CHO did not affect mRNA expression of *MTTP* in this study. In contrast to results reported by Preynat et al. (2010), who showed an increase in *MTTP* mRNA expression when Met was fed to periparturient cows, there were no changes in *MTTP* mRNA expression in association with MET in the current study. Microsomal triglyceride transfer protein is an enzyme involved in packaging TG into VLDL in the liver (Bernabucci et al., 2004). An increase in *MTTP* mRNA expression would indicate a potential increase in the rate of VLDL formation, which is the proposed route by which choline could reduce hepatic TG concentration in dairy cows (Cooke et al., 2007). However, results of the current study do not support this, and these results are in line with a lack of a MET or CHO effect on liver TG content (presented in Chapter 4).

## CONCLUSION

The results from this study underscore that choline and Met metabolism are interconnected, and suggest that each affect AA and choline metabolism differently when supplemented during the periparturient period. Results for Met suggest an improvement in AA status to support protein synthesis, while changes in plasma lactate and pyruvate in response to choline suggest potential alterations in muscle glucose metabolism. Changes in the PC profile of both plasma and milk were associated with feeding both choline and Met, and suggest that both nutrients can modulate the balance of PC synthesis via the CDP-

choline and PEMT pathways during the periparturient period. However, responses were quite variable between primi- and multiparous cows, which could reflect differences in choline and Met requirements during this time. Further investigation into the response differences between primi- and multiparous cows fed choline and Met during the periparturient period is warranted.

### **ACKNOWLEDGMENTS**

The author would like to acknowledge the staff the Central Maryland Research and Education Center Dairy Unit for their assistance with animal care and management. The authors are also grateful to Claudia Gomez and Emily Davis (University of Maryland, Department of Animal and Avian Sciences) for providing assistance with sample and data collection. Partial funding for this study was provided by Balchem Corporation (New Hampton, NY).



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**Table 5.1.** Forward and reverse primers used for RT-qPCR

Gene Symbol	Description	GenBank Accession	Primer	Sequence (5' to 3') <sup>1</sup>	Length <sup>2</sup>
<b>Choline and Methionine Metabolism</b>					
<i>BHMT</i>	betaine-homocysteine S-methyltransferase	NM_001011679.1	F	GCTGTATGGGCAGTTGAAGC	70
			R	AATGGCAGTTTACCCCCACG	
<i>GSS</i>	glutathione synthetase	NM_001015630.1	F	GAAGATCGAACCCGAGCCTT	157
			R	GGTTCGAAGCAGATGTCCCA	
<i>MTR</i>	5-methyltetrahydrofolate-homocysteine methyltransferase	NM_001030298.1	F	AGCCTTTCTTCGACGTCTGG	264
	phosphate		R	CGTAGAAGGTGGCTATGGGC	
<i>PCYT1A</i>	cytidyltransferase 1, choline, $\alpha$	NM_001105052.2	F	GCTCCAACACAGAGGACAGAA	384
			R	ACTCTGCCTTGGACTGATGG	
<i>PEMT</i>	phosphatidylethanolamine N-methyltransferase	NM_182989.3	F	TCCGAGCGGGCATGACAAC	197
			R	CAAAGTGGGGTTCCGAGAGGT	
<b>Lipid Metabolism</b>					
<i>CPT1A</i>	carnitine palmitoyltransferase 1A	NM_001304989.1	F	TCGTCACCATGCGTTACTCC	131
			R	GGGTTTTTCGGCCTGAGAAGA	
<i>DGAT1</i>	diacylglycerol O-acyltransferase 1	NM_174693	F	TGGCCTTTCTCCTCGAGTCT	214
			R	TAGGTCAGGTTGTCGGGGTA	
<i>HMGCS2</i>	3-hydroxy-3-methylglutaryl-CoA synthase 2	NM_001045883.1	F	GCAACACTGACATTGAGGGC	125
			R	ACAGACCACCAGTGCATAGC	
<i>MTTP</i>	microsomal triglyceride transfer protein	NM_001101834.1	F	GCTTCAGCTGCAATCTGGAC	157
			R	CACCGTGATGCCACCAGTTA	
<i>PPAR<math>\alpha</math></i>	peroxisome proliferator activated receptor alpha	NM_001034036.1	F	CCGAGGAGTCATCCAGCATC	129
			R	TCAGCCGAATCGTTCTCCTAAA	
<b>Housekeeping</b>					
<i>PGK1</i>	phosphoglycerate kinase 1	NM_001034299.1	F	GAATGGGAAGCTTTTGCCCG	176
			R	AGCTCTAAACTGGCACCACC	

<sup>1</sup>Primer direction (F=Forward; R=Reverse).<sup>2</sup>Amplicon length in base pairs.

**Table 5.2.** Postpartum plasma amino acid and organic acid concentrations ( $\mu\text{M}$ ) for multiparous cows ( $n = 27$ ) fed the control (CON) diet or the control diet plus RPC (CHO), RPM (MET), or both (CHO + MET)<sup>1,2</sup>

Item	Treatment				SEM	Week Postpartum			P-value			
	CON	CHO	MET	CHO + MET		1	2	SEM	CHO	MET	MET x CHO	Week
Alanine	234	194	215	216	17.4	196	228	9.18	0.38	0.67	0.42	<0.01
Aspartate	4.63	3.90	4.44	4.36	0.35	4.30	4.36	0.20	0.22	0.71	0.33	0.80
Glutamate <sup>‡‡</sup>	34.8	30.5	36.5	34.2	2.29	33.6	34.4	1.24	0.13	0.26	0.63	0.55
Glutamine	54.8	49.5	54.0	59.1	10.5	52.9	55.8	5.3	0.99	0.66	0.61	0.54
Glycine	633	591	747	662	109	714	603	68.7	0.54	0.38	0.84	0.24
Histidine	63.0	59.7	66.7	65.5	4.11	62.5	65.0	2.26	0.57	0.24	0.80	0.29
Isoleucine <sup>†</sup>	196	198	224	179	19.7	195	204	12.3	0.26	0.79	0.22	0.56
Leucine <sup>†</sup>	186	172	213	161	15.7	177	189	9.72	0.04	0.58	0.21	0.37
Lysine	76.8	68.2	88.3	74.4	7.61	74.5	79.3	4.46	0.12	0.23	0.72	0.39
Methionine	23.1	21.8	35.3	34.6	2.30	29.0	28.4	1.26	0.65	<0.01	0.90	0.68
Phenylalanine	53.7	56.9	58.7	48.6	2.76	54.0	55.0	1.64	0.20	0.54	0.03	0.63
Proline	99.8	89.2	100.1	95.8	8.63	91	102	4.55	0.38	0.68	0.72	0.02
Serine <sup>††</sup>	126	124	108	118	7.40	114	125	5.34	0.57	0.12	0.45	0.20
Threonine	91.6	91.3	98.4	97.5	10.5	89.6	99.9	5.49	0.96	0.52	0.98	0.04
Tyrosine	43.6	44.8	46.6	43.2	3.74	39.9	49.2	2.03	0.75	0.84	0.54	<0.01
Valine <sup>††</sup>	257	248	301	236	23.7	247	274	13.7	0.11	0.49	0.24	0.11
BCAA <sup>3†</sup>	639	618	737	578	58.5	618	667	35.3	0.11	0.60	0.23	0.29
EAA <sup>3</sup>	973	908	1120	927	77.0	958	1006	48.2	0.10	0.30	0.43	0.41

<sup>1</sup>Prepartum, cows were supplemented with 60 g/d RPC (ReaShure<sup>®</sup>, Balchem Corp., New Hampton, NY; CHO), 12 g/d RPM (Smartamine<sup>®</sup> M; Adisseo USA, Inc., Alpharetta, GA; MET), or both (CHO + MET). Postpartum, cows were supplemented with 60 g/d RPC, 18 g/d RPM, or both. Cows were monitored and treatments were applied from 21 days before expected calving through 35 DIM.

<sup>2</sup>Blood samples were obtained during week 1 and week 2 postpartum.

<sup>3</sup>BCAA = branched chain AA; EAA = essential AA.

<sup>††</sup>MET x Week interaction,  $P \leq 0.05$ ; <sup>†</sup>MET x Week interaction,  $P \leq 0.10$ ; <sup>‡‡</sup>MET x CHO x Week interaction,  $P \leq 0.05$ .

**Table 5.3.** Postpartum plasma amino acid concentrations ( $\mu\text{M}$ ) for primiparous cows ( $n = 24$ ) fed the control (CON) diet or the control diet plus RPC (CHO), RPM (MET), or both (CHO + MET)<sup>1, 2</sup>

Item	Treatment				Week Postpartum			P-value				
	CON	CHO	MET	CHO + MET	SEM	1	2	SEM	CHO	MET	MET x CHO	Week
Alanine	191	213	209	209	15.2	196	215	8.98	0.44	0.63	0.45	0.14
Aspartate	4.96	5.51	5.50	4.69	0.42	5.15	4.94	0.22	0.78	0.32	0.24	0.42
Glutamate	45.1	46.4	47.2	45.2	2.83	47	45	1.68	0.90	0.86	0.54	0.39
Glutamine**	72.4	78.1	77.3	87.3	7.4	81	77	4.7	0.25	0.33	0.76	0.50
Glycine*	725	989	638	562	166	757	700	93.5	0.53	0.12	0.26	0.64
Histidine	64.9	67.5	63.7	64.8	3.11	64.5	65.9	2.05	0.52	0.50	0.80	0.64
Isoleucine	161	171	184	176	16.1	163	183	9.05	0.95	0.35	0.52	0.08
Leucine	158	164	170	159	15.1	155	171	8.17	0.88	0.79	0.53	0.09
Lysine	63.5	71.0	74.1	76.6	9.56	65.7	76.9	4.74	0.55	0.32	0.78	0.04
Methionine	22.2	24.1	36.2	35.3	2.21	29.4	29.5	1.15	0.80	<0.01	0.49	0.90
Phenylalanine	51.3	56.2	53.1	49.8	2.69	53.3	51.9	1.67	0.75	0.35	0.12	0.55
Proline	87.2	96.3	88.2	94.2	7.06	86.2	96.8	3.86	0.26	0.94	0.81	0.02
Serine	113	127	106	95	7.85	111	110	4.72	0.90	0.01	0.08	0.96
Threonine	75.8	77.6	70.7	69.7	7.0	64.7	82.2	3.90	0.95	0.31	0.84	<0.01
Tyrosine†	41.6	41.5	40.3	34.6	3.69	35.8	43.2	2.15	0.40	0.23	0.40	0.01
Valine	225	219	231	216	18.5	205	241	9.92	0.55	0.93	0.77	<0.01
BCAA <sup>3</sup>	543	556	585	552	48.5	523	595	26.3	0.82	0.67	0.61	0.03
EAA <sup>3</sup>	829	861	895	909	96.7	820	927	46.0	0.80	0.48	0.91	0.04

<sup>1</sup>Prepartum, cows were supplemented with 60 g/d RPC (ReaShure<sup>®</sup>, Balchem Corp., New Hampton, NY; CHO), 12 g/d RPM (Smartamine<sup>®</sup> M; Adisseo USA, Inc., Alpharetta, GA; MET), or both (CHO + MET). Postpartum, cows were supplemented with 60 g/d RPC, 18 g/d RPM, or both. Cows were monitored and treatments were applied from 21 days before expected calving through 35 DIM.

<sup>2</sup>Blood samples were obtained during week 1 and week 2 postpartum.

<sup>3</sup>BCAA = branched chain AA; EAA = essential AA.

\*\*CHO x Week interaction,  $P \leq 0.05$ ; \*CHO x Week interaction,  $P \leq 0.10$ ; †MET x Week interaction,  $P \leq 0.10$ .



**Table 5.4.** Postpartum plasma organic acid concentrations ( $\mu\text{M}$ ) for multiparous ( $n = 27$ ) and primiparous cows ( $n = 24$ ) fed the control (CON) diet or the control diet plus RPC (CHO), RPM (MET), or both (CHO + MET)<sup>1,2</sup>

Item	Treatment				SEM	Week Postpartum			P-value			
	CON	CHO	MET	CHO + MET		1	2	SEM	CHO	MET	MET x CHO	Week
<b>Multiparous</b>												
Citrate	667	619	709	818	76.0	674	733	39.0	0.67	0.11	0.29	0.10
Fumarate <sup>‡‡</sup>	11.9	12.0	12.2	12.2	0.67	12.1	12.1	0.37	0.92	0.73	0.96	0.94
Lactate	1053	570	1089	671	181	936	756	102	<0.01	0.64	0.86	0.25
Malate <sup>‡</sup>	4.27	3.91	4.08	3.85	0.26	4.12	3.94	0.15	0.27	0.61	0.80	0.34
Oxaloacetate <sup>††</sup>	42.0	36.1	55.8	29.8	6.58	37.6	44.2	3.45	0.01	0.52	0.14	0.11
Pyruvate	66.4	36.5	50.5	50.4	7.20	52.3	49.7	4.24	0.02	0.88	0.04	0.68
Succinate	70.4	69.3	70.0	69.7	0.71	70.0	69.8	0.42	0.30	0.99	0.53	0.65
a-ketoglutarate	10.15	9.65	9.21	9.63	0.57	10.0	9.3	0.28	0.94	0.37	0.37	0.03
<b>Primiparous</b>												
Citrate	710	729	787	725	50	707	768	26.6	0.64	0.45	0.41	0.04
Fumarate	10.4	12.1	10.7	11.8	0.65	11.4	11.1	0.28	0.02	0.98	0.58	0.31
Lactate	903	654	601	752	152	743	712	82.1	0.73	0.47	0.17	0.74
Malate	3.59	4.26	3.62	4.13	0.32	4.04	3.76	0.15	0.05	0.84	0.75	0.08
Oxaloacetate <sup>†</sup>	41.5	45.8	43.9	39.9	6.45	39.1	46.4	3.91	0.98	0.77	0.49	0.18
Pyruvate	51.9	46.6	42.7	51.9	3.72	51.0	45.6	2.71	0.59	0.58	0.05	0.22
Succinate <sup>*</sup>	70.8	71.2	70.6	70.0	0.53	70.6	70.7	0.28	0.82	0.16	0.30	0.88
a-ketoglutarate	10.10	11.17	10.82	10.60	0.95	11.1	10.3	0.38	0.63	0.92	0.40	<0.01

<sup>1</sup>Prepartum, cows were supplemented with 60 g/d RPC (ReaShure<sup>®</sup>, Balchem Corp., New Hampton, NY; CHO), 12 g/d RPM (Smartamine<sup>®</sup> M; Adisseo USA, Inc., Alpharetta, GA; MET), or both (CHO + MET). Postpartum, cows were supplemented with 60 g/d RPC, 18 g/d RPM, or both. Cows were monitored and treatments were applied from 21 days before expected calving through 35 DIM.

<sup>2</sup>Blood samples were obtained during week 1 and week 2 postpartum.

\*CHO x Week interaction,  $P \leq 0.10$ ; <sup>††</sup>MET x Week interaction,  $P \leq 0.05$ ; <sup>‡</sup>MET x Week interaction,  $P \leq 0.10$ ; <sup>‡‡</sup>MET x CHO x Week interaction,  $P \leq 0.05$ ; <sup>‡</sup>MET x CHO x Week interaction,  $P \leq 0.10$ .

**Table 5.5.** Postpartum plasma choline metabolite concentrations ( $\mu\text{M}$ ) for multiparous ( $n = 27$ ) and primiparous cows ( $n = 24$ ) fed the control (CON) diet or the control diet plus RPC (CHO), RPM (MET), or both (CHO + MET)<sup>1, 2</sup>

Item <sup>3</sup>	Treatment				SEM	Week Postpartum			P-value			Week
	CON	CHO	MET	CHO + MET		1	2	SEM	CHO	MET	MET x CHO	
<b>Multiparous</b>												
Betaine	49.7	41.0	42.3	44.8	4.75	49.7	39.2	2.41	0.49	0.69	0.21	<0.01
Free Choline	4.27	4.85	4.10	3.63	0.32	4.54	3.88	0.20	0.85	0.03	0.11	0.03
GPC	11.9	20.6	11.5	17.2	3.79	11.2	19.4	2.55	0.07	0.60	0.69	0.03
SM	325	280	329	282	29.6	281	327	14	0.10	0.91	0.99	<0.01
Total LPC	249	319	245	255	32.0	217	317	21.9	0.23	0.28	0.36	<0.01
Total PC	2328	2778	2687	2389	276	2185	2906	163	0.78	0.96	0.18	<0.01
<b>Primiparous</b>												
Betaine	52.6	51.2	45.8	48.3	6.50	52.7	46.2	3.20	0.92	0.42	0.75	0.03
Free Choline	4.18	3.85	4.68	4.29	0.33	4.34	4.16	0.20	0.26	0.17	0.92	0.48
GPC <sup>‡</sup>	10.5	14.0	13.6	14.9	2.59	13.1	13.4	1.92	0.36	0.46	0.69	0.90
SM	298	298	308	303	26.61	261	343	13	0.92	0.77	0.93	<0.01
Total LPC <sup>*</sup>	230	269	246	226	25.13	219	267	14.9	0.67	0.57	0.22	0.02
Total PC <sup>††</sup>	2574	2337	2316	2151	221	1923	2766	124	0.34	0.29	0.86	<0.01

<sup>1</sup>Prepartum, cows were supplemented with 60 g/d RPC (ReaShure<sup>®</sup>, Balchem Corp., New Hampton, NY; CHO), 12 g/d RPM (Smartamine<sup>®</sup> M; Adisseo USA, Inc., Alpharetta, GA; MET), or both (CHO + MET). Postpartum, cows were supplemented with 60 g/d RPC, 18 g/d RPM, or both. Cows were monitored and treatments were applied from 21 days before expected calving through 35 DIM.

<sup>2</sup>Blood samples were obtained during week 1 and week 2 postpartum.

<sup>3</sup>GPC = glycerophosphocholine; SM = sphingomyelin; LPC = lysophosphatidylcholine; PC = phosphatidylcholine.

\*CHO x Week interaction,  $P \leq 0.10$ .

††MET x Week interaction,  $P \leq 0.05$ .

‡MET x CHO x Week interaction,  $P \leq 0.10$ .

**Table 5.6.** Milk choline metabolite yields ( $\mu\text{mol/d}$ ) for multiparous ( $n = 27$ ) and primiparous cows ( $n = 24$ ) fed the control (CON) diet or the control diet plus RPC (CHO), RPM (MET), or both (CHO + MET)<sup>1, 2</sup>

Item <sup>3</sup>	Treatment				SEM	Week Postpartum			P-value			
	CON	CHO	MET	CHO + MET		1	2	SEM	CHO	MET	MET x CHO	Week
<b>Multiparous</b>												
Betaine**	3957	3437	3993	2982	377	4270	2914	213	0.05	0.57	0.50	<0.01
Free Choline	2749	1923	1480	1221	754	1515	2172	361	0.45	0.18	0.70	<0.01
GPC <sup>†</sup>	74791	74847	75579	80981	4884	71383	81716	2992	0.56	0.47	0.57	0.01
SM	1271	1444	1363	1277	91	1395	1283	60.2	0.62	0.67	0.15	0.19
Total LPC	101	99	107	107	10.2	93.6	114	5.98	0.96	0.47	0.94	<0.01
Total PC	4211	4636	3839	4491	424	4108	4480	293	0.20	0.53	0.78	0.38
<b>Primiparous</b>												
Betaine	2497	3239	2534	2815	294	3399	2143	173	0.09	0.50	0.43	<0.01
Free Choline	1061	1212	1049	1158	104	804	1435	67.0	0.21	0.74	0.83	<0.01
GPC	45550	44377	43049	49441	4143	43457	47751	2323	0.52	0.75	0.36	0.07
SM*‡	910	948	855	876	87	890	904	48.5	0.73	0.47	0.92	0.77
Total LPC*	71.7	101	84.1	73.2	9.1	73.8	91.2	5.13	0.31	0.39	0.04	<0.01
Total PC	2887	2730	2966	2354	391	2658	2811	243	0.32	0.70	0.55	0.61

<sup>1</sup>Prepartum, cows were supplemented with 60 g/d RPC (ReaShure<sup>®</sup>, Balchem Corp., New Hampton, NY; CHO), 12 g/d RPM (Smartamine<sup>®</sup> M; Adisseo USA, Inc., Alpharetta, GA; MET), or both (CHO + MET). Postpartum, cows were supplemented with 60 g/d RPC, 18 g/d RPM, or both. Cows were monitored and treatments were applied from 21 days before expected calving through 35 DIM.

<sup>2</sup>Milk samples were obtained during week 1 and week 2 postpartum.

<sup>3</sup>GPC = glycerophosphocholine; SM = sphingomyelin; LPC = lysophosphatidylcholine; PC = phosphatidylcholine.

\*\*CHO x Week interaction,  $P \leq 0.05$ .

\*CHO x Week interaction,  $P \leq 0.10$ .

<sup>†</sup>MET x Week interaction,  $P \leq 0.10$ .

<sup>‡</sup>MET x CHO x Week interaction,  $P \leq 0.10$ .

**Table 5.7.** Postpartum covariate-adjusted gene expression fold-changes relative to CON for multiparous cows ( $n = 20$ ) fed the control (CON) diet or the control diet plus RPC (CHO), RPM (MET), or both (CHO + MET)<sup>1,2</sup>

Gene Symbol <sup>3</sup>	Treatment					P-value		
	CON	CHO	MET	CHO + MET	SEM	CHO	MET	MET x CHO
Choline & Methionine Metabolism								
<i>BHMT</i>	0.94	2.10	1.07	1.03	0.36	0.13	0.17	0.09
<i>GSS</i>	1.52	1.73	1.59	1.51	0.31	0.72	0.69	0.43
<i>MTR</i>	1.01	1.38	0.82	0.79	0.26	0.51	0.14	0.42
<i>PCYT1A</i>	0.96	1.65	0.65	0.66	0.20	0.10	<0.01	0.10
<i>PEMT</i>	1.60	2.01	1.80	1.66	0.46	0.65	0.79	0.32
Lipid Metabolism								
<i>CPT1A</i>	1.04	1.37	1.01	0.66	0.26	0.97	0.16	0.18
<i>DGAT1</i>	1.01	1.21	0.71	0.71	0.22	0.64	0.08	0.66
<i>HMGCS2</i>	1.04	0.52	0.82	0.51	0.25	0.10	0.64	0.69
<i>MTTP</i>	1.00	1.23	1.01	0.88	0.25	0.82	0.48	0.44
<i>PPAR<math>\alpha</math></i>	0.93	0.63	1.16	0.72	0.20	0.08	0.43	0.75

<sup>1</sup>Prepartum, cows were supplemented with 60 g/d RPC (ReaShure<sup>®</sup>, Balchem Corp., New Hampton, NY; CHO), 12 g/d RPM (Smartamine<sup>®</sup> M; Adisseo USA, Inc., Alpharetta, GA; MET), or both (CHO + MET). Postpartum, cows were supplemented with 60 g/d RPC, 18 g/d RPM, or both. Cows were monitored and treatments were applied from 21 days before expected calving through 35 DIM.

<sup>2</sup>Prepartum (-21 d relative to calving) gene expression fold-change was used as a covariate in the statistical model.

<sup>3</sup>BHMT = betaine-homocysteine S-methyltransferase; GSS = glutathione synthetase; MTR = 5-methyltetrahydrofolate-homocysteine methyltransferase; PCYT1A = phosphate cytidyltransferase 1, choline,  $\alpha$ ; PEMT = phosphatidylethanolamine N-methyltransferase; CPT1A = carnitine palmitoyltransferase 1A; DGAT1 = diacylglycerol O-acyltransferase 1; HMGCS2 = 3-hydroxy-3-methylglutaryl-CoA synthase 2; MTTP = microsomal triglyceride transfer protein; PPAR $\alpha$  = peroxisome proliferator activated receptor  $\alpha$ .

**Table 5.S1.** Postpartum plasma concentrations ( $\mu\text{M}$ ) of lysophosphatidylcholine (LPC) and phosphatidylcholine (PC) species for multiparous cows ( $n = 27$ ) fed control (CON) diet or control diet plus RPC (CHO), RPM (MET), or both (CHO + MET)<sup>1,2</sup>

Item	Treatment				SEM	Week Postpartum			P-value			Week
	CON	CHO	MET	CHO + MET		1	2	SEM	CHO	MET	MET x CHO	
Lysophosphatidylcholine Species												
LPC 16:0	68.1	85.2	70.0	69.7	8.32	65.4	81.1	4.64	0.27	0.37	0.26	0.02
LPC 18:2	128	169	124	133	21.1	108	169	12.6	0.21	0.30	0.40	<0.01
LPC 18:1	4.28	6.43	4.94	5.03	0.63	4.18	6.16	0.39	0.07	0.53	0.10	<0.01
LPC 18:0	43.8	56.0	43.8	50.0	6.75	40.4	55.0	4.00	0.25	0.81	0.81	<0.01
Phosphatidylcholine Species												
PC 16:0/16:1	16.8	15.9	17.4	14.2	2.64	14.2	17.9	1.43	0.40	0.83	0.63	0.03
PC 16:0/16:0	14.3	16.7	16.0	12.4	2.09	12.5	17.1	1.27	0.78	0.49	0.14	0.02
PC 16:0/18:2 <sup>‡</sup>	588	732	671	526	69.8	544	715	35.1	0.99	0.35	0.03	<0.01
PC 16:0/18:1	274	288	269	251	28.1	251	290	15.4	0.94	0.44	0.54	0.04
PC 16:0/20:5,16:1/20:4	5.16	6.47	5.73	5.83	1.09	5.23	6.37	0.66	0.49	0.97	0.55	0.22
PC 16:0/20:4	101	130	113	95	15.9	97	122	9.04	0.70	0.45	0.12	0.05
PC 16:0/20:3	181	216	237	201	26.0	177	240	13.8	0.98	0.42	0.16	<0.01
PC 18:0/18:2,18:1/18:1	583	712	719	631	75.1	552	771	41.3	0.78	0.69	0.13	<0.01
PC 18:0/18:1	211	217	221	235	26.9	191	250	15.4	0.69	0.59	0.87	<0.01
PC 16:0/22:6,18:1/22:5,18:2/20:4	10.8	13.7	13.4	11.7	2.27	11.5	13.3	1.31	0.76	0.91	0.28	0.33
PC 18:1/20:4,18:0/20:5,16:0/22:5	67.4	83.6	80.3	73.9	13.5	67.8	84.8	7.74	0.69	0.89	0.37	0.11
PC 18:0/20:4	132	148	164	147	29.1	121	174	17.7	0.97	0.56	0.55	0.05
PC 18:0/20:3	99.4	92.4	141	114	25.8	85	139	15.0	0.48	0.20	0.68	<0.01
PC 18:1/22:6	2.88	3.88	3.36	3.26	0.52	2.99	3.70	0.34	0.37	0.89	0.28	0.14
PC 18:0/22:6,18:1/22:5	10.3	12.5	11.2	11.9	2.49	10.2	12.8	1.50	0.51	0.96	0.75	0.22

PC 18:0/22:5	39.7	48.6	48.0	47.5	9.36	37.3	54.7	5.75	0.62	0.66	0.59	0.05
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<sup>1</sup>Prepartum, cows were supplemented with 60 g/d RPC (ReaShure<sup>®</sup>, Balchem Corp., New Hampton, NY; CHO), 12 g/d RPM (Smartamine<sup>®</sup> M; Adisseo USA, Inc., Alpharetta, GA; MET), or both (CHO + MET). Postpartum, cows were supplemented with 60 g/d RPC, 18 g/d RPM, or both. Cows were monitored and treatments were applied from 21 days before expected calving through 35 DIM.

<sup>2</sup>Blood samples were obtained during week 1 and week 2 postpartum.

<sup>‡</sup>MET x CHO x Week interaction,  $P \leq 0.10$ .

**Table 5.S2.** Postpartum plasma concentrations ( $\mu\text{M}$ ) of lysophosphatidylcholine (LPC) and phosphatidylcholine (PC) species for primiparous cows ( $n = 24$ ) fed control (CON) diet or control diet plus RPC (CHO), RPM (MET), or both (CHO + MET)<sup>1, 2</sup>

Item	Treatment				SEM	Week Postpartum			P-value			
	CON	CHO	MET	CHO + MET		1	2	SEM	CHO	MET	MET x CHO	Week
Lysophosphatidylcholine Species												
LPC 16:0**	73.3	79.6	73.0	68.1	6.12	68.6	78.4	4.03	0.89	0.32	0.33	0.11
LPC 18:2**	102	121	120	111	12.8	98	129	7.7	0.70	0.77	0.26	<0.01
LPC 18:1*	3.94	4.80	4.29	4.08	0.48	3.65	4.91	0.29	0.46	0.69	0.24	<0.01
LPC 18:0**	46.8	51.5	52.7	43.1	5.70	43.1	53.9	3.17	0.64	0.81	0.19	<0.01
Phosphatidylcholine Species												
PC 16:0/16:1††	16.6	19.5	16.8	15.1	2.69	14.4	19.7	1.41	0.81	0.41	0.35	<0.01
PC 16:0/16:0††	14.5	15.8	14.3	12.1	2.01	11.7	16.7	1.12	0.80	0.31	0.38	<0.01
PC 16:0/18:2††	558	541	518	492	58.8	439	615	30.1	0.70	0.42	0.93	<0.01
PC 16:0/18:1†	307	275	303	276	32.2	261	320	16.9	0.33	0.98	0.93	<0.01
PC 16:0/20:5,16:1/20:4	5.51	5.21	5.29	3.42	1.02	4.28	5.43	0.57	0.25	0.33	0.40	0.11
PC 16:0/20:4†	99	100	90	84	11.2	77	110	6.63	0.80	0.25	0.76	<0.01
PC 16:0/20:3††	204	182	190	176	21.8	149	227	11.5	0.39	0.61	0.84	<0.01
PC 18:0/18:2,18:1/18:1††	657	554	583	551	64.4	466	707	34.1	0.27	0.52	0.55	<0.01
PC 18:0/18:1††	289	227	259	238	23.2	216	290	13.4	0.07	0.66	0.36	<0.01
PC 16:0/22:6,18:1/22:5,18:2/20:4	16.9	14.2	15.8	12.3	2.49	12.6	17.0	1.43	0.19	0.52	0.85	0.02
PC 18:1/20:4,18:0/20:5,16:0/22:5	87.2	86.8	69.7	64.1	8.46	62.8	91.1	6.11	0.70	0.03	0.74	<0.01
PC 18:0/20:4†	135	123	128	104	21.1	99	145	12.3	0.37	0.52	0.76	<0.01
PC 18:0/20:3††	99.8	89.8	89	79	16.1	69	110	9.0	0.51	0.47	1.00	<0.01
PC 18:1/22:6	4.27	3.51	3.24	2.84	0.35	2.85	4.08	0.26	0.09	0.03	0.60	<0.01
PC 18:0/22:6,18:1/22:5	20.6	19.2	18.6	12.6	2.69	14.7	20.8	1.75	0.15	0.11	0.37	0.03

PC 18:0/22:5                      62.0      58.7      53.1      33.7      9.36      41.7      62.0      5.66      0.19      0.08      0.35      0.02

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<sup>1</sup>Prepartum, cows were supplemented with 60 g/d RPC (ReaShure<sup>®</sup>, Balchem Corp., New Hampton, NY; CHO), 12 g/d RPM (Smartamine<sup>®</sup> M; Adisseo USA, Inc., Alpharetta, GA; MET), or both (CHO + MET). Postpartum, cows were supplemented with 60 g/d RPC, 18 g/d RPM, or both. Cows were monitored and treatments were applied from 21 days before expected calving through 35 DIM.

<sup>2</sup>Blood samples were obtained during week 1 and week 2 postpartum.

\*\*CHO x Week interaction,  $P \leq 0.05$ ; \*CHO x Week interaction,  $P \leq 0.10$ ; ††MET x Week interaction,  $P \leq 0.05$ ; †MET x Week interaction,  $P \leq 0.10$ ; ‡MET x CHO x Week interaction,  $P \leq 0.10$ .



**Table 5.S3.** Milk yields ( $\mu\text{mol/d}$ ) of lysophosphatidylcholine (LPC) and phosphatidylcholine (PC) species for multiparous cows ( $n = 27$ ) fed the control (CON) diet or the control diet plus RPC (CHO), RPM (MET), or both (CHO + MET)<sup>1,2</sup>

Item	Treatment				SEM	Week Postpartum			P-value			
	CON	CHO	MET	CHO + MET		1	2	SEM	CHO	MET	CHO x MET	Week
Lysophosphatidylcholine Species												
LPC 16:0	79.2	76.3	82.1	81.7	8.73	72.9	86.7	5.03	0.84	0.63	0.88	0.02
LPC 18:2	9.9	8.0	10.7	8.99	1.13	7.5	11.3	0.68	0.11	0.44	0.90	<0.01
LPC 18:1	1.55	2.08	1.76	1.63	0.20	1.42	2.10	0.12	0.32	0.55	0.11	<0.01
LPC 18:0	10.6	13.6	13.1	14.9	1.07	12.5	13.7	0.75	0.03	0.08	0.60	0.28
Phosphatidylcholine Species												
PC 16:0/16:1	164	169	144	167	17.6	140	181	12.8	0.43	0.51	0.60	0.04
PC 16:0/16:0	571	574	540	585	55.0	511	624	39.9	0.65	0.85	0.70	0.07
PC 16:0/18:2	474	568	442	480	54	479	504	34.8	0.22	0.26	0.60	0.59
PC 16:0/18:1	1309	1512	1202	1371	136	1307	1390	96.2	0.17	0.35	0.90	0.56
PC 16:0/20:5,16:1/20:4	0.12	0.24	0.15	0.39	0.07	0.33	0.12	0.05	0.01	0.18	0.32	0.02
PC 16:0/20:4	147	160	137	138	13.9	142	149	9.81	0.59	0.25	0.63	0.60
PC 16:0/20:3	359	407	358	391	39.7	370	387	26.8	0.30	0.83	0.84	0.64
PC 18:0/18:2,18:1/18:1	767	840	676	800	84.8	766	776	55.7	0.24	0.43	0.76	0.89
PC 18:0/18:1	262	304	263	324	28.2	304	273	20.1	0.07	0.72	0.73	0.31
PC 16:0/22:6,18:1/22:5,18:2/20:4	14.1	17.0	15.5	18.4	1.93	17.2	15.3	1.42	0.14	0.46	1.00	0.39
PC 18:1/20:4,18:0/20:5,16:0/22:5	45.3	52.2	45.7	55.1	5.12	51.7	47.5	3.80	0.11	0.74	0.80	0.48
PC 18:0/20:4	29.3	35.1	34.8	41.2	4.12	37.6	32.7	2.80	0.14	0.16	0.94	0.22
PC 18:0/20:3	10.6	12.6	13.1	14.5	1.65	13.7	11.7	1.02	0.29	0.17	0.85	0.11
PC 18:1/22:6**	1.66	2.41	2.56	4.02	0.45	2.79	2.54	0.30	0.02	0.01	0.42	0.56
PC 18:0/22:6,18:1/22:5	3.36	4.64	4.16	6.82	0.67	5.23	4.26	0.52	<0.01	0.03	0.29	0.24

PC 18:0/22:5 <sup>††</sup>	2.97	4.40	4.20	6.61	0.63	5.64	3.45	0.46	<0.01	0.01	0.43	<0.01
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<sup>1</sup>Prepartum, cows were supplemented with 60 g/d RPC (ReaShure<sup>®</sup>, Balchem Corp., New Hampton, NY; CHO), 12 g/d RPM (Smartamine<sup>®</sup> M; Adisseo USA, Inc., Alpharetta, GA; MET), or both (CHO + MET). Postpartum, cows were supplemented with 60 g/d RPC, 18 g/d RPM, or both. Cows were monitored and treatments were applied from 21 days before expected calving through 35 DIM.

<sup>2</sup>Milk samples were obtained during week 1 and week 2 postpartum.

\*\*CHO x Week interaction,  $P \leq 0.05$ ; <sup>††</sup>MET x Week interaction,  $P \leq 0.05$ ; <sup>†</sup>MET x Week interaction,  $P \leq 0.10$ .

**Table 5.S4.** Milk yields ( $\mu\text{mol/d}$ ) of lysophosphatidylcholine (LPC) and phosphatidylcholine (PC) species for primiparous cows ( $n = 24$ ) fed the control (CON) diet or the control diet plus RPC (CHO), RPM (MET), or both (CHO + MET)<sup>1,2</sup>

Item	Treatment				SEM	Week Postpartum			P-value			
	CON	CHO	MET	CHO + MET		1	2	SEM	CHO	MET	CHO x MET	Week
Lysophosphatidylcholine Species												
LPC 16:0*	58.1	81.5	67.1	57.7	7.87	59.7	72.5	4.4	0.97	0.35	0.05	<0.01
LPC 18:2**	4.59	5.38	4.88	5.39	0.58	4.15	5.97	0.31	0.27	0.80	0.81	<0.01
LPC 18:1	0.97	1.11	0.97	1.33	0.12	0.96	1.23	0.07	0.05	0.36	0.35	<0.01
LPC 18:0	8.9	12.9	10.7	8.3	1.30	9.34	11.1	0.74	0.53	0.28	0.02	0.03
Phosphatidylcholine Species												
PC 16:0/16:1	128	112	119	86.4	15.4	97.8	124	10.5	0.12	0.25	0.59	0.08
PC 16:0/16:0	378	366	380	294	53.1	324	385	35.2	0.35	0.49	0.48	0.22
PC 16:0/18:2	286	270	261	209	42.8	249	264	25.9	0.43	0.31	0.66	0.64
PC 16:0/18:1	916	899	930	752	137	852	896	85.1	0.47	0.62	0.55	0.68
PC 16:0/20:5,16:1/20:4	0.17	0.07	0.13	0.26	0.09	0.25	0.06	0.06	0.90	0.41	0.21	0.02
PC 16:0/20:4	79.7	78.4	81.0	65.3	12.8	75.1	77.1	7.69	0.50	0.64	0.57	0.82
PC 16:0/20:3	218	242	250	195	36.4	224	228	22.0	0.67	0.84	0.28	0.89
PC 18:0/18:2,18:1/18:1	538	540	608	478	81.6	557	525	49.9	0.43	0.96	0.41	0.59
PC 18:0/18:1	222	199	231	173	31.0	215	198	18.5	0.19	0.77	0.56	0.43
PC 16:0/22:6,18:1/22:5,18:2/20:4	9.6	10.6	12.7	9.59	2.09	11.3	9.9	1.23	0.60	0.60	0.32	0.33
PC 18:1/20:4,18:0/20:5,16:0/22:5	34.9	30.2	37.5	30.4	5.53	36.3	30.2	3.32	0.28	0.79	0.82	0.13
PC 18:0/20:4	21.7	20.5	24.9	19.2	3.65	23.6	19.5	2.21	0.34	0.80	0.53	0.14
PC 18:0/20:3	7.25	7.14	9.02	7.08	1.24	8.22	7.03	0.73	0.40	0.48	0.45	0.16
PC 18:1/22:6	2.48	2.58	3.42	2.45	0.68	3.03	2.44	0.40	0.52	0.55	0.43	0.19
PC 18:0/22:6,18:1/22:5	4.10	4.17	5.59	4.18	0.97	5.01	4.00	0.57	0.49	0.43	0.44	0.12

PC 18:0/22:5                      3.39      2.80      4.02      3.34      0.85      4.08      2.69      0.49      0.45      0.48      0.95      0.01

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<sup>1</sup>Prepartum, cows were supplemented with 60 g/d RPC (ReaShure<sup>®</sup>, Balchem Corp., New Hampton, NY; CHO), 12 g/d RPM (Smartamine<sup>®</sup> M; Adisseo USA, Inc., Alpharetta, GA; MET), or both (CHO + MET). Postpartum, cows were supplemented with 60 g/d RPC, 18 g/d RPM, or both. Cows were monitored and treatments were applied from 21 days before expected calving through 35 DIM.

<sup>2</sup>Milk samples were obtained during week 1 and week 2 postpartum.

\*CHO x Week interaction,  $P \leq 0.10$ ; \*\*MET x CHO x Week interaction,  $P \leq 0.05$ ; †MET x CHO x Week interaction,  $P \leq 0.10$ .

**Table 5.S5.** Milk choline metabolite concentrations ( $\mu\text{M}$ ) for multiparous cows ( $n = 27$ ) fed the control (CON) diet or the control diet plus RPC (CHO), RPM (MET), or both (CHO + MET)<sup>1, 2</sup>

Item <sup>3</sup>	Treatment				SEM	Week Postpartum			P-value			
	CON	CHO	MET	CHO + MET		1	2	SEM	CHO	MET	CHO	MET x Week
Betaine <sup>††</sup>	120	96	116	114	13.4	142	80	7.18	0.33	0.61	0.42	<0.01
Free Choline	80.4	52.6	41.0	39.9	23.3	47.9	59.1	11.2	0.52	0.25	0.55	<0.01
GPC	2221	2150	2115	2745	135	2372	2243	79.0	0.04	0.08	0.01	0.17
SM <sup>**</sup>	38.1	41.2	37.7	47.0	3.64	47.1	34.8	1.91	0.09	0.44	0.38	<0.01
Total LPC	2.95	2.76	2.94	3.66	0.26	3.09	3.07	0.14	0.29	0.09	0.08	0.86
LPC 16:0	2.32	2.12	2.25	2.79	0.22	2.41	2.34	0.12	0.43	0.17	0.09	0.54
LPC 18:2	0.29	0.22	0.30	0.30	0.03	0.24	0.31	0.02	0.28	0.14	0.25	0.01
LPC 18:1	0.046	0.056	0.049	0.053	0.005	0.045	0.056	0.003	0.12	0.96	0.47	0.02
LPC 18:0	0.32	0.38	0.36	0.52	0.03	0.42	0.37	0.02	<0.01	<0.01	0.17	0.08
Total PC	125	131	107	162	16.5	141	122	9.28	0.07	0.70	0.14	0.07
PC 16:0/16:1	4.83	4.80	3.92	5.72	0.63	4.72	4.91	0.36	0.15	0.99	0.14	0.64
PC 16:0/16:0	16.7	16.3	14.9	20.2	1.91	17.1	16.9	1.13	0.20	0.58	0.13	0.88
PC 16:0/18:2 <sup>†</sup>	14.0	16.2	12.4	17.8	2.22	16.4	13.8	1.21	0.09	1.00	0.46	0.03
PC 16:0/18:1	38.9	42.9	33.5	48.8	4.97	44.3	37.8	2.87	0.06	0.95	0.25	0.06
PC 16:0/20:5,16:1/20:4	0.004	0.007	0.005	0.016	0.0003	0.012	0.004	0.002	0.03	0.12	0.16	0.01
PC 16:0/20:4 <sup>††</sup>	4.31	4.52	3.89	5.14	0.61	4.85	4.08	0.34	0.22	0.86	0.38	0.04
PC 16:0/20:3	10.6	11.5	10.0	14.3	1.64	12.7	10.6	0.90	0.11	0.49	0.30	0.03
PC 18:0/18:2,18:1/18:1	23.0	23.9	19.0	29.4	3.33	26.3	21.2	1.80	0.09	0.82	0.15	<0.01
PC 18:0/18:1	7.88	8.79	7.37	11.8	1.17	10.5	7.46	0.66	0.03	0.27	0.13	<0.01
PC 16:0/22:6,18:1/22:5,18:2/20:4 <sup>††</sup>	0.43	0.49	0.45	0.69	0.09	0.60	0.43	0.05	0.09	0.20	0.29	<0.01
PC 18:1/20:4,18:0/20:5,16:0/22:5 <sup>††</sup>	1.38	1.49	1.30	2.03	0.22	1.79	1.31	0.13	0.06	0.29	0.16	<0.01

PC 18:0/20:4 <sup>††</sup>	0.90	1.02	0.98	1.54	0.16	1.33	0.90	0.10	0.04	0.07	0.18	<0.01
PC 18:0/20:3 <sup>††</sup>	0.32	0.36	0.37	0.55	0.06	0.48	0.32	0.04	0.08	0.06	0.25	<0.01
PC 18:1/22:6 <sup>††</sup>	0.05	0.07	0.08	0.15	0.02	0.10	0.07	0.01	0.02	<0.01	0.12	0.03
PC 18:0/22:6,18:1/22:5 <sup>†</sup>	0.11	0.14	0.12	0.25	0.03	0.19	0.12	0.02	<0.01	0.02	0.07	<0.01
PC 18:0/22:5 <sup>††</sup>	0.10	0.13	0.12	0.26	0.03	0.21	0.10	0.02	<0.01	0.01	0.09	<0.01

<sup>1</sup>Prepartum, cows were supplemented with 60 g/d RPC (ReaShure<sup>®</sup>, Balchem Corp., New Hampton, NY; CHO), 12 g/d RPM (Smartamine<sup>®</sup> M; Adisseo USA, Inc., Alpharetta, GA; MET), or both (CHO + MET). Postpartum, cows were supplemented with 60 g/d RPC, 18 g/d RPM, or both. Cows were monitored and treatments were applied from 21 days before expected calving through 35 DIM.

<sup>2</sup>Milk samples were obtained during week 1 and week 2 postpartum.

<sup>3</sup>GPC = glycerophosphocholine; SM = sphingomyelin; LPC = lysophosphatidylcholine; PC = phosphatidylcholine.

\*\*CHO x Week interaction,  $P \leq 0.05$ ; <sup>††</sup>MET x Week interaction,  $P \leq 0.05$ ; <sup>†</sup>MET x Week interaction,  $P \leq 0.10$ .

**Table 5.S6.** Milk choline metabolite concentrations ( $\mu\text{M}$ ) for primiparous cows ( $n = 24$ ) fed the control (CON) diet or the control diet plus RPC (CHO), RPM (MET), or both (CHO + MET)<sup>1, 2</sup>

Item <sup>3</sup>	Treatment					Week Postpartum			P-value			
	CON	CHO	MET	CHO +	SEM	1	2	SEM	CHO	MET	MET x	Week
				MET							CHO	
Betaine	122	133	124	136	21.84	166	92	11.8	0.60	0.88	0.98	<0.01
Free Choline	51.7	48.0	49.6	47.3	5.76	38.8	59.5	3.35	0.60	0.80	0.89	<0.01
GPC <sup>‡</sup>	2174	1778	1994	2153	112	2095	1955	71.4	0.28	0.38	0.02	0.15
SM <sup>‡</sup>	45.3	38.1	40.1	39.1	4.77	43.8	37.5	2.60	0.38	0.66	0.51	0.01
Total LPC	3.46	4.12	3.85	3.21	0.44	3.54	3.78	0.24	0.99	0.55	0.15	0.28
LPC 16:0	2.80	3.33	3.07	2.56	0.38	2.86	3.02	0.21	0.99	0.51	0.18	0.40
LPC 18:2	0.22	0.22	0.21	0.23	0.02	0.20	0.25	0.01	0.87	0.76	0.83	<0.01
LPC 18:1	0.045	0.044	0.045	0.056	0.004	0.045	0.050	0.003	0.25	0.16	0.16	0.14
LPC 18:0	0.44	0.53	0.49	0.36	0.07	0.45	0.46	0.036	0.72	0.35	0.11	0.70
Total PC	145	108	137	107	17.7	131	118	10.5	0.07	0.79	0.82	0.30
PC 16:0/16:1	6.53	4.39	5.43	3.75	0.73	4.88	5.17	0.49	0.01	0.22	0.74	0.68
PC 16:0/16:0	18.8	14.5	17.3	13.1	2.24	16.0	15.3	1.49	0.06	0.51	0.96	0.99
PC 16:0/18:2	14.6	10.6	12.0	9.8	2.17	12.4	11.1	1.25	0.15	0.42	0.68	0.32
PC 16:0/18:1	45.9	35.5	43.0	34.3	6.46	41.9	37.4	3.81	0.14	0.74	0.89	0.30
PC 16:0/20:5,16:1/20:4	0.010	0.003	0.006	0.013	0.006	0.013	0.003	0.004	1.00	0.57	0.23	0.03
PC 16:0/20:4	4.07	3.09	3.73	2.94	0.59	3.70	3.22	0.35	0.14	0.67	0.87	0.25
PC 16:0/20:3	11.0	9.6	11.4	8.8	1.55	11.0	9.4	0.93	0.19	0.89	0.68	0.18
PC 18:0/18:2,18:1/18:1	27.1	21.4	28.1	22.1	3.65	27.3	22.0	2.16	0.11	0.80	0.96	0.04
PC 18:0/18:1	11.4	7.94	10.67	7.98	1.53	10.59	8.38	0.87	0.06	0.83	0.81	0.02
PC 16:0/22:6,18:1/22:5,18:2/20:4	0.50	0.43	0.58	0.43	0.10	0.55	0.42	0.06	0.26	0.67	0.66	0.03
PC 18:1/20:4,18:0/20:5,16:0/22:5	1.78	1.23	1.74	1.37	0.28	1.77	1.28	0.16	0.10	0.86	0.74	<0.01

PC 18:0/20:4	1.12	0.84	1.14	0.86	0.18	1.16	0.82	0.11	0.13	0.90	0.99	<0.01
PC 18:0/20:3	0.37	0.29	0.41	0.32	0.06	0.40	0.30	0.03	0.16	0.53	0.90	<0.01
PC 18:1/22:6	0.13	0.11	0.15	0.11	0.03	0.14	0.10	0.02	0.31	0.65	0.69	0.04
PC 18:0/22:6,18:1/22:5	0.21	0.17	0.26	0.19	0.05	0.24	0.17	0.03	0.25	0.56	0.80	0.01
PC 18:0/22:5	0.19	0.12	0.19	0.15	0.05	0.20	0.12	0.03	0.27	0.72	0.73	<0.01

<sup>1</sup>Prepartum, cows were supplemented with 60 g/d RPC (ReaShure<sup>®</sup>, Balchem Corp., New Hampton, NY; CHO), 12 g/d RPM (Smartamine<sup>®</sup> M; Adisseo USA, Inc., Alpharetta, GA; MET), or both (CHO + MET). Postpartum, cows were supplemented with 60 g/d RPC, 18 g/d RPM, or both. Cows were monitored and treatments were applied from 21 days before expected calving through 35 DIM.

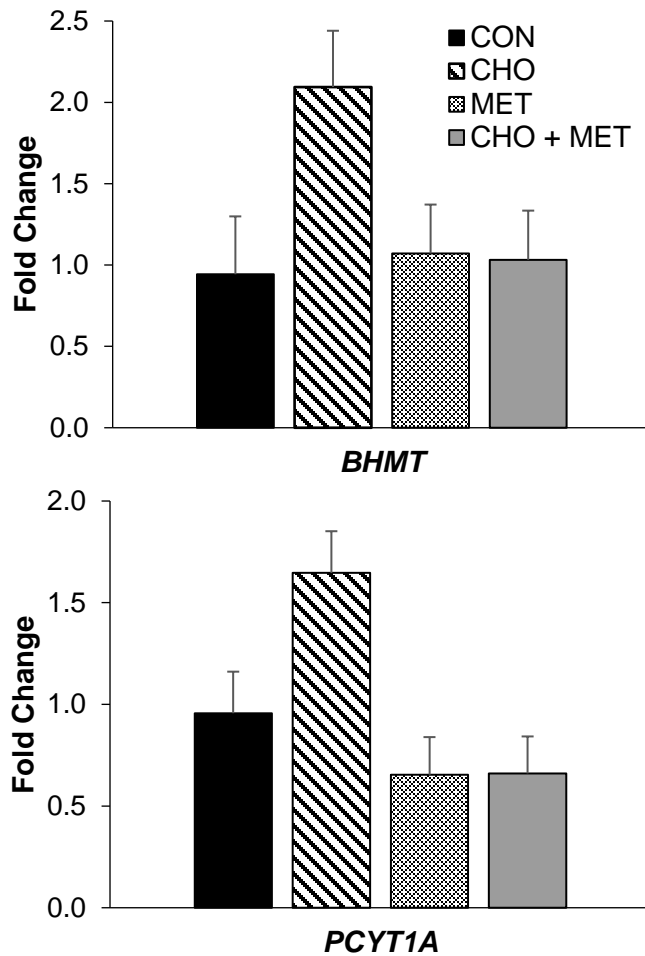
<sup>2</sup>Milk samples were obtained during week 1 and week 2 postpartum.

<sup>3</sup>GPC = glycerophosphocholine; SM = sphingomyelin; LPC = lysophosphatidylcholine; PC = phosphatidylcholine.

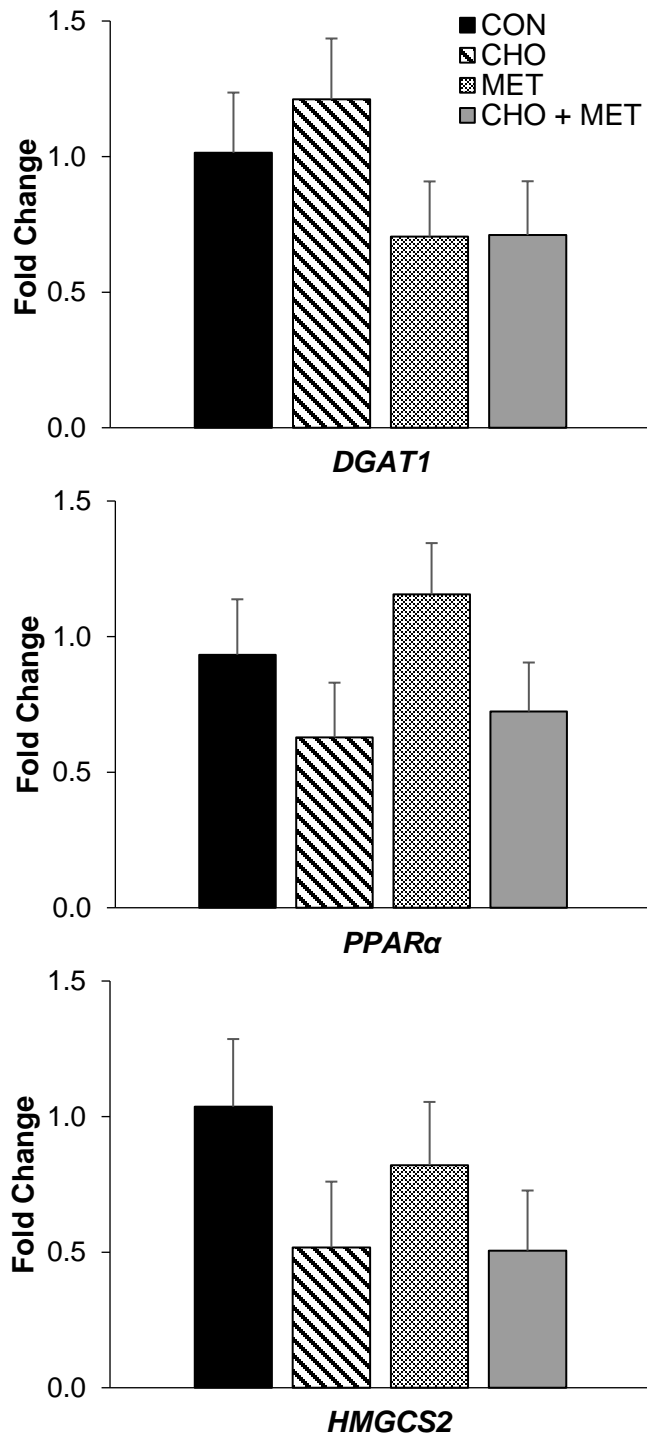
<sup>‡</sup>MET x CHO x Week interaction,  $P \leq 0.10$ .



**Figure 5.1.** Relative postpartum hepatic mRNA expression of genes related to Met and choline metabolism of multiparous cows ( $n = 20$ ) fed the control (CON) diet or the control diet plus RPC (60 g/d; CHO), RPM (12 g/d prepartum, 18 g/d postpartum; MET), or both (CHO + MET) from samples taken ~7 d after calving. Data are expressed as covariate-adjusted fold-changes relative to the CON treatment. For both genes, MET x CHO tended to be significant ( $P = 0.09$  and  $P = 0.10$ , respectively).



**Figure 5.2.** Relative postpartum hepatic mRNA expression of genes related to lipid metabolism of multiparous cows ( $n = 20$ ) fed the control (CON) diet or the control diet plus RPC (60 g/d; CHO), RPM (12 g/d prepartum, 18 g/d postpartum; MET), or both (CHO + MET) from samples taken ~7 d after calving. Data are expressed as covariate-adjusted fold-changes relative to the CON treatment. The main effect of MET tended to be significant ( $P = 0.08$ ) for *DGAT1*. The main effect of CHO tended to be significant for *PPAR $\alpha$*  and *HMGCS2* ( $P = 0.08$  and  $P = 0.10$ , respectively).



## **CHAPTER 6: SUMMARY AND FUTURE DIRECTIONS**

## **CHAPTER 6: SUMMARY AND FUTURE DIRECTIONS**

The first objective of this work was to identify blood and milk markers that are responsive to post-ruminal choline supplied via an abomasal infusion and rumen-protected choline (RPC). Results from the first study (Chapter 3) provide additional support for the use of betaine and free choline in blood and milk as indicators of post-ruminal choline supply; however, these markers were not affected by feeding RPC, even at a very high feeding rate (>100 g/d choline chloride). This suggested that either choline chloride supplied as RPC is absorbed differently than when it is supplied as an abomasal infusion or that the RPC product is over-protected, such that it is unavailable to the cow post-ruminally. It is likely that both scenarios contribute to the observations of the first study. Because RPC has been shown to elicit physiological effects in several different studies, both in feed-restricted cows and lactating cows (Chapters 4 and 5), it is unreasonable to suggest that the RPC was completely unavailable to the cow. Perhaps the protective coating of RPC is broken down slowly, such that its release into the small intestine is delayed, and subsequent absorption efficiency is reduced.

The second objective of this work was to determine the effects of providing rumen-protected Met (RPM) and RPC on production, hepatic gene expression, and indicators of choline and amino acid metabolism of periparturient dairy cows. Results from the third study utilizing periparturient cows (Chapter 4) suggest that RPM and RPC affect milk production responses in different ways, and that these effects vary depending on the age of the cow. Primiparous cows responded more favorably to RPC, where milk yield was increased. In contrast, positive responses caused by RPM for multiparous cows manifested as increases in milk component concentrations and yield, as well as fat-corrected milk.

These results suggest that the physiological state as well as the magnitude of the choline or Met deficiency are likely important factors that determine how cows respond to supplemental choline or Met. The absence of a synergistic effect when both RPC and RPM were supplemented conflicts with the hypothesis that providing these two nutrients together during the periparturient period augments production responses.

Both RPM and RPC caused slight changes in the blood AA and organic acid profile, and RPM, but not RPC, increased blood Met concentrations for both primi- and multiparous cows (Chapter 5). Interestingly, RPC consistently reduced circulating lactate concentrations, which could be indicative of a choline-induced modification of the Cori Cycle. Alterations in blood choline metabolites were also apparent for both RPM and RPC which supports the hypothesis that both RPM and RPC have effects on choline metabolism. Additionally, alterations in the phosphatidylcholine (PC) profile of blood and milk by both RPC and RPM suggested potential shifts in the PC synthesis pathways. Specifically, several PC species that contained poly-unsaturated fatty acids were elevated by RPC and RPM, indicating a potential increase in de novo PC synthesis from phosphatidylethanolamine. Somewhat contradictory to this observation was that RPC also increased hepatic expression of the gene that regulates the synthesis of PC from dietary choline in multiparous cows. However, this change did coincide with an increase in milk PC 18:0/18:1 yield, a PC species presumably synthesized via this pathway, for multiparous cows. The results from this study demonstrated that RPM and RPC each have the ability to modify AA and choline metabolism, but that these effects are probably achieved through different avenues.

Future studies should continue to investigate the mechanisms by which choline and Met influence cow health and performance not only during the early postpartum period, but also throughout lactation. Additionally, establishing the conditions under which feeding supplemental choline and Met would have the most benefit is also of interest from a practical standpoint. Results reported in the third and fourth studies (Chapters 4 and 5) suggest a potential interaction between these nutrients and parity. Given that a large portion of the U.S. dairy herd is comprised of primiparous cows, this potential interaction warrants further exploration.

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