ABSTRACT

DAVIDSON, SHANNON. Supplementation of rumen-protected forms of methionine, betaine, and choline to early lactation Holstein cows. (Under the direction of Dr. B.A. Hopkins and Dr. J. Odle.)

Methionine (**Met**) is frequently the first limiting amino acid or co-limiting with lysine in dairy rations, and Met metabolism is closely linked to that of betaine and choline. Methionine, betaine, and choline are all degraded by microbes in the rumen, so rumen-protected (**RP**) forms were used to perform two experiments. The objective of these experiments was to investigate the impact of supplementing RP forms of methionine, betaine, and choline to a Met-limited total mixed ration (**TMR**) on performance, metabolism and ruminal fermentation in early lactation Holstein cows.

Experiment 1 utilized 80 lactating Holstein cows from 21 to 91 days in milk (**DIM**) that were fed a corn silage-based TMR formulated to meet National Research Council (2001) recommendations, except the Met content was limited (42 g/d). One of four supplements was blended into the TMR to produce four dietary treatments: 1.) control, 2.) 20 g/d RP-Met, 3.) 45 g/d RP-betaine, and 4.) 40 g/d RP-choline. The RP-Met supplement was protected by encapsulation. Calcium salts of fatty acids were used to protect the RP-betaine and RP-choline supplements; therefore the same Ca salts of fatty acids were added to the control and RP-Met supplements in order to supply equal amounts of fat to all treatments. Dry matter (**DM**) intake, body weight, and body condition score were not significantly different among treatments ($P > 0.2$). Feeding RP-choline to MP cows that received a Met-limited diet improved milk yield and increased

milk CP yield, but not in primiparous cows. In this study, supplementing RP-betaine was not beneficial. Cows fed RP-Met or RP-choline had higher milk crude protein yield than cows fed control or RP-betaine ($P = 0.02$). However, there were no differences in milk fat yield or milk urea nitrogen ($P > 0.2$).

Experiment 2 utilized 4 dual-flow continuous culture fermentors (700 ml) to determine the effects of supplementation of rumen-protected forms of methionine, betaine, and choline to a Met-limited corn silage-based TMR on microbial metabolism by mixed ruminal cultures. Fermentors were inoculated with rumen fluid and allowed to stabilize for 2 days. Treatments were added for 5 days of adaptation followed by 3 days of sample collection. One of 4 supplements was blended into the TMR to produce 4 dietary treatments with a composition that was similar to those used in Experiment 1. Here, treatments are described as a percent of dietary DM: 1.) control, 2.) RP-Met (0.09% of DM as Met), 3). RP-betaine (0.20% of DM as betaine), or 4.) RP-choline (0.18% of DM as choline). Fat was added to all treatments as in Experiment 1. Four replicates were performed with each fermentor receiving each of the 4 treatments for one replicate. Total volatile fatty acids (**VFA**) and individual VFA concentrations were not affected by dietary treatment, except for propionate and isobutyrate concentration. Propionate production was significantly lower in RP-choline than in control fermentors ($P = 0.05$). Methane production and pH were similar across treatments ($P > 0.2$). Ruminal ammonia concentration was lower for fermentors receiving RP-choline than those receiving control ($P = 0.04$). There were no significant differences in microbial N %, flow, or efficiency between treatments ($P > 0.2$).

Overall, cows fed RP-choline produced more milk and milk protein than those fed the Met-limited control, and there were no beneficial effects of RP-betaine supplementation to a Met-limited TMR. Also, RP-Met, RP-betaine, and RP-choline affected ruminal fermentation minimally which suggests that they were protected from degradation. The responses to RP-choline could be the result of increasing phosphatidylcholine, supplying methyl groups, or providing methionine.

SUPPLEMENTATION OF RUMEN-PROTECTED FORMS OF METHIONINE, BETAINE, AND CHOLINE TO EARLY LACTATION HOLSTEIN COWS

By

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BIOGRAPHY

Shannon Davidson was born in Memphis, Tennessee on October 14, 1975 to Mr. and Mrs. Junius Edward Davidson, III. She graduated from St. Mary's Episcopal School of Memphis in 1994 and began her studies at the University of Kentucky in Lexington, Kentucky in the fall of 1994. Shannon received a B.S. degree in Animal Science in May of 1998. Then, she entered the North Carolina State University animal science program in the spring of 1999 in pursuit of a M.S. degree in Nutrition under the direction of Dr. Brinton Hopkins and Dr. Lon Whitlow, which she completed in December of 2001. At that time, Shannon began working towards a Ph.D. degree in Nutrition at North Carolina State University in Raleigh, North Carolina under the direction of Dr. Hopkins and Dr. Jack Odle. She married Mr. Jeremy Edward Pflasterer in September of 2005. Upon the completion of her degree, Shannon intends to work in the animal nutrition industry.

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ABBREVIATIONS

2AMP	2-amino-2-methyl-l-propanol
AA	amino acid
ADF	acid detergent fiber
ADG	average daily gain
ADICP	acid detergent insoluble crude protein
ATP	adenosine triphosphate
BHBA	beta-hydroxybutyric acid
BCS	body condition score
BHMT	betaine:homocysteine methyltransferase
BUN	blood urea nitrogen
BW	body weight
CCT	CTP:phosphocholine cytidylyltransferase
CDP	cytidine diphosphate
CNCPS	Cornell net carbohydrate and protein system
CP	crude protein
CTP	cytidine triphosphate
DIM	days in milk
DM	dry matter
DMI	dry matter intake
DNA	deoxyribonucleic acid
EAA	essential amino acid
ECM	energy-corrected milk
FCM	fat-corrected milk
HDL	high density lipoprotein
LDL	low density lipoprotein
Lys	lysine
Met	methionine
MP	multiparous
MUN	milk urea nitrogen
N	nitrogen
NDF	neutral detergent fiber
NDICP	neutral detergent insoluble crude protein
NEFA	nonesterified fatty acids
NE_L	net energy of lactation
NFC	nonfiber carbohydrate
NPN	non-protein nitrogen
OM	organic matter
PC	phosphatidylcholine
PEMT	phosphatidylethanolamine *N*-methyltransferase
PP	primiparous
RDP	rumen degradable protein

RNA	ribonucleic acid
RP	rumen-protected
RUP	rumen undegradable protein
SAM	*S*-adenosylmethionine
TMR	total mixed ration
VFA	volatile fatty acids
VLDL	very low density lipoprotein

CHAPTER ONE

LITERATURE REVIEW

INTRODUCTION

The pathways of methionine (**Met**), betaine, and choline metabolism are interrelated. An improved understanding of the mechanisms that regulate these overlapping pathways is needed so that these compounds may be fed to lactating dairy cows in order to improve lactation performance and reduce the incidence of ketosis and fatty liver.

When choline is oxidized irreversibly to betaine, it supplies methyl groups that recycle Met from homocysteine. Also, Met is the source of the methyl donor *S*-adenosylmethionine (**SAM**), the metabolite that provides methyl groups for the *de novo* synthesis of choline. Because of these metabolic relationships, dietary supply of either choline or betaine affects Met requirements and vice versa.

Choline deficiency results in fatty liver because inadequate choline supply limits the export of triglycerides from the liver by reducing very low density lipoprotein (**VLDL**) formation. Therefore, optimizing the feeding of Met, betaine, and choline could reduce the incidence of fatty liver in early lactation dairy cattle. Clinical ketosis often results in the development of fatty liver, so it has been speculated that choline or Met supplementation could play a role in ketosis prevention as well (Erdman, 1991).

Research has suggested that early lactation dairy cattle can produce more milk when they receive supplemental choline (Erdman, 1991). However, because choline is susceptible to extensive degradation in the rumen (Atkins et al., 1988; Sharma and

Erdman, 1989), rumen-protected forms are needed. Rumen-protected forms of Met also have been fed to early lactation dairy cattle and have increased milk yield as well as milk protein and milk fat content (NRC, 2001). Betaine also is degraded rapidly by rumen microorganisms and should be ruminally-protected when fed to dairy cattle. However, very little research has investigated the use of rumen-protected betaine in ruminants, and this research has focused on the ability of betaine to improve carcass traits and not lactation performance (Fernandez et al., 2000; Löest et al., 2002). Nevertheless, if milk yield in dairy cows is limited as a result of a methyl group deficiency, then supplying betaine should increase milk production.

Optimizing the feeding levels of these supplements has the potential to improve lactation performance by increasing milk, milk protein, and milk fat synthesis, as well as improve health by reducing the incidence of the metabolic disorders fatty liver and ketosis. It also may reduce nitrogen excretion by increasing the efficiency of nitrogen secretion into milk.

AMINO ACID REQUIREMENTS

Essential amino acids (**EAA**) are required by lactating dairy cattle for milk production. However, these requirements vary with the amount and composition of milk produced. Microbial protein and rumen undegradable protein (**RUP**) are the primary sources of EAA that can be digested and later absorbed by the intestine. Ruminal microorganisms utilize N from rumen degradable protein (**RDP**) and nonprotein nitrogen (**NPN**) for microbial protein synthesis, while RUP passes intact from the rumen. In the

intestine, amino acids and small peptides from RUP and microbial protein are absorbed into the bloodstream where they supply amino acids and carbon skeletons for metabolic functions, which include utilization by the mammary gland for milk protein synthesis.

Microbial protein provides a better profile of EAA than most sources of RUP, because microbial protein reflects the EAA profile of milk. Data adapted from Clark et al. (1992), Mantysaari et al. (1989), and Jacobson et al. (1970) illustrate that rumen bacteria, body tissue, and milk contain similar proportions of EAA (Table 1.1).

Table 1.1. Amino acid content of bacteria, tissue and milk as a percentage of the total essential amino acids (**EAA**) in each.

AA, % of total EAA	Bacteria[1]	Tissue[2]	Milk[3]
Arginine	10.5	14.2	7.2
Histidine	4.1	6.3	5.6
Isoleucine	11.7	11.5	12.1
Leucine	16.7	15.1	20.0
Lysine	16.3	17.2	16.7
Methionine	5.4	5.6	5.3
Phenylalanine	10.5	9.6	10.1
Threonine	12.0	9.6	9.5
Valine	12.8	10.9	13.6

[1]Clark et al., 1992.
[2]Adapted from Mantysaari et al., 1989.
[3]Adapted from Jacobson et al., 1970.

When formulating rations for early lactation dairy cattle, it is important to formulate diets that optimize microbial protein synthesis while supplying RUP in an amount and of such quality that will complement microbial protein in meeting the cow's AA requirements. Stern et al. (1994) used values from the National Research Council's

Nutrient Requirements of Dairy Cattle (NRC, 1989) to approximate the contribution of microbial protein to total protein requirements of the lactating dairy cow. Their calculations emphasize the need to supply RUP to lactating cows because as milk production increases, RUP supplies a larger proportion of the protein requirement (Table 1.2).

Table 1.2 Theoretical contribution of the microbial protein to the total protein requirement of the lactating dairy cow[1].

Efficiency of microbial synthesis[2]	Daily milk yield		
	25 kg	35 kg	45 kg
(g of N/kg of OM truly digested)	----------%----------		
20	49	42	39
30	73	64	59
40	98	85	79

[1]Requirements determined using NRC (1989) values for a 600 kg cow during second lactation producing 4% fat-corrected milk.
[2]Assumed that 55% of organic matter (OM) intake is truly digested in the rumen.

Until recently, diets have been formulated to meet the dietary EAA requirements of lactating dairy cows by overfeeding protein to ensure that the cow's minimum needs have been met. Currently, many researchers are focusing on defining the EAA requirements of lactating dairy cows more clearly in order to improve the efficiency of nitrogen (**N**) utilization by increasing secretion of N into milk and decreasing the excretion of N in feces and urine. Overall, EAA requirements are affected by a variety of factors including age, stage of lactation, level of milk production, and the milk's nutrient

composition. These factors must be considered in order to balance rations that effectively meet the AA needs of lactating dairy cows.

FUNCTIONS OF METHIONINE

Methionine is a sulfur-containing AA that is involved in many pathways including the synthesis of phospholipids, carnitine, creatine and the polyamines (Bequette et al., 1998). In addition to being used for protein synthesis, it can be used to provide methyl groups for a variety of reactions and to provide sulfur groups from the synthesis of cysteine.

Methionine frequently is considered to be a limiting amino acid for milk protein synthesis in corn-based diets for lactating dairy cattle (NRC, 2001). According to Schingoethe (1996), when dairy cows are producing more than 45 kg/d of milk, roughly 90% of their protein requirement is used for milk protein synthesis. Schingoethe (1996) suggested that to optimize N utilization by the dairy cow, amino acids absorbed by the intestine should be provided in a profile similar to that required by the animal for milk protein synthesis. Therefore, adequate Met must be supplied to lactating dairy cattle receiving corn-based diets in order to avoid limiting the protein concentration of milk.

Lysine (**Lys**) and Met are present in body tissue, rumen bacteria, and milk in approximately a 3:1 ratio (NRC, 2001). Schwab (1996) suggested that supplying Lys and Met as 15 and 5% of the duodenally digestible EAA profile or in approximately a 3:1 ratio should optimize Lys and Met availability for milk protein production. Many of the studies that have evaluated production responses of dairy cattle to protein

supplementation have not considered the Lys and Met content across treatments, but have used diets formulated for crude protein (**CP**) or RUP content only (NRC, 2001). In such studies, it was not clear whether production responses were the effect of RUP content or AA supply. Therefore, future studies need to consider AA supply in order to determine the causes of responses to changes in protein feeding.

FUNCTIONS OF BETAINE

Betaine functions as an osmolyte or a methyl donor. Betaine is a zwitterionic compound that has three chemically reactive methyl groups that can be donated to other molecules. As an osmolyte, betaine has been shown to protect cells stressed by dehydration, high salinity, or extreme temperatures (Craig, 2004). In experimentally induced stress, plants have been shown to accumulate betaine resulting in effects such as increasing water retention, replacing inorganic salts, or increasing the stability of intracellular enzymes (Craig, 2004). As a methyl donor, betaine is converted to dimethylglycine for the transmethylation of homocysteine to methionine. This reaction is catalyzed by the enzyme betaine:homocysteine methyltransferase (**BHMT**), and it occurs primarily in the liver and kidney. Dimethylglycine can donate a methyl group to become methylglycine, and methylglycine can donate a methyl group to become the amino acid glycine. Homocysteine also can be converted to methionine by the enzyme methionine synthase. This reaction utilizes a methyl group derived from 5-methyltetrahydrofolate. This methyl group is transferred from 5-methyltetrahydrofolate to cobalamin (vitamin B_{12}) to form methylcobalamin. Methylcobalamin then transfers the methyl group to

homocysteine to form methionine. Methylenetetrahydrofolate reductase then is involved in reforming methyltetrahydrofolate from methylenetetrahydrofolate. Although both systems are important, methionine synthase is located primarily in peripheral tissues while BHMT is located in the kidney and liver which may make BHMT more pivotal in regulating methyl group supply when methyl groups may be needed in the liver (Finkelstein et al., 1984).

Betaine is rapidly absorbed in the small intestine where it is transported by amino acid transport systems via active Na^+/Cl^- coupled transport or Na^+ independent transport systems. However, betaine is rapidly degraded to trimethylamine, methane, and carbon dioxide by ruminal microorganisms, so intestinal supply in dairy cattle is very limited compared to other livestock species (Mitchell, 1979).

Betaine is found in a wide variety of animal and plant products, but wheat and wheat products are the best grain sources (Eklund et al., 2005). However, the most concentrated natural source is sugar beets, which are often used as an animal feed in a variety of ways. When sugar is extracted from beet molasses, a high betaine concentration by-product is produced that is often referred to as condensed molasses solubles or concentrated separator by-product. Concentrated separator by-product used in experiments with beef steers conducted by Löest et al. (2002) contained 62 g/kg betaine, but processing results in a range of betaine contents. Betaine is most commonly added to animal diets as anhydrous betaine, but betaine monophosphate and betaine hydrochloride have been used (Eklund et al., 2005).

FUNCTIONS OF CHOLINE

Choline is sometimes classified as a B vitamin, even though it does not fulfill the standard vitamin definition, and is an essential nutrient when excess Met and folate are not available (NRC, 1998). It has been estimated that cows require gram quantities of choline for milk production (Erdman, 1991). Choline has two general functions: it can be used to supply the methyl donor betaine or it can be used as choline, primarily as a component of phospholipids or acetylcholine.

Choline is a small water-soluble molecule that has been found in all mammalian cells. It is a quaternary amine that features three methyl groups, which enable it to function as a methyl donor when choline is converted to betaine. Choline is required not only for the synthesis of betaine, but also for the synthesis of acetylcholine and the choline phospholipids (Shin et al., 1997). The primary forms of choline phospholipids are phosphatidylcholine (**PC**), lysophosphatidylcholine, choline plasmalogen, platelet-activating factor, sphingosylphosphorylcholine, and sphingomyelin (Zeisel, 1993; Figure 1.1). Phosphatidylcholine, sometimes referred to as lecithin, is the predominant form and makes up more than 50% of phospholipids in mammalian cell membranes (Zeisel, 1993). Overall, choline is required for methyl metabolism, maintenance of cellular membrane integrity, cholinergic neurotransmission, transmembrane signaling, as well as lipid and cholesterol transport and metabolism (NRC, 1998). It is also an essential component of VLDL and cannot be substituted with other phospholipids (Pinotti, 2002).

Soybean meal, rapeseed meal, fishmeal, and dried yeast have the highest contents of choline of commonly used animal feed ingredients (Pinotti et al., 2002). However,

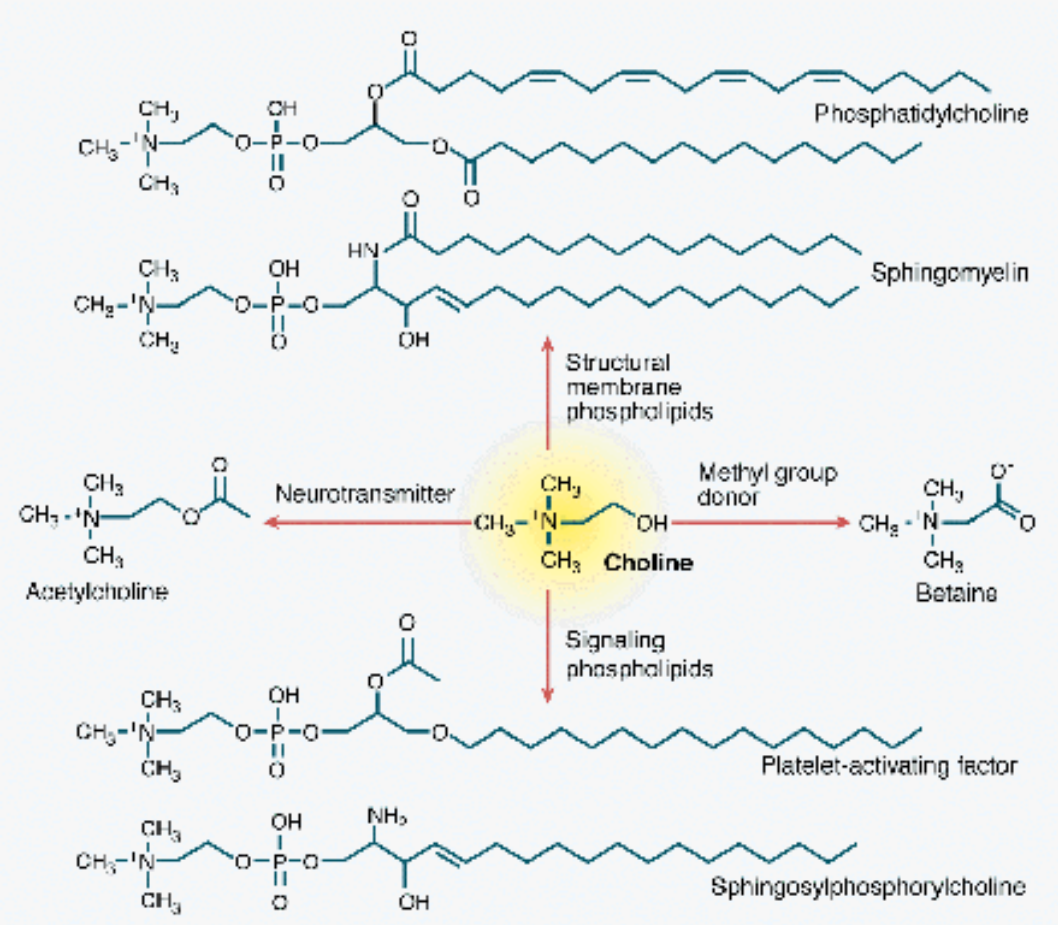

Figure 1.1 Choline's structure and other compounds synthesized from it (Zeisel and Blustajn, 1994).

choline is found as free choline, acetylcholine, and phosphatidylcholine in a wide variety of plant and animal products (Zeisel, 1988). Choline chloride is the most commonly fed source of supplemental choline (Pinotti et al., 2002), but choline stearate has also been evaluated as a feed ingredient (Sharma and Erdman, 1989).

FATTY LIVER AND KETOSIS IN CHOLINE DEFICIENCY

Choline deficiency results in fatty liver in many mammals (Zeisel, 1993). Fatty liver results in reduced reproductive and lactation performance that led Bobe et al. (2004) to estimate that the cost of fatty liver in the US dairy industry was over $60 million dollars annually.

In choline deficiency, fatty liver occurs because the export of triglycerides from the liver is limited by VLDL production causing triglycerides to accumulate in the liver (Zeisel, 1993). During early lactation, dairy cattle are in a negative energy balance and must mobilize fat stores from adipose tissue. This increases blood nonesterified fatty acid (**NEFA**) concentrations which are taken up by the liver and esterified to triglycerides or oxidized to ketone bodies (Van den Top et al., 1995).

Erdman (1991) suggested that since clinical ketosis often leads to fatty liver, choline may be useful in reducing the incidence of clinical ketosis. Choline potentially could affect the incidence of subclinical ketosis as well, which is defined by high levels of circulating ketone bodies when no clinical symptoms are evident (Andersson, 1988). Estimates of the prevalence of subclinical ketosis range from 6.9 to 34% (Carrier et al., 2004), and it most commonly occurs during the first two weeks in lactation (Duffield et al, 1997). Economic losses from cases of subclinical ketosis are due to lower milk production and poor reproductive performance (Duffield, 2000). Also, there is a higher risk for developing displaced abomasum or clinical ketosis in cows with subclinical ketosis (Duffield, 2000).

Ruminants have a limited ability to secrete VLDL from the liver compared to other mammals. Kleppe et al. (1988) determined that rat hepatocytes secreted 25 times more triglycerides synthesized from NEFA than goat hepatocytes. Van den Top et al. (1995) suggested that the ruminant liver is unable to respond to increased NEFA with increased VLDL production in cases of fatty liver because hepatic synthesis of either apolipoprotein B or phospholipids, especially PC, may be limiting. Choline supplementation routinely increases VLDL secretion from the liver in rats (Zeisel, 1993), and Met supplementation has been shown to increase VLDL synthesis in the liver of calves (Auboiron et al., 1995). Overall, identifying the required amount of choline and determining how to supply adequate amounts to the liver should reduce the incidence of fatty liver and ketosis in early lactation dairy cattle.

RELATIONSHIPS IN METHIONINE, BETAINE, AND CHOLINE METABOLISM

When choline is irreversibly oxidized to betaine, the three methyl groups from choline become available for use in one-carbon metabolism. In one-carbon metabolism, methyl groups (CH_3) contribute to a pool that is used to for a variety of metabolic reactions that include the synthesis of methionine from homocysteine, purine synthesis, as well as deoxyribonucleic acid (**DNA**) and ribonucleic acid (**RNA**) methylation (Arinze, 2005).

The pathways of one-carbon metabolism intersect at a reaction where betaine donates a methyl group to homocysteine to form Met. This reaction is catalyzed by the

enzyme betaine:homocysteine methyltransferase (**BHMT**), and it occurs primarily in the liver and kidney. Methionine also can be regenerated from homocysteine by 5-methyltetrahydrofolate:homocysteine methyltransferase (commonly known as Met synthase) which requires vitamin B_{12} as a cofactor. This pathway uses methyl groups derived from the one-carbon pool and is affected by both methylfolate and B_{12} supply. Methionine's methyl group becomes labile when it is converted to SAM by an ATP-dependent reaction catalyzed by methionine adenosyltransferase. Betaine and SAM are the two principal methyl donors in animals.

Choline can be synthesized *de novo* through the sequential methylation of phosphatidylethanolamine by phosphatidylethanolamine *N*-methyltransferase (**PEMT**) using methyl groups donated by SAM to result in the synthesis of PC (NRC, 1998). In rats, the highest level of PEMT activity has been found in the liver, where it is reported to supply anywhere from 15-40% of the PC in the liver (Yao and Vance, 1988). Therefore, the amount of choline required is dependent on the supply of other methyl donors available, such as Met, folate, and betaine (Figure 1.2).

In nonruminants, other methyl donors and *de novo* choline synthesis can reduce the amount of dietary choline required, but choline cannot be completely supplied by these sources. Zeisel et al. (1991) showed that healthy men with normal folate and vitamin B_{12} status fed a choline-deficient diet developed liver damage and had lower plasma choline and PC concentrations than men fed a choline-adequate diet. These data suggest that the requirement for choline cannot be completely replaced by other methyl donors. In other studies, choline-deficient rats had undermethylated DNA and 30-40%

lower hepatic and brain folate concentrations than rats receiving a choline-sufficient diet (Combs, 1992).

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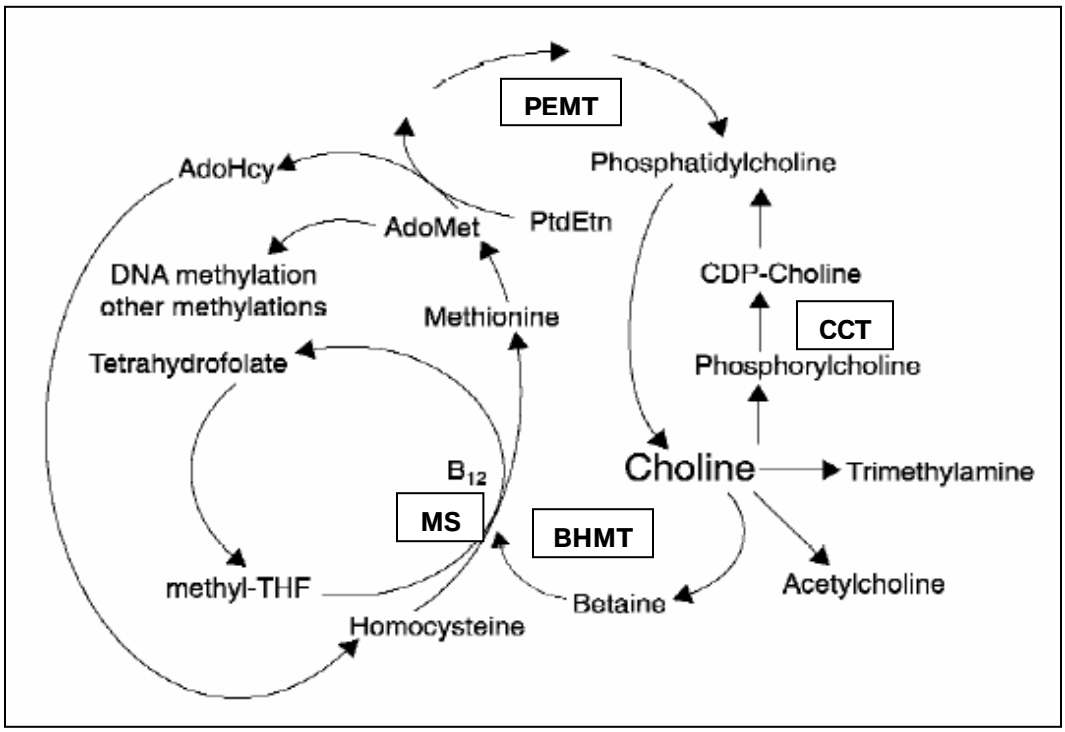

Figure 1.2. Interrelationship of choline with betaine, folate, vitamin B_{12}, and methionine. These molecules all supply sources of labile methyl groups for synthesis of *S*-adenosylmethionine (SAM or AdoMet), the primary methylating molecule for most enzymatic methylations. Other abbreviations: AdoHyc=*S*-adenosylhomocysteine, CDP-choline=cytidine diphosphocholine, PtdEtn=phosphatidylethanolamine, THF=tetrahydrofolate (Zeisel and Blusztajn, 1994). Enzymes have been added to the figure so that PEMT= phosphatidylethanolamine-N-methyltransferase, CCT=CTP:phosphocholine cytidylyltransferase, BHMT=betaine:homocysteinetransferase, and MS=methionine synthase

In rats, the primary pathway for PC synthesis is the cytidine diphosphate (**CDP**)-choline pathway which forms from 70-80% of their PC supply, while the remainder is derived from phosphatidylethanolamine (Yao and Vance, 1988). The enzyme that regulates the CDP-choline pathway is cytidine triphosphate (**CTP**):phosphocholine

cytidylyltransferase (**CCT**) and is responsible for the conversion of phosphocholine to CDP-choline.

Ruminants absorb very few dietary methyl groups compared to nonruminants because choline (Erdman et al., 1984) and betaine (Mitchell et al., 1979) are both rapidly degraded in the rumen. Walkey et al. (1998) developed a PEMT knockout mouse line to investigate the importance of PEMT derived choline in choline deficiency. As a result of only 3 days of dietary choline withdrawal, these mice developed severe liver pathology. These authors determined that the PEMT pathway which uses methyl groups from SAM for PC synthesis must exist in order to provide choline when dietary choline is insufficient (Walkey et al., 1998). Since cattle absorb very little dietary choline, it is likely that a larger percentage of their PC supply comes from the PEMT pathway instead of the CDP-choline pathway compared to that of nonruminants.

The activity of BHMT also should be different in ruminants compared to nonruminants. Research has shown that Met deficiency increases hepatic BHMT activity in rats and chickens and that supplementation of choline or betaine further elevates BHMT when Met is deficient (Emmert et al., 1996; Park et al., 1997). However, pigs did not respond to Met-deficient conditions with increased hepatic BHMT activity perhaps because they have a higher BHMT activity than rats or chicks when Met is adequate (Emmert et al., 1998).

Overall the metabolism of Met, choline, and betaine is closely related in all mammals, but the metabolic supplies of the compounds are different in ruminants and nonruminants because of ruminal degradation. Therefore, this metabolism potentially is

regulated quite differently in ruminants and nonruminants, and further research investigating the regulation of this metabolism in ruminants is needed to better understand how to supply Met, choline, and betaine to the cow.

SUPPLEMENTATION OF RUMEN-PROTECTED METHIONINE, BETAINE, AND CHOLINE

Free amino acids are rapidly degraded in the rumen. Although Met is not as rapidly degraded as many EAA, supplementation of Met to dairy cattle is more effective when dietary Met sources are ruminally-protected (Onodera, 1993). Feeding rumen-protected Met often results in increased milk and milk protein yield (Illg et al., 1987; Casper and Schingoethe, 1988; Donkin et al., 1989). In some cases, RP-Met has increased fat-corrected milk (**FCM**) and milk fat yield (Overton et al., 1996). For the purposes of this review, only research that relates RP-Met supplementation to either choline or betaine metabolism will be discussed.

Two mechanisms have been proposed to explain how Met supply increases milk fat synthesis. Methionine may increase *de novo* short and medium chain fatty acid synthesis (Pisulewski et al., 1996) or Met may increase VLDL synthesis, possibly by supplying PC (Auboiron et al., 1995). Although the reason that Met supplementation increases milk fat synthesis is unknown (Overton et al., 1996; NRC, 2001), Emmanuel and Kennelly (1984) reported that 28% of absorbable Met was used for choline synthesis in lactating goats, which could explain increases in milk fat associated with Met supplementation. Because Met frequently is a limiting AA for milk production in

lactating dairy cattle (NRC, 2001), excess Met may not be available for use in supplying choline.

Rulquin and Delaby (1997) fed 24 Holstein cows (58 DIM) either 0 or 21 g/d RP-Met (Smartamine) and either a low or normal energy diet (87% or 100% of requirement). The authors reported that milk protein content increased when either energy level was increased or Met was supplemented to the diet and that the effect of each was additive with no interaction between the two effects. The results of the study by Rulquin and Delaby (1997) support the suggestion that Met has a role in liver gluconeogenesis especially when cows are in negative energy balance.

Choline supplementation routinely increases VLDL secretion from the liver in rats (Zeisel, 1993), and Met supplementation has been shown to increase VLDL synthesis in the livers of calves (Auboiron et al., 1995). Research has suggested that early lactation dairy cattle can produce more milk when they receive supplemental choline (Erdman, 1991; Pinotti, 2002). However, choline is rapidly degraded in the rumen (Atkins et al., 1988), so it is necessary to feed rumen-protected forms.

Sharma and Erdman (1988b) attempted to saturate the ability of rumen microbes to degrade choline by supplementing choline chloride up to levels that contained 326 g/d choline. Doing this increased duodenal choline flow from 1.2 to 2.5 g/d (Sharma and Erdman, 1988b). Sharma and Erdman (1989) also measured the choline content and rumen degradability of several feedstuffs commonly fed to dairy cows as well as synthetic choline sources using *in vitro* incubations in rumen fluid (Table 1.3). They reported that all of the feedstuffs analyzed contained very little choline with fish meal

containing the highest level of choline (4.17 ± 0.57 mg/g). Also, all of the sources analyzed were degraded to the extent that rumen-protected forms would be required to provide choline in gram quantities to the cow (Sharma and Erdman, 1989).

Table 1.3 Choline content and *in vitro* degradability of selected feedstuffs (Sharma and Erdman (1989).

Feedstuff	Choline (mg/g)	Degradation (%) Standard error = 1.55
Corn silage	0.38 ± 0.09	
Barley	1.84 ± 0.05	79.4[a]
Corn	0.68 ± 1.10	
Corn gluten meal	0.60 ± 0.11	
Cottonseed meal	2.60 ± 0.31	84.7 [a]
Fish meal	4.17 ± 0.57	82.9[a]
Soybean meal	2.95 ± 0.23	83.8 [a]
Alfalfa hay	0.43 ± 0.44	
Timothy hay	0.36 ± 0.08	
Choline chloride	162.8 ± 17.57	98.6[b]
Choline stearate	357.9 ± 32.61	98.0[b]

[a,b] Means within column are significantly different ($P < 0.05$).

Sharma and Erdman (1988a) compared the effects of Met and choline abomasal infusions and investigated the effects of 2-amino-2-methyl-1-propanol (**2AMP**) on choline metabolism in lactating dairy cows. Studies with rats have shown that 2AMP inhibits the methylation of phosphatidylethanolamine and limits the contribution of Met to PC synthesis (Wells, 1955). Sharma and Erdman (1988a) used four ruminally cannulated multiparous Holstein cows producing an average of 28.9 kg/d of milk that were between 89-125 days in milk (**DIM**). They infused two cows with 45.6 g/d Met and

two cows with 30 g/d choline for 5 weeks and then switched the treatments for an additional five weeks. During weeks 4 and 5 of each period, 40 g/d 2AMP was infused in addition to either the Met or choline treatment. As a result, choline infusion increased milk, protein, and fat yield when 2AMP was infused duodenally, but not when 2AMP was not infused. Sharma and Erdman (1988a) concluded that 2AMP appeared to block the ability of Met to supply PC. Even when 2AMP was not infused, choline infusion resulted in higher milk fat yield compared to Met infusion, which indicated an observable requirement for choline in lactating dairy cattle.

Overall, the research in dairy cattle with rumen-protected forms of choline typically has resulted in increased milk yield and milk components, as well as some improvements in liver health and negative energy balance (Pinotti et al., 2002). Table 1.4 includes data adapted from a review by Pinotti et al. (2002) and includes additional data not reported by Pinotti et al. (2002). Brusemeister and Sudekum (2006) also reviewed several of the same studies that were evaluated by Pinotti et al. (2002), but their review included calculations of the adequacy of the supply of metabolizable energy, metabolizable protein, and Met in the diets used in six studies including eleven treatments that either infused choline or fed RP-choline. The studies evaluated by Brusemeister and Sudekum (2006) were Sharma and Erdman (1988a), Sharma and Erdman (1989), Erdman and Sharma (1991), Hartwell et al. (2000), Pinotti et al. (2003), and Piepenbrink and Overton (2003). Using the Cornell Net Carbohydrate and Protein System (**CNCPS**), eight of the eleven treatments were adequate in Met content, however the author suspects

Table 1.4 Effects of RP-choline in dairy cows adapted from Pinotti et al. (2002).

Stage of lactation	Total choline supplemented	Choline effects	Reference
Mid-lactation	From 18.5 to 56.9 g/d and 13.0% CP	↑ Milk yield (linear response)	Erdman and Sharma (1991)
Mid-lactation	From 19.6 to 57.8 g/d and 16.5% CP	↑ Milk yield ↑ Milk protein content	Erdman and Sharma (1991)
Early lactation	33 g/d	↑ Milk yield ↑ Milk fat content ↑ 4% FCM	Erdman 1994
From 20 d prepartum to 100 DIM	From 5 to 45 g/d	No effects	DiCostanzo and Spain (1995)
Weeks 4-24 in lactation	10 g/d	↑ Milk yield ↑ Milk fat content and yield ↑ Milk protein content and yield ↓ Plasma Met and glucose ↓Plasma NEFA ↓ Plasma GOT, γ-GT activities	Bonomi et al. (1996)
Mid-lactation	50 g/d	– Milk yield* ↑ Choline secretion in milk ↑ Higher choline availability	Deuchler et al. (1998)
From 28 d prepartum to 63 DIM	12 g/d 4.0% RUP during prepartum	↑ Milk yield ↑ Body weight loss after calving	Hartwell et al, (2000)
From 21 d prepartum to 63 DIM	45, 60 or 75 g/d	↑ Liver glycogen content (linear response) ↑ Liver fatty acid metabolism ↑ Milk fat content	Piepenbrink and Overton (2003)
From 14 d prepartum to 30 DIM	20 g/d	↑ Milk yield ↑ 3.5% FCM ↓NEFA on parturition ↓ NEFA:cholesterol ratio on parturition ↑ α-Tocopherol in plasma	Pinotti et al. (2000) Pinotti et al. (2001) Pinotti et al. (2003)
From 21 d prepartum to 21 DIM	15 g/d as protected choline chloride	– DMI – Milk yield – Milk fat and protein yield	Janovick-Guretzky et al. (2006)

↑ = Increase relative to control (no supplemented); ↓ = decrease relative to control (no supplemented); – = no effect; DIM = days in milk; FCM = fat-corrected milk; NEFA = non-esterified fatty acids; GOT = glutamate-oxalacetate transaminase; γ-GT = γ-glutamate transaminase; RUP = percentage of rumen undegradable protein in diet. * The study was not designed to measure milk production effect or change.

that this supply is overestimated by the CNCPS model and that Met may actually have been limiting in many of these diets and that Met adequacy may not reflect sufficient methyl group supply (Brusemeister and Sudekum, 2006).

Supplementing betaine has the potential to produce results similar to those obtained from supplementing choline or Met in cases where additional methyl donors are needed (Loest et al., 2002). Like choline, betaine is rapidly degraded in the rumen, so ruminants have a limited dietary supply of methyl groups compared to nonruminants. Mitchell et al. (1979) reported that betaine is involved in rumen digestion by adding [methyl-^{14}C] betaine to the rumens of sheep. They found that within the rumen, microbes convert betaine to acetate and could trace nearly 100% of the added betaine as acetate and other compounds in body tissues. The methyl groups of betaine were primarily converted to trimethylamine, and the authors suggest that this occurs through cleavage of betaine by microbes to produce acetate and trimethylamine. A similar reaction has been reported involving choline, where choline cleavage results in either acetate or ethanol production and trimethylamine (Bradbeer, 1965). Neill et al. (1978) reported that trimethylamine derived from choline degradation by ruminal microorganisms resulted in methane production in the rumen. Therefore, increased methane production in the rumen occurs when unprotected sources of betaine or choline are fed to ruminants (Mitchell et al., 1978; Neill et al., 1978). The work of Mitchell et al. (1978) suggests that betaine supplementation in ruminants could provide acetate for fatty acid production in mammary gland, but that unprotected betaine is rapidly degraded in the rumen and does not contribute substantially in methyl donor metabolism.

Betaine is commonly added to diets for pigs and poultry because it has been shown to improve meat quality (Sillence, 2005). Matthews et al. (2001) reported that dietary betaine supplementation in pigs resulted in increased carcass leanness, as well as increased initial pH and decreased drip loss in fresh pork (Matthews et al., 2001). However, Overland et al. (1999) saw no effects of betaine supplementation on meat quality. Dunshea et al. (2005) suggest that there are a limited number of studies that report effects of dietary betaine on meat quality for its use to be widespread in animal production.

Loest et al. (2002) used 5 Holstein steers (158 kg initial body weight) in a 5×5 Latin square experiment. They abomasally infused methionine, betaine and choline in addition to feeding a soyhull-based diet that was formulated to minimize the supply of AA to the intestine, especially Met. Glucose, VFA, and 12 non-sulfur AA were infused abomasally to supply energy and protein. They reported increases in N retention in steers infused with three different levels of betaine (1.6, 8, or 16 g/d) that were not statistically significant compared to the increases in N retention in steers receiving Met infusion, which suggested that betaine was an inefficient means of replacing Met. Also, choline infused at 8 g/d did not produce a beneficial response. As a result, they concluded that they succeeded in limiting Met supply in the control diet and that the differences that they recorded between Met-infused steers (2 g/d) and the others were in response to correcting a deficiency of Met rather than a result of its role as a methyl group donor.

Although there are some studies that evaluate the effects of betaine in ruminants, very little research has been conducted where betaine was infused or fed to lactating

ruminants. Fernandez et al. (2004) conducted a study with lactating goats where anhydrous betaine was added to the diet so that 4 g/kg of diet dry matter (**DM**) betaine was fed to the goats from approximately one week prepartum and continued throughout the lactation. Betaine-fed goats were compared to control goats that did not receive betaine and as a result milk production for betaine-fed goats was higher during late lactation (3^{rd}, 4^{th} and 5^{th} months of lactation). Milk fat percentage also was increased by betaine supplementation during the 5^{th} month of lactation.

Minimal research has been conducted with rumen-protected betaine supplementation in ruminants, and all of the research has focused on the effects of betaine on growth and not on lactation performance. In an abstract reported by Puchala et al. (1994), goats supplemented with rumen-protected betaine had higher average daily gains (**ADG**) than goats that were not supplemented. Another study conducted with growing goats (43 ± 5.1 kg) investigated the effects of both protein level (9% or 15% CP) and the supplementation of rumen protected betaine or rumen protected choline (both fed at 0.9% of diet DM) on plasma metabolites (Banskalieva et al., 2005). In this study, the authors reported that the metabolic responses to both RP-betaine and RP-choline were affected by the level of CP in the diet, and that it appeared that the dietary supply of both compounds affected N metabolism and lipid metabolism (Banskalieva et al., 2005). Because the amount of protein required for milk production is substantially larger than the amount required for growth, it is likely that both responses to betaine and the level of betaine supplementation needed would not be the same for growing and lactating ruminants.

SUMMARY

More research with rumen-protected forms of betaine and choline is necessary to improve our understanding of the metabolism of these compounds and their effects on Met metabolism. The goal of this research is to gain information that will help define the role of betaine and choline in Met metabolism in lactating dairy cows in order to improve lactation performance and reduce N excretion by improving the efficiency of N secretion into milk. In summary, because the metabolism of Met, choline, and betaine is interrelated, we need to improve our understanding of how dietary supply of Met, choline, and betaine affects lactation performance and metabolism in dairy cattle.

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CHAPTER TWO

Supplementing Limited Methionine Diets with Rumen-protected Methionine, Betaine, and Choline in Early Lactation Holstein Cows. *Davidson et al*. Eighty lactating Holstein cows from 21 to 91 days in milk were fed a corn silage-based total mixed ration formulated to meet National Research Council (2001) recommendations except the methionine content was limited (42 g/d) in order to investigate the impact of supplementing rumen-protected forms of methionine, betaine, and choline on performance and metabolism. Supplementing rumen-protected choline to multiparous cows that received a methionine-limited diet improved milk yield, and supplementing rumen-protected choline and rumen-protected methionine resulted in increased milk protein yield. However, supplementing rumen-protected betaine was not beneficial.

RUNNING HEAD: METHYL DONORS IN EARLY LACTATION COWS

Supplementing Limited Methionine Diets with Rumen-protected Methionine, Betaine, and Choline in Early Lactation Holstein Cows

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ABSTRACT

Eighty lactating Holstein cows from 21 to 91 days in milk were fed a corn silage-based total mixed ration (**TMR**) formulated to meet National Research Council (2001) recommendations except the Met content was limited (42 g/d) in order to investigate the impact of supplementing rumen-protected (**RP**) forms of Met, betaine, and choline on performance and metabolism. One of four supplements was blended into the TMR to produce four dietary treatments: 1.) control, 2.) 20 g/d RP-Met, 3.) 45 g/d RP-betaine, and 4.) 40 g/d RP-choline. Calcium salts of fatty acids were used to protect both RP-betaine and RP-choline supplements and were added to both control and RP-met supplements so that equal amounts of fat were supplied to all treatments. Intake of DM was not different among treatments ($P > 0.2$). Body weight and body condition score (**BCS**) also were not different among treatments ($P > 0.2$). The treatment by parity interaction tended to be different ($P = 0.06$) for milk yield with 44.3 kg/d produced in multiparous (**MP**) cows fed RP-choline compared to MP cows fed all other treatments (37.8, 40.0, and 38.7, respectively) while there were no differences among treatments in primiparous (**PP**) cows. Cows fed RP-met or RP-choline had higher milk CP yield than cows fed control or RP-betaine ($P = 0.02$). There were no differences in milk fat yield or milk urea nitrogen (**MUN**) ($P > 0.2$). Overall, cows fed RP-choline produced more milk and milk protein than those fed the Met-limited control. There were no beneficial effects of RP-betaine supplementation to a Met-limited TMR.

(**Key words:** choline, betaine, methionine, dairy)

INTRODUCTION

Methionine is frequently the first limiting amino acid or co-limiting with lysine in dairy rations, and Met metabolism is closely linked to that of betaine and choline. An improved understanding of the mechanisms that regulate these overlapping pathways is needed because these compounds can be fed to lactating dairy cows in a way that potentially will improve lactation performance and reduce the incidence of ketosis and fatty liver.

When choline is oxidized irreversibly to betaine, it supplies methyl groups that recycle Met from homocysteine. Also, Met is the source of the methyl donor *S*-adenosylmethionine (**SAM**), the metabolite that provides methyl groups for the *de novo* synthesis of choline. Because of these metabolic relationships, dietary supply of either choline or betaine affects Met requirements and vice versa.

Choline deficiency results in fatty liver because the export of triglycerides from the liver is limited by reduced very low density lipoprotein (**VLDL**) formation that results from inadequate choline supply (NRC, 2001). Therefore, optimizing the feeding of Met, betaine, and choline could reduce the incidence of fatty liver in early lactation dairy cattle. Clinical ketosis often results in the development of fatty liver, so it has been speculated that choline or Met supplementation could play a role in ketosis prevention as well (Erdman, 1991).

Research has suggested that early lactation dairy cattle can produce more milk when they receive supplemental choline (Pinotti, 2002). Because choline is susceptible to extensive degradation in the rumen (Atkins et al., 1988; Sharma and Erdman, 1989),

rumen-protected forms are needed. Rumen-protected forms of Met also have been fed to early lactation dairy cattle and have increased milk yield as well as milk protein and milk fat content (NRC, 2001). Betaine also is degraded rapidly by rumen microorganisms and should be ruminally-protected when fed to dairy cattle. However, very little research has investigated the use of rumen-protected betaine in ruminants, and this research has focused on the ability of betaine to improve carcass traits and not lactation performance (Fernandez et al., 2000; Löest et al., 2002). Nevertheless, if milk yield in dairy cows is limited as a result of a methyl group deficiency, then supplying betaine should increase milk production.

Therefore, the objective of this study was to evaluate the effects on the performance and metabolism of early lactation Holstein cows fed a limited methionine diet supplemented with rumen-protected forms of methionine, betaine, or choline.

MATERIALS AND METHODS

Diets and Cow Management

The Institutional Animal Care and Use Committee of North Carolina State University approved all procedures under approval number 01-63-A. Eighty Holstein cows from the Piedmont Research Station in Salisbury, NC were assigned randomly to one of four treatment groups within either primiparous (**PP**) or multiparous (**MP**) blocks. Each treatment group consisted of eight PP and twelve MP cows. Four three-quartered MP cows were included in the study with one assigned to each of the four treatments. Cows were added to the study individually over approximately a 12 month period at the

time each calved. Following calving, cows were trained to Calan feeding stations and adjusted to their treatment diets (American Calan Inc., Northwood, NH). By 21 DIM, cows were adjusted to the feeding stations and consuming experimental diets fed as a TMR. In order to provide adequate adaptation to feeding stations and diets, data collection began at 28 DIM and continued through 91 DIM. Cows were housed in a free stall barn, fed for *ad libitum* consumption, and daily feed allocations and orts were recorded for each cow. Overall, the orts percentage was 15% when averaged from all intake measurements taken throughout the study.

A corn silage-based TMR was formulated to meet the NRC (2001) recommendations for NE_L, MP, RDP, RUP, macrominerals, microminerals and the vitamins A, D, and E (Table 2.1). In addition, the unsupplemented TMR was formulated to contain a limited amount of methionine, but adequate lysine, so that this basal diet supplied approximately 44g Met and 165g Lys (Lys to Met ratio of 3.75:1) according to the Mepron Ration Evaluator (Version 2.6) (Table 2.3).

All diets contained approximately 58.3% DM, 17.6% CP, and 23.7% ADF (Table 2.2). One of four supplements was added to this Met-limited TMR to form the four experimental diets: 1.) control with no rumen-protected compound (CON), 2.) 20 g/d rumen-protected methionine (RP-MET), 3.) 45 g/d rumen-protected betaine (RP-BET), or 4.) 40 g/d rumen-protected choline (RP-CHOL). All four supplements provided similar amounts of Ca salts of soy fatty acids to the diet. Calcium salts of fatty acids needed to be included as the primary fat source in the treatment diets because both the betaine and choline supplements were protected with this source of fat. The supplements containing

betaine and choline were fed at levels that supplied equivalent amounts of the compounds on a molecular weight basis, so that equal amounts of methyl groups would be supplied in each treatment. Unlike the betaine and choline supplements, the rumen-protected Met used was an encapsulated product instead of fat-protected and provided substantially fewer methyl groups. The level of rumen-protected Met supplementation was chosen so that enough Met was provided to result in approximately a 3:1 postruminal Lys to Met ratio in that treatment (Table 2.3). As a result, the RP-MET treatment contained adequate dietary Met while the CON, RP-BET, and RP-CHOL treatments all contained limited amounts of Met. All four supplements were mixed thoroughly into the TMR.

Sample Collection and Analysis

The four treatment TMR were sampled once a week and composited by month for analysis (n = 54). In addition, individual feed ingredients were sampled monthly and analyzed so that the TMR formulation could be adjusted to reduce variation in the content of DM, CP, and ADF throughout the study (Constable Laboratory, North Carolina Department of Agriculture, Raleigh, NC). After collection, weekly TMR samples were frozen at -20 C until they were thawed and dried for 48 h in a 60 C oven. Then, dried weekly samples were ground through a Wiley mill fitted with a 1 mm screen (Arthur H. Thomas, Philadelphia, PA) and composited by month. The composited TMR samples were analyzed for DM, CP, NDF, ADF, protein fractions, and minerals by the Cumberland Valley Analytical Laboratory (Hagerstown, MD). The ingredient and

nutrient compositions of the treatment diets are reported in Table 2.1 and Table 2.2 respectively.

All cows were weighed and body condition scored weekly prior to the AM feeding. Body condition score was assessed according to the guidelines of Ferguson et al. (1994). Mean BW changes were calculated as the difference between beginning and final body weight predicted by linear regression over the weeks of the trial.

Cows were milked twice daily at 0100 and 1300 h and milk yields were recorded at each milking. Milk samples were composited once weekly from consecutive AM and PM milkings and frozen at -20 C until analysis. These composited samples were analyzed for milk fat, milk protein, and milk urea nitrogen by the United Federation of DHIA Laboratory (Blacksburg, VA). Milk fat and CP were analyzed according to AOAC (1990) procedures while the Bentley ChemSpec 150 analyzer (Chaska, MN) was used to determine MUN concentrations by means of a modified Berthelot reaction (Chaney and Marbach, 1962).

Blood was collected from the coccygeal vein or artery prior to AM feeding on 28, 49, 70, and 91 DIM. Two samples were collected from each cow into vacutainers containing either EDTA or no additive. After collection, all samples were immediately placed on ice for transport to the laboratory. The EDTA-containing samples were centrifuged immediately for 15 min at 2500 × g, plasma was harvested and frozen until analysis. Vacutainers containing blood with no additive were kept on ice for at least 2 h to allow samples to clot and were then centrifuged for 15 min at 2500 × g. Total serum cholesterol, triglycerides, urea N, and BHBA were analyzed at the Texas Veterinary

Medical Diagnostic Laboratory (Amarillo, TX). High density lipoproteins (**HDL**) in serum were analyzed by the Michigan State University Diagnostic Center for Population and Animal Health (Lansing, MI). Very low density lipoproteins (**VLDL**) were calculated from serum triglycerides so that VLDL (mg/dl) = triglycerides (mg/dl) ÷ 5 (Friedewald et al., 1972). Low density lipoproteins (**LDL**) were calculated using the Friedewald equation where LDL (mg/dl) = Cholesterol (mg/dl) – [HDL (mg/dl) + VLDL (mg/dl)] (Friedewald et al., 1972). Plasma was analyzed for NEFA using WAKO reagent kits (Anonymous, Wako Chemicals USA, Inc., Richmond, VA).

Statistical Analyses

This experiment used a factorial arrangement of treatments, the factors being dietary treatment, parity (PP or MP), and time. Cow within treatment and parity provided replication with measurements over time on the same cow. Data were analyzed by repeated measures ANOVA as recommended by Littell et al. (1998) using the MIXED procedure with the autoregressive (1) covariance structure (SAS, 2004). The slice option was used to compare the treatment effects on milk production and composition within either primiparous or multiparous cows because parity was highly significant. Least square means for treatments were compared using the least significant difference procedure (LSD) with statistical significance declared at $P < 0.05$.

RESULTS AND DISCUSSION

Intake

There were no significant dietary treatment effects on daily DMI (Table 2.4). As expected, there was a significant parity effect on intake with MP cows consuming more DM ($P < 0.01$) than PP cows (Table 2.4). In addition, there were no significant treatment effects on daily DMI within either PP or MP cows (Tables 2.5 and 2.6), and the interaction between treatment and parity was not significant for DMI.

Body Weights and Body Condition Scores

There were no significant treatment differences in mean BW or mean BCS for PP, MP, or all cows (Tables 2.4, 2.5, and 2.6). Body condition scores indicate that the cows used in this study were not overconditioned, and therefore not at high risk for developing fatty liver. However, there was a significant parity effect ($P < 0.01$) for BW with MP cows weighing more than PP cows as would be expected. Again, the interaction between treatment and parity was not significant.

Milk Yield and Composition

Milk yield was significantly higher for cows fed the RP-CHOL diet than cows fed either the CON or BET diets while cows fed the RP-MET diet produced an amount of milk that was not statistically different from the other three treatments (Table 2.4). The treatment by parity interaction for milk yield was $P = 0.06$. Multiparous cows fed RP-CHOL produced 44.1 kg/d of milk which was significantly more than MP cows fed

CON, RP-MET, or RP-BET (37.7, 39.8, and 38.6, respectively). However, there were no significant differences in milk yield among treatments in PP cows. Therefore, it appears that the overall treatment effect on milk production was the result of increased milk yield by MP cows fed RP-CHOL.

Milk CP yield was significantly higher from cows fed either RP-MET or RP-CHOL than from cows fed either CON or RP-BET. However, milk CP content was higher in cows fed RP-MET than in cows fed CON or RP-BET, but was not significantly different from cows fed RP-CHOL. Multiparous cows fed RP-CHOL produced significantly more milk CP (kg/d) than MP cows fed CON. Multiparous cows fed RP-MET also produced significantly more milk CP (kg/d) than MP cows fed CON, but not more than MP cows fed RP-BET or RP-CHOL. In addition, MP cows fed RP-BET produced significantly less milk CP (kg/d) than MP cows fed RP-CHOL, but not less than MP cows fed CON or RP-MET. There were no significant differences in milk CP % between the dietary treatments the MP cows. Also, in PP cows there were no significant differences in either milk CP yield or content. Milk fat yield was not affected by dietary treatment when compared across all cows or PP cows, but was significantly different among MP cows. Multiparous cows fed RP-CHOL produced significantly more milk fat (kg/d) than MP cows fed RP-MET, while MP cows fed CON or RP-BET produced amounts of milk fat that were similar to both MP cows fed RP-CHOL or RP-MET. Over all cows, Milk fat content was lower in cows fed RP-MET than in cows fed CON or BET, but was not significantly different from cows fed RP-CHOL. However, within either PP or MP cows, there were no significant differences in milk fat content. As

expected, yields of milk, fat, and protein were significantly lower in PP cows than in MP cows. Also, concentrations of both milk fat and milk protein also were higher in PP cows (2.91 ± 0.07 kg/d; 2.68 ± 0.03 kg/d) than in MP cows (2.70 ± 0.07 kg/d; 2.50 ± 0.03 kg/d). The dietary treatments did not result in differences in MUN concentrations for all, PP, or MP cows, which suggests that the nitrogen utilization efficiency was similar between the treatments.

Fat-corrected milk was calculated so that 3.5% FCM (kg/d) = [Milk (kg/d) × 0.4324] + [Fat (kg/d) × 16.2162]. Yields of FCM were not significantly different between dietary treatments for all cows or PP cows, but FCM yield was significantly higher for MP cows fed RP-CHOL compared to those fed CON, RP-MET, or RP-BET. Also, energy-corrected milk (**ECM**) was calculated so that ECM = [Milk (kg/d) × 0.3246] + [Fat (kg/d) × 12.86] + [Protein (kg/d) × 7.04]. Like FCM, ECM yield was not significantly different between dietary treatments for all cows or PP cows, but ECM yield was significantly higher for MP cows fed RP-CHOL than MP cows on all other treatments. Feed efficiency is reported as kg of ECM per kg of DMI and was not significantly different as a result of dietary treatment for all, PP, or MP cows.

Blood Metabolites

There were no significant differences in plasma NEFA as a result of dietary treatment (Table 2.7). There were significant differences ($P < 0.01$) in plasma NEFA between PP (0.351 ± 0.032 Meq/L) and MP (0.546 ± 0.026 Meq/L) cows. This difference in plasma NEFA between parities suggests that MP cows mobilized more

stored energy to support milk production than PP cows, which partially may account for the differences in milk yield seen between the parities in response to the dietary treatments.

The concentrations of SUN were not different in response to either dietary treatment or parity, which suggests that overall nitrogen utilization was similar across cows receiving all dietary treatments. Concentrations of serum BHBA were not significantly different in response to dietary treatment, but were significantly higher in MP cows (585 ± 33 μmol/L) than in PP cows (452 ± 40 μmol/L). This difference between parities in BHBA concentration also supports that MP cows responded to dietary treatments. Ketosis incidence was determined to be the number of cows from each treatment with BHBA > 1400 μmol/L at any of the four sampling times. This calculation was based on the work of Duffield et al. (1997) who suggested 1400 μmol/L as the level of BHBA that indicated that a cow was subclinically ketotic. These data were not analyzed statistically because the study did not contain enough animals to detect differences in the incidence of a disorder, but numerically no PP cows had a BHBA sample that indicated that they were subclinically ketotic.

Serum triglycerides were not significantly different in response to either dietary treatment or parity (Table 2.7). Serum total cholesterol was significantly higher in cows fed RP-BET and RP-CHOL than in cows fed RP-MET, but was not significantly different from cows fed CON. Multiparous cows (197.0 ± 4.6 mg/dl) had significantly higher serum cholesterol than PP cows (169.1 ± 5.7 mg/dl). Serum HDL was not significantly different as a result of either dietary treatment or parity. Serum LDL was higher in cows

fed RP-BET than in cows fed CON or MET, however serum LDL in cows fed RP-CHOL was not significantly different from any of the other three treatments. Multiparous cows (95.4 ± 4.2 mg/dl) had significantly higher serum LDL concentrations than PP cows (59.2 ± 5.2 mg/dl). Since serum VLDL was calculated from serum triglycerides using the Friedewald equation, there was no effect of either dietary treatment or parity on serum VLDL. Reports of blood lipid profiles in dairy cattle fed RP-choline are limited, and these authors are not aware of reports of lipid profiles in dairy cattle fed RP-betaine. Janovick-Guretzky et al. (2006) reported that feeding RP-choline to periparturient cows did not alter lipid metabolism possibly because the cows were not overconditioned and not at a high risk of developing fatty liver. The cows utilized in this study were not overconditioned, which may have contributed to the responses seen in their lipoprotein and lipid profiles.

CONCLUSIONS

Feeding RP-choline to MP cows that received a Met-limited diet improved milk yield and increased milk CP yield. In this study, supplementing RP-betaine was not beneficial. Overall, it appears that there was no enhancement of methionine production from homocysteine. This suggests that RP-choline's effect was due to increased supply of phosphatidylcholine. However, responses to RP-choline could be the result of increasing phosphatidylcholine, supplying methyl groups, or providing methionine.

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Table 2.1 Ingredient composition of dietary treatments (% of DM).

Ingredient, % of DM	Basal Total Mixed Ration
Corn silage, unprocessed	40.7
Cottonseed hulls	10.3
Soybean hulls	12.7
Ground, dry shelled corn	7.2
48% soybean meal	9.9
Porcine blood meal, ring dried	1.9
Citrus pulp	10.4
Cane molasses, dried	0.48
Sodium bicarbonate	0.83
Salt	0.31
Dicalcium phosphate	0.48
Calcitic limestone	0.39
Vit-TM premix[1]	0.51
Dyna-mate[2]	0.10
Urea	0.64
Ca salts of fatty acids + Treatment[3]	3.1

[1]Vitamin-trace mineral premix. Contained 21.5% Ca; 5.5% S; 3.87% Zn; 3.87% Mn; 1.18% Cu; 9650 ppm Fe; 700 ppm I; 590 ppm Co; 250 ppm Se; 1,215,420 IU/kg Vitamin A; 304,545 IU/kg Vitamin D-3; 3,646 IU/kg Vitamin E.

[2]IMC-AGRICO, Bannockburn, IL.

[3]0.68 kg/d of calcium salts of fatty acids fed alone or blended with RP-met, RP-choline or RP-betaine.

Table 2.2 Chemical composition of dietary treatments. All means are reported as a % of DM unless otherwise indicated.

Item[1]	Mean	SD
DM, % of diet	58.3	2.6
CP	17.6	1.1
Soluble CP	5.4	5.4
NDICP[2]	2.56	0.67
ADICP[3]	1.00	0.20
NDF	36.0	1.7
ADF	23.7	1.5
NFC[4]	36.4	2.0
Starch	19.9	1.7
NE_L, Mcal/kg[5]	1.61	0.22
Fat	3.50	0.27
Ash	6.68	0.69
Ca	0.87	0.13
P	0.38	0.03
Mg	0.28	0.03
K	1.31	0.10

[1]Analysis conducted with n=54 TMR samples.

[2] NDICP = neutral detergent insoluble crude protein.

[3] ADICP = acid detergent insoluble crude protein.

[4] NFC = 100 – (NDF + CP + fat + ash).

[5] $NE_L = 0.866 – [0.007 \times$ ADF (% of DM)].

Table 2.3 Formulated content of Lys to Met ratios in treatment diets.

	Dietary Treatments[1]			
Item	CON	RP-MET	RP-BET	RP-CHOL
Lys to Met ratio[2]	3.88:1	2.96:1	3.88:1	3.88:1
Lys, g/d[3]	163	163	163	163
Met, g/d[3]	42	55	42	42
Lys, % of EAA[4]	14.8	14.6	14.8	14.8
Met, % of EAA[4]	3.8	4.9	3.8	3.8
Lys to Met ratio[5]	3.75:1	2.89:1	3.75:1	3.75:1
Lys, g/d[6]	165	165	165	165
Met, g/d[6]	44	57	44	44
Lys, % of EAA[7]	15.1	14.9	15.1	15.1
Met, % of EAA[7]	4.0	5.1	4.0	4.0

[1]CON = Ca salts of fatty acids; RP-MET = 20 g/d Met; RP-BET = 40 g/d betaine; RP-CHOL = 45 g/d choline.

[2]Calculated from predicted flows of digestible Lys and Met to the small intestine (g/d) according to the NRC (2001).

[3]Predicted flow of digestible Lys and Met to the small intestine (g/d) as calculated by the NRC (2001).

[4]Calculated from NRC (2001) using the predicted flow of digestible Lys, Met, and EAA to the small intestine (g/d).

[5]Calculated from predicted flows of Lys and Met according to the Mepron Dairy Ration Evaluator (Version 2.1, 1999; Degussa-Hüls Corp., Bannockburn, IL).

[6]Predicted flow to intestine formulated according to the Mepron Dairy Ration Evaluator (Version 2.1, 1999; Degussa-Hüls Corp., Bannockburn, IL).

[7]Calculated from the Mepron Dairy Ration Evaluator (Version 2.1, 1999; Degussa-Hüls Corp., Bannockburn, IL) using the predicted flow of digestible Lys, Met, and EAA to the small intestine (g/d).

Table 2.4 Daily milk yield, milk composition, intake, body weight, and body condition score as affected by dietary treatment or parity.

	Dietary Treatments[1]					Effect ($P \leq$)	
Item	CON	RP-MET	RP-BET	RP-CHOL	SEM	Treatment	Parity
Milk							
Yield, kg/d	32.8[a]	33.9[a,b]	32.3[a]	35.8[b]	0.9	0.04	0.01
CP, %	2.54[b]	2.69[a]	2.55[b]	2.59[a,b]	0.04	0.05	0.01
CP, kg/d	0.83[b]	0.90[a]	0.83[b]	0.92[a]	0.03	0.02	0.01
Fat, %	2.89[a]	2.59[b]	2.97[a]	2.78[a,b]	0.10	0.05	0.04
Fat, kg/d	0.93	0.88	0.95	0.98	0.04	0.26	0.01
MUN, mg/dl	17.1	15.8	16.6	16.2	0.6	0.44	0.09
DMI, kg/d	20.8	20.4	20.2	22.3	1.0	0.46	0.01
ECM[2], kg/d	28.9	29.1	29.0	31.3	0.8	0.12	0.01
FCM[3], kg/d	29.3	28.9	29.4	31.4	0.8	0.16	0.01
ECM/DMI, kg/kg	1.42	1.51	1.47	1.45	0.08	0.89	0.01
BW, kg	527	546	527	546	12	0.48	0.01
BCS[4]	2.21	2.29	2.16	2.14	0.08	0.60	0.60

[a,b,c,d]Means within a row lacking a common superscript differ ($P < 0.05$).

[1]CON = Ca salts of fatty acids; RP-MET = 20 g/d Met; RP-BET = 40 g/d betaine; RP-CHOL = 45 g/d choline.

[2]ECM (kg/d) = [milk yield (kg/d) × 0.3246] + [milk fat (kg/d) × 12.86] + [milk CP (kg/d) × 7.04]

[3]FCM (kg/d) = [milk yield (kg/d) × 0.4324] + [milk fat (kg/d) × 16.2162]

[4]Body condition score (five-point scale where 1 = very thin to 5 = obese (Ferguson et al., 1994).

Table 2.5 Daily milk yield, milk composition, intake, body weight, and body condition score as affected by dietary treatment for primiparous cows.

	Dietary Treatments[1]					
Item	CON	RP-MET	RP-BET	RP-CHOL	SEM	$P \leq$
Milk						
Yield, kg/d	27.9	28.0	26.1	27.5	1.3	0.73
CP, %	2.60	2.77	2.67	2.68	0.06	0.28
CP, kg/d	0.73	0.76	0.70	0.74	0.04	0.72
Fat, %	2.97	2.71	3.03	2.93	0.15	0.46
Fat, kg/d	0.84	0.77	0.79	0.79	0.05	0.81
MUN, mg/dl	17.2	15.6	15.8	15.2	0.9	0.40
DMI, kg/d	19.7	20.0	18.8	20.2	1.4	0.90
ECM[2], kg/d	25.5	24.6	23.8	24.8	1.2	0.81
FCM[3], kg/d	25.8	24.4	24.0	24.7	1.3	0.78
ECM/DMI, kg/kg	1.29	1.32	1.30	1.24	0.12	0.97
BW, kg	479	485	476	485	19	0.98
BCS[4]	2.18	2.25	2.16	2.14	0.13	0.93

[a,b,c,d]Means within a row lacking a common superscript differ ($P < 0.05$).

[1]CON = Ca salts of fatty acids; RP-MET = 20 g/d Met; RP-BET = 40 g/d betaine; RP-CHOL = 45 g/d choline.

[2]ECM (kg/d) = [milk yield (kg/d) × 0.3246] + [milk fat (kg/d) × 12.86] + [milk CP (kg/d) × 7.04]

[3]FCM (kg/d) = [milk yield (kg/d) × 0.4324] + [milk fat (kg/d) × 16.2162]

[4]Body condition score (five-point scale where 1 = very thin to 5 = obese (Ferguson et al., 1994).

Table 2.6 Daily milk yield, milk composition, intake, body weight, and body condition score as affected by dietary treatment for multiparous cows.

	Dietary Treatments[1]					
Item	CON	RP-MET	RP-BET	RP-CHOL	SEM	$P \leq$
Milk						
Yield, kg/d	37.7[b]	39.8[b]	38.6[b]	44.1[a]	1.2	0.01
CP, %	2.47	2.61	2.44	2.49	0.06	0.18
CP, kg/d	0.92[c]	1.04[a,b]	0.96[b,c]	1.10[a]	0.04	0.01
Fat, %	2.80	2.46	2.91	2.64	0.13	0.11
Fat, kg/d	1.03[a,b]	0.99[b]	1.11[a,b]	1.16[a]	0.05	0.05
MUN, mg/dl	17.0	16.0	17.4	17.2	0.8	0.58
DMI, kg/d	21.9	20.8	21.7	24.3	1.4	0.32
ECM[2], kg/d	32.3[b]	33.6[b]	34.2[b]	37.8[a]	1.1	0.01
FCM[3], kg/d	32.8[b]	33.3[b]	34.9[b]	38.0[a]	1.1	0.01
ECM/DMI, kg/kg	1.54	1.69	1.64	1.66	0.11	0.79
BW, kg	574	606	577	607	15	0.26
BCS[4]	2.25	2.32	2.17	2.14	0.10	0.59

[a,b,c,d]Means within a row lacking a common superscript differ ($P < 0.05$).

[1]CON = Ca salts of fatty acids; RP-MET = 20 g/d Met; RP-BET = 40 g/d betaine; RP-CHOL = 45 g/d choline.

[2]ECM (kg/d) = [milk yield (kg/d) × 0.3246] + [milk fat (kg/d) × 12.86] + [milk CP (kg/d) × 7.04]

[3]FCM (kg/d) = [milk yield (kg/d) × 0.4324] + [milk fat (kg/d) × 16.2162]

[4]Body condition score (five-point scale where 1 = very thin to 5 = obese (Ferguson et al., 1994).

Table 2.7 Plasma and serum metabolites as affected by dietary treatment or parity.

	Dietary Treatments[1]					Effect ($P \leq$)	
Item	CON	RP-MET	RP-BET	RP-CHOL	SEM	Treatment	Parity
SUN[2], mg/dl	15.2	14.3	15.3	14.3	0.6	0.57	0.18
Plasma NEFA[3], Meq/l	0.473	0.466	0.439	0.417	0.040	0.74	0.01
Serum Total Cholesterol[4], mg/dl	180.9[a,b]	165.6[b]	198.3[a]	189.7[a]	7.2	0.01	0.01
HDL, mg/dl	107.4	96.8	101.6	104.1	2.9	0.08	0.09
LDL[5], mg/dl	68.1[b]	66.2[b]	92.3[a]	82.5[a,b]	6.5	0.02	0.01
VLDL[6], mg/dl	2.93	2.83	2.70	2.86	0.11	0.56	0.93
Serum Triglycerides, mg/dl	14.7	14.1	13.5	14.3	0.6	0.56	0.93
Beta-hydroxybutyric acid, µmol/L	609	449	510	507	51	0.17	0.01
Ketosis Incidence[7]							
Primiparous cows	0	0	0	0			
Multiparous cows	4	2	3	1			

[a,b,c]Means within a row lacking a common superscript differ ($P < 0.05$).

[1]CON = Ca salts of fatty acids; RP-MET = 20 g/d Met; RP-BET = 40 g/d betaine; RP-CHOL = 45 g/d choline.

[2]SUN = Serum urea nitrogen.

[3]NEFA = Nonesterified fatty acids.

[4]HDL = high density lipoproteins; LDL = low density lipoproteins; VLDL = very low density lipoproteins.

[5]LDL (mg/dl) = cholesterol (mg/dl) – [HDL (mg/dl) + VLDL (mg/dl)] (Friedewald et al., 1972).

[6]VLDL (mg/dl) = triglycerides (mg/dl) ÷ 5 (Friedewald et al., 1972).

[7]Ketosis incidence is calculated as the number of cows from each treatment where BHBA > 1400 µmol/L for any of the four sampling times.

CHAPTER THREE

Fermentation of Rumen-protected Forms of Methionine, Betaine, and Choline by Ruminal Microorganisms in Dual-Flow Continuous Culture. *Davidson et al.* Methionine is frequently the first limiting amino acid or co-limiting with lysine in dairy rations, and methionine metabolism is closely linked to that of betaine and choline. Therefore, rumen-protected methionine, betaine, or choline was added to a methionine-limited control diet and fermented in continuous culture in order to evaluate the effects of these products on ruminal microorganisms when compared to the control. Overall, there were limited effects of rumen-protected methionine, betaine, and choline suggesting that all products were protected from ruminal degradation.

RUNNING HEAD: FERMENTATION OF METHYL DONORS

Fermentation of Rumen-protected Forms of Methionine, Betaine, and Choline by Ruminal Microorganisms in Dual-Flow Continuous Culture

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ABSTRACT

Four dual-flow continous culture fermentors (700 ml) were used to determine the effects of supplementation of rumen-protected forms of methionine, betaine, and choline to a Met-limited corn silage-based total mixed ration (**TMR**) on microbial metabolism by mixed ruminal cultures. Fermentors were inoculated with rumen fluid and allowed to stabilize for 2 days. Treatments were added for 5 days of adaptation followed by 3 days of sample collection. One of four supplements was blended into the TMR to produce four dietary treatments: 1.) CON: control supplement (fat only), 2.) RP-MET: rumen-protected methionine (0.09% of DM as methionine), 3.) RP-BET: rumen-protected betaine (0.20% of DM as betaine), or 4.) RP-CHOL: rumen-protected choline (0.18% of DM as choline). Calcium salts of fatty acids were used to protect both RP-betaine and RP-choline supplements and were added to both control and RP-met supplements so that equal amounts of fat were supplied to all treatments. Four replicates were performed with each fermentor receiving each of the four treatments for one replicate. Total volatile fatty acids (**VFA**) and individual VFA concentrations were not affected by dietary treatment, except for propionate and isobutyrate. Propionate production was significantly lower in RP-CHOL than in CON fermentors ($P = 0.05$). Isobutyrate production was significantly higher in CON fermentors than in the ones supplemented with either RP-MET or RP-BET, but was not significantly different from RP-CHOL fermentors ($P = 0.05$). Methane production and pH were similar across treatments ($P > 0.2$). Rumen ammonia concentration was lower for fermentors receiving RP-CHOL than those receiving CON ($P = 0.04$). There were no significant differences in microbial N %, flow,

or efficiency as a result of treatment addition ($P > 0.2$). Overall, there were limited effects of RP-MET, RP-BET, or RP-CHOL suggesting that all products were protected from ruminal degradation.

(**Key words:** fermentation, choline, betaine, methionine)

INTRODUCTION

Methionine (**Met**) is frequently the first limiting amino acid or co-limiting with lysine in dairy rations, and Met metabolism is closely linked to that of betaine and choline. An improved understanding of the mechanisms that regulate these overlapping pathways is needed because these compounds can be fed to lactating dairy cows in a way that potentially will improve lactation performance and reduce the incidence of ketosis and fatty liver (Erdman, 1991). Methionine (Onodera, 1993), betaine (Mitchell et al., 1979), and choline (Atkins et al., 1988) are degraded by microorganisms in the rumen, so rumen-protected (**RP**) forms are more effective at supplying the compounds to the cow than forms that are not protected.

There has been extensive research conducted to develop and determine the effectiveness of technologies for protecting Met (Robinson, 1996; Schwab, 1996). However, very little research has been conducted that investigates the effectiveness of RP-betaine and RP-choline, even though some workers have studied the effects of free choline (Neill et al., 1978; Sharma and Erdman, 1989) and betaine (Mitchell et al., 1979) on ruminal fermentation in cannulated cows.

The objective of this study was to use dual-flow continuous culture fermentors to

determine the effects of supplementation of rumen-protected forms of methionine, betaine, and choline to a Met-limited corn silage-based total mixed ration (**TMR**) on microbial metabolism by mixed ruminal cultures

MATERIALS AND METHODS

Apparatus, Diets, and Design

A corn silage-based TMR was formulated to meet the NRC (2001) recommendations for NE_L, MP, RDP, RUP, macrominerals, microminerals and the vitamins A, D, and E (Table 3.1). This TMR was formulated to contain a limited amount of Met, but adequate Lys, so that the diet supplied a Lys to Met ratio of 3.7:1 according to the Mepron Ration Evaluator (Version 2.6).

All fermentors received the same Met-limited TMR with the addition of one of four supplements: 1.) CON: control supplement (fat only), 2.) RP-MET: rumen-protected methionine (0.09% of DM as methionine), 3). RP-BET: rumen-protected betaine (0.20% of DM as betaine), or 4.) RP-CHOL: rumen-protected choline (0.18% of DM as choline).

All four supplements provided equivalent amounts of Ca salts of fatty acids to the diet. It was necessary to use Ca salts of fatty acids as the primary dietary fat source because both the betaine and choline supplements were protected with this fat source. The supplements containing betaine and choline were fed at levels that supplied equivalent amounts of the compounds on a molecular weight basis, so that equal amounts of methyl groups would be supplied in each treatment. Unlike the betaine and choline supplements, the rumen-protected Met used was an encapsulated product instead of fat-

protected and provided substantially fewer methyl groups. The level of RP-Met supplementation was chosen so that enough Met was provided to result in a 3:1 postruminal Lys to Met ratio in that treatment. As a result, the RP-MET treatment contained adequate dietary Met while the CON, RP-BET, and RP-CHOL treatments all contained limited amounts of Met. All four supplements were mixed thoroughly into the TMR.

To prepare the diets for use in the fermentors, the four treatment diets were chopped for 5 minutes (Blakeslee and Co. FC-19, Cicero, IL). Ruminal fluid was obtained from a fistulated non-lactating Holstein cow fed a 100% forage diet. Samples of rumen contents were taken from several sites within the reticulo-rumen, collected with a vacuum pump fitted with a copper filter, and transported to the laboratory in a sealed, pre-heated container. In the laboratory, the contents were filtered through double-layered cheesecloth into a wide mouth beaker and mixed thoroughly before 700 mL of rumen fluid was transferred into each of four fermentors (Teather and Sauer, 1988). Several hours before introduction of the rumen fluid, the fermentor system was purged with CO_2 gas in order to displace O_2 and maintain anaerobic conditions. The rate of CO_2 gas was fixed at 20 ml/min and a circulating water bath maintained the temperatures of the fermentors at 39º C. A central paddle that was set at a speed of 10 rpm was used to stir the fermentors continuously. Artificial saliva with a pH of 6.8 was prepared as described by Slyter et al. (1966) and delivered through a precision pump at a flow rate of 0.73 ml/min to yield a fractional dilution rate of 6.3%/h. Effluent from each fermentor was collected into containers that were maintained in an ice bath (4° C) to inhibit further

microbial activity.

Following addition of rumen fluid, the fermentors were stabilized for 48 h. During this stabilization period, all four fermentors received the same diet of pelleted alfalfa in the amount of 15 g/d of DM divided into two equal feedings. After stabilization, the fermentors were adjusted to the treatment diets over a 5 day period. Following adjustment, the sampling period lasted an additional 3 days. During the sampling period, approximately 15 g of DM of treatment diets (Table 3.4) were added to the fermentors on a DM basis daily divided into two equal feedings. The entire experiment was repeated four times. The design was balanced with respect to the fermentors so that each of the four fermentors received each of the four treatment diets exactly one time over the course of the four replicates.

Sample Collection and Analysis

Methane was measured five times daily by collecting 10 µl gas samples from the headspace of the fermentor into a gas tight syringe which was analyzed using gas chromatography. The pH of the cultures was monitored continuously and recorded when methane samples were collected. Fermentor contents were sampled twice daily, two hours after both the am and pm feedings. To sample fermentor contents, the fermentors first were mixed thoroughly by temporarily increasing the speed of the central paddle to approximately 100 rpm, and then 5 ml samples of fermentor contents were collected twice daily. These samples were analyzed for volatile fatty acids (**VFA**), ammonia N, DM, NDF, and ADF. Effluent was sampled once on each day of the sampling period and

50 ml were collected for analysis of DM and NDF. Effluent containers were kept on ice and contents were weighed and discarded daily. At the conclusion of the experiment, the entire fermentor contents were collected in order to obtain microbial pellets and determine microbial N content.

Statistical Analyses

The design used was a 4 × 4 Latin square. The four fermentors were used in four replicates so that each of the four treatments was applied to exactly one fermentor in each replicate. In addition, each fermentor received each of the four treatments exactly once during the experiment.

Since all data were collected as repeated measures, they were analyzed using ANOVA according to the mixed procedure of SAS (2004). Dietary treatment and sampling day were fixed effects in the model, and replicate and replicate by treatment were included as random effects. Preliminary analyses accounting for fermentor and replicate showed that fermentor was not important, and this factor was eliminated from the final analyses. Interactions of the main effects with sampling day originally were included in the model, but then were removed since none were significant for any parameter measured. Least squares means for treatments were reported with statistical significance declared at $P < 0.05$. Tendencies for differences are discussed where $0.05 < P < 0.10$.

RESULTS AND DISCUSSION

As reported in Table 3.2, total VFA concentrations were not significantly different as a result of the dietary treatments ($P = 0.17$). Also, molar proportions (mol/100 mol) and production (mmol/d) of acetate, butyrate, isovalerate, and valerate were not significantly different as a result of dietary treatment. However, RP-CHOL fermentors had a significantly lower ($P = 0.05$) molar proportion of propionate and tended to have less daily production of propionate ($P = 0.07$) than fermentors containing CON, RP-MET, or RP-BET. As a result, the ratio of acetate to propionate tended to be higher ($P = 0.10$) for RP-CHOL than for CON, RP-MET, or RP-BET fermentors. Fermentors receiving CON had a significantly higher molar proportion and greater daily production of isobutyrate compared to those receiving RP-MET and RP-BET, but neither value was significantly different from those of RP-CHOL fermentors. Atkins et al. (1988) investigated the effects of unprotected choline supplementation on rumen fermentation and reported a higher percentage of acetate in choline-supplemented cows than in unsupplemented cows. Sharma and Erdman (1988) investigated whether or not the ability of rumen bacteria to degrade choline could be overwhelmed by supplementing up to 326 g/d dietary choline. In their study, there was a quadratic effect of supplementing unprotected choline on both production and molar percentages of propionate such that the cows receiving the intermediate level of choline supplementation (177 g/d) had significantly lower propionate molar percentages and production in their rumens than in those of the unsupplemented cows. Also, the acetate to propionate ratio increased linearly with increased choline supplementation (Sharma and Erdman, 1988).

Overall, previous authors have concluded that choline supplementation results in very limited effects on ruminal fermentation (Atkins et al., 1988; Sharma and Erdman, 1988).

There was no significant difference in pH or methane production as a result of dietary treatment (Table 3.3). The end product of ruminal degradation of the methyl groups of methionine, choline and betaine is methane (Zikakis et al., 1969; Neill et al., 1978). Therefore any ruminal degradation of the three products that occurred was not substantial enough to result in a significant increase in methane production. Dietary supplementation of unprotected choline has resulted in decreased rumen pH (Sharma and Erdman, 1988), so inadequate protection could result in lowered rumen pH, which did not occur in this experiment.

Rumen ammonia concentration was significantly lower in RP-CHOL fermentors than in CON fermentors, but neither was significantly different from RP-MET or RP-BET fermentors (Table 3.3). Studies that have supplemented unprotected forms of Met, choline or betaine have reported either no change or increases in rumen ammonia concentrations as a result of supplementation (Sharma and Erdman, 1988). Reduced rumen ammonia concentration in RP-CHOL fermentors suggests that postruminal N availability was enhanced as a result of RP-choline addition to a Met-limited TMR (CON).

There were no significant differences in fermentability or NDF digestibility as a result of dietary treatment. In addition, there were no significant differences in the N content of microbial cells, flow of cells, or N efficiency as a result of treatment. Because there were no significant effects of treatment on the fermentability of the diets or changes

in methane production or pH, it may be concluded that all products were adequately protected from ruminal degradation.

CONCLUSIONS

Overall, there were very limited effects on ruminal fermentation in continuous culture as a result of supplementing RP-Met, betaine, or choline to a Met-limited corn silage-based TMR. Previous research has shown little effect on ruminal fermentation when either source of these compounds were evaluated intraruminally in cannulated cows (Atkins et al., 1988; Sharma and Erdman, 1988). Because there were minimal effects on *in vitro* fermentation, these results suggest that the RP-Met, betaine, and choline products tested in this experiment were adequately protected from ruminal degradation.

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Table 3.1 Ingredient and chemical composition of the basal diet.

Item	Basal Total Mixed Ration
Ingredient, % of DM	
Corn silage, unprocessed	40.7
Cottonseed hulls	10.3
Soybean hulls	12.7
Ground, dry shelled corn	7.2
48% soybean meal	9.9
Porcine Blood meal, ring dried	1.9
Citrus Pulp	10.4
Cane Molasses, dried	0.48
Sodium bicarbonate	0.83
Salt	0.31
Dicalcium phosphate	0.48
Calcitic limestone	0.39
Vit-TM premix[1]	0.51
Dyna-mate[2]	0.10
Urea	0.64
Ca salts of fatty acids + Treatment[3]	3.1
Nutrients	
DM, % of diet	57.1
CP, % of DM	17.5
Soluble CP, % of DM	5.58
NDICP[4], % of DM	2.91
ADICP[5], % of DM	0.91
NDF, % of DM	35.2
ADF, % of DM	23.1
NFC[6], % of DM	37.2
Fat, % of DM	3.42
NE_L, Mcal/kg	1.61

[1]Vitamin-trace mineral premix. Contained 21.5% Ca; 5.5% S; 3.87% Zn; 3.87% Mn; 1.18% Cu; 9650 ppm Fe; 700 ppm I; 590 ppm Co; 250 ppm Se; 1,215,420 IU/kg Vitamin A; 304,545 IU/kg Vitamin D-3; 3,646 IU/kg Vitamin E.
[2]IMC-AGRICO, Bannockburn, IL.
[3]Equal amounts of Ca salts of fatty acids were provided with either no rumen-protected supplement, RP-Met, RP-betaine, or RP-choline.
[4]NDICP = neutral detergent insoluble crude protein.
[5]ADICP = acid detergent insoluble crude protein.
[6]NFC = 100 – (NDF + CP + fat + ash).

Table 3.2 Volatile fatty acid (VFA) production and concentration as affected by rumen-protected supplement addition.

	Dietary Treatments[1]					
VFA	CON	MET	BET	CHOL	SEM	$P \leq$
Total, m*M*	70.57	64.11	67.02	65.80	1.97	0.17
A: P[2]	2.40	2.38	2.46	2.96	0.17	0.10
Individual, mol/100 mol						
Acetate	50.91	52.43	51.52	54.24	1.18	0.24
Propionate	21.47[a]	22.38[a]	21.16[a]	19.03[b]	0.70	0.05
Isobutyrate	0.99[a]	0.76[b]	0.83[b]	0.89[a,b]	0.06	0.05
Butyrate	19.72	18.12	20.23	19.12	1.05	0.54
Isovalerate	4.96	4.84	4.97	4.94	0.12	0.87
Valerate	1.95	1.48	1.32	1.96	0.25	0.21
Individual, mmol/d[3]						
Acetate	37.84	35.21	36.32	37.38	1.24	0.41
Propionate	15.86	15.04	14.89	13.22	0.65	0.07
Isobutyrate	0.73[a]	0.51[b]	0.58[b]	0.62[a,b]	0.04	0.01
Butyrate	14.63	12.25	14.27	13.17	0.89	0.28
Isovalerate	3.68	3.26	3.50	3.43	0.13	0.22
Valerate	1.45	0.99	0.93	1.36	0.18	0.15
Total, mmol/d[3]	74.19	67.40	70.46	69.17	2.07	0.17

[a,b,c,d]Means within a row lacking a common superscript differ ($P < 0.05$).
[1]CON = no rumen-protected supplement; MET = 0.09% of DM Met as RP-methionine; BET = 0.20% of DM betaine as RP-betaine; CHOL = 0.18% of DM choline as RP-choline.
[2]A: P = ratio of acetate to propionate.
[3]Production of VFA is based on 700 mL of ruminal cultures.

Table 3.3 Methane (CH_4) production, pH, and ruminal ammonia (NH_3-N) concentration as affected by dietary treatment.

	Dietary Treatments[1]					
Item	CON	MET	BET	CHOL	SEM	$P \leq$
CH_4, mmol/d	38.8	33.5	36.8	34.9	4.8	0.87
pH	5.4	5.5	5.5	5.5	0.1	0.86
NH_3-N, mg/dl	93.4[a]	87.7[a,b]	86.1[a,b]	75.1[b]	5.4	0.04

[a,b,c,d]Means within a row lacking a common superscript differ ($P < 0.05$).

[1]CON = no rumen-protected supplement; MET = 0.09% of DM Met as RP-methionine; BET = 0.20% of DM betaine as RP-betaine; CHOL = 0.18% of DM choline as RP-choline.

Table 3.4 Digestion, fermentability and microbial growth as affected by dietary treatment.

	Dietary Treatments[1]					
Item	CON	MET	BET	CHOL	SEM	$P \leq$
DM fed, g/d	14.9	14.9	15.0	14.8	0.1	0.44
Fermentability[2], %	58.6	53.2	55.9	54.1	1.9	0.27
NDF digestibility[3], %	28.9	29.1	30.8	28.9	0.7	0.25
NH_3-N flow[4], g/d	0.98[a]	0.92[a,b]	0.90[a,b]	0.79[b]	0.06	0.04
Microbial N						
%[5]	7.70	8.22	8.16	7.87	0.35	0.62
Flow[6], g/d	0.021	0.026	0.022	0.021	0.003	0.62
Efficiency[7], g/kg	1.26	2.01	1.53	1.42	0.34	0.43

[a,b,c,d]Means within a row lacking a common superscript differ ($P < 0.05$).
[1]CON = no rumen-protected supplement; MET = 0.09% of DM Met as RP-methionine; BET = 0.20% of DM betaine as RP-betaine; CHOL = 0.18% of DM choline as RP-choline.
[2]Total substrate fermented expressed as a percentage of DM fed.
[3]NDF digestibility (%) = {diet NDF (% of DM) – [fermentor NDF (% of DM) × diet NDF (% of DM)]} ÷ diet NDF (% of DM).
[4]NH_3-N flow (g/d) = (NH_3-N concentration (mg/100 ml) × fermentor volume (700 ml) × 15.98 (turnover rate of fermentor)) ÷ 1,000.
[5]Microbial N (%) = microbial N (g) ÷ microbial DM (g) × 100.
[6]Microbial N flow (g/d) = total microbial DM in fermentor × 0.0455 (flow rate) × 24 hr/d × microbial N (% of DM) ÷ 100.
[7]Efficiency of microbial protein synthesis expressed as g of N per kg OM fermented.

CHAPTER FOUR

OVERALL SUMMARY

The overall objective of these experiments was to gain information that will help define the role of betaine and choline in Met metabolism in lactating dairy cows in order to improve lactation performance by increasing the production of milk and milk components and by improving heath by reducing the incidence of fatty liver and ketosis.

The metabolism of Met, betaine, and choline is interrelated; therefore dietary supply of Met, betaine, and choline is dependent on the supply of each of the other two compounds as well as other factors such as the production level of the cow and the supply of other components of the diet. More research with RP forms of betaine and choline is necessary to improve our understanding of the metabolism of these compounds and their effects on Met metabolism.

The *in vitro* experiment reported in Chapter 3 illustrated that because there were minimal effects on ruminal fermentation by the RP-Met, RP-betaine, and RP- choline products, the products were adequately protected from ruminal degradation. As a result of the experiment reported in Chapter 2, feeding RP-choline to cows that received a Met-limited diet improved milk yield and feeding RP-choline and RP-methionine resulted in increased milk CP yield, while supplementing RP-betaine was not beneficial. Therefore, it appeared that there was no enhancement of methionine production from homocysteine which suggested that the effect of RP-choline was due to increased supply of phosphatidylcholine. However, these responses to RP-choline could be the result of increasing phosphatidylcholine, supplying methyl groups, or providing methionine.

Further research that investigates the effects of changes in supply of Met, betaine, and choline on the regulation of the enzymes that control the pathways of Met, betaine, and choline metabolism is necessary to improve our understanding of how to supply these compounds to the lactating dairy cow effectively.

APPENDICES

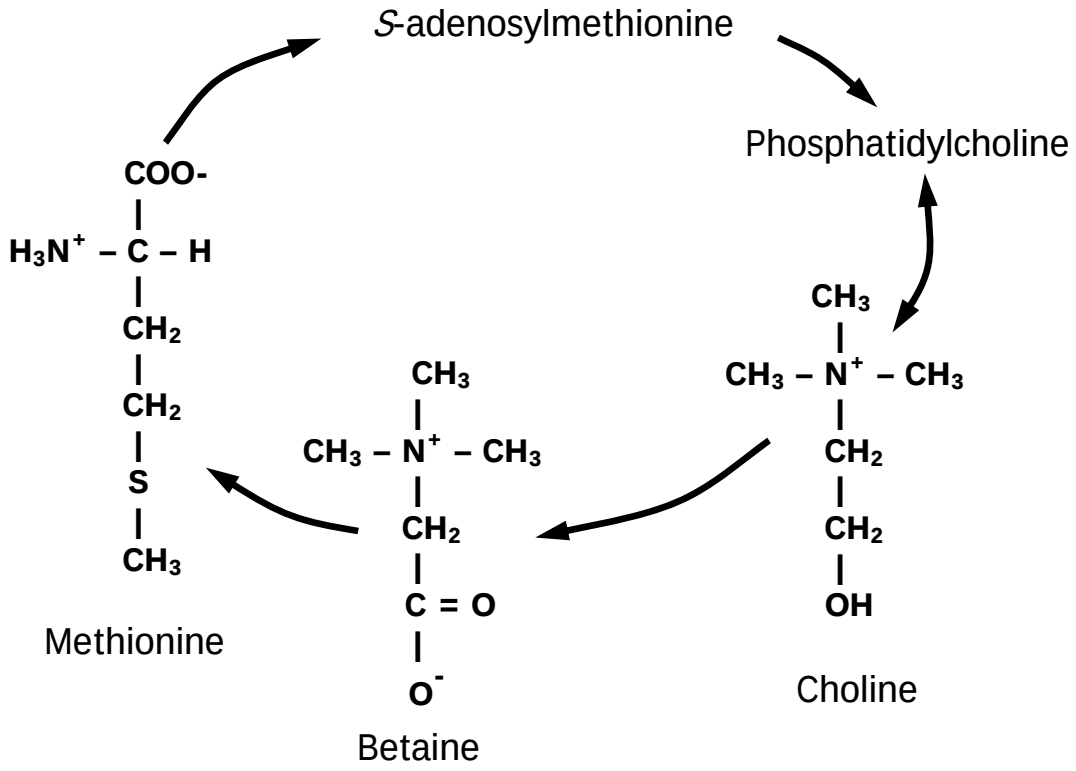

Figure A.1 The structures of methionine, betaine, and choline and their metabolic relationship.

Table A.1 Composition of calcium salts of fatty acid product used in the experiments described in Chapter 2 and Chapter 3 (provided by Robt. Morgan, Inc).

Date of Analysis	**September 2002**	**October 2002**	**November 2002**
Fatty acid:	**----------molar %----------**		
$C_{8:0}$	0.020	0.005	0.016
$C_{10:0}$	0.014	0.061	0.003
$C_{12:0}$	0.055	0.062	0.040
$C_{14:0}$	0.064	0.008	0.060
$C_{15:0}$	0.022	0.026	0.007
$C_{16:0}$	4.108	4.307	4.201
$C_{16:1}$	0.044	0.091	0.051
$C_{17:0}$	0.070	0.030	0.026
$C_{17:1}$	0.110	0.031	0.034
$C_{18:0}$	3.832	6.446	4.673
$C_{18:1}$	28.864	31.478	30.138
$C_{18:2}$	55.377	50.222	54.208
$C_{18:3}$	4.736	4.323	4.793
$C_{20:0}$	0.462	0.510	0.294
$C_{20:1}$	1.468	1.534	0.796
$C_{22:0}$	0.179	0.110	0.115
$C_{22:1}$	0.421	0.460	0.214
$C_{24:0}$	0.127	0.294	0.282
Monounsaturated	30.932	33.602	31.261
Polyunsaturated	60.114	54.546	59.001
Saturated	8.954	11.852	9.737

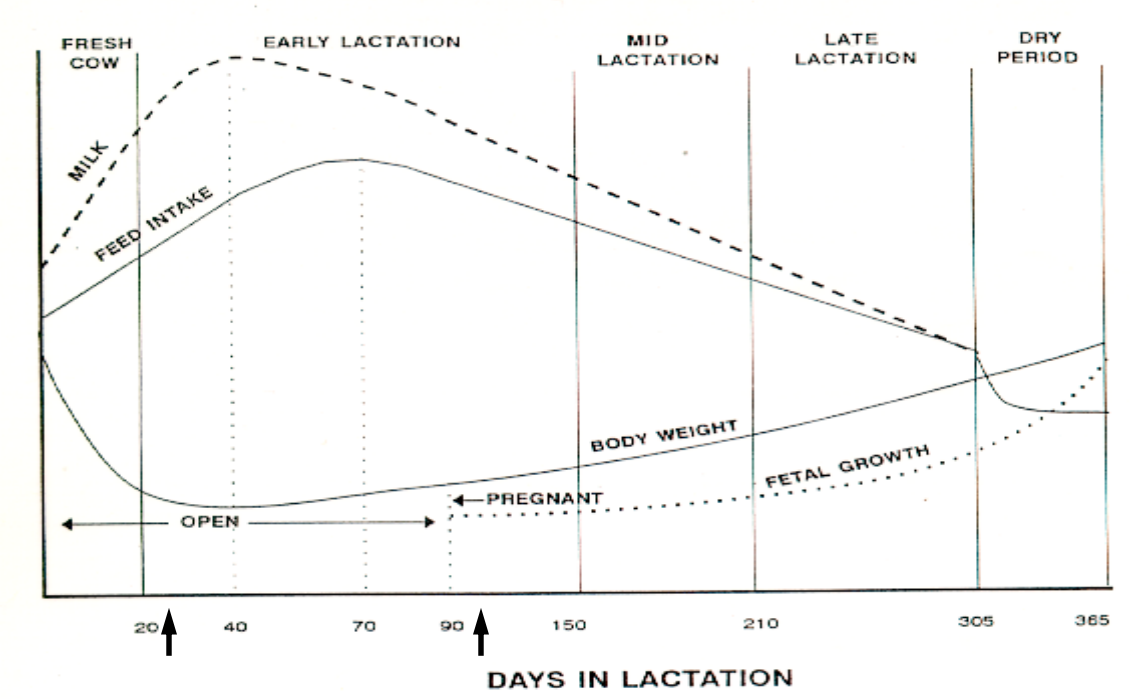

Figure A.2 The typical lactation cycle and reproductive cycle of high producing dairy cows with days in milk (DIM) on the x-axis and relative levels of milk yield, feed intake, and body weight on the y-axis [Figure 34.1, *Large Dairy Herd Management*, H. H. Van Horn and C. J. Wilcox (ed.)]. Bold arrows beneath the figure indicate the beginning (21 DIM) and endpoint (91 DIM) of the experiment reported in Chapter 2.

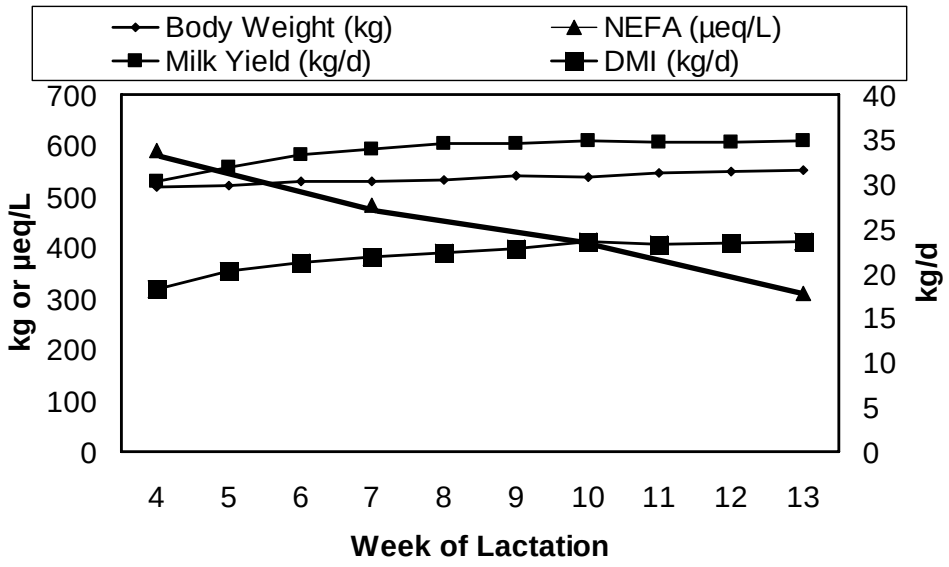

Figure A.3. Milk yield (kg/d), dry matter intake (DMI) (kg/d), body weight (kg), nonesterified fatty acid concentration (NEFA) (µeq/L) reported by week in lactation for the experiment reported in Chapter 2. The weeks are significantly different for NEFA and DMI ($P < 0.01$).