The effects of methyl donors and modulated methyl group status on health and performance in growing cattle

by

Madeline Sue Grant

B.S., University of Missouri, 2017

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Animal Sciences and Industry
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2020

Approved by:

Major Professor
Evan C. Titgemeyer

Copyright

Abstract

Two experiments were conducted to evaluate the effects of methyl donor supplementation to growing cattle. In the first experiment, 384 crossbred heifers (222 kg initial BW) were utilized to study effects of methionine supplementation on high-risk receiving cattle. Heifers were limit-fed at 2.2% BW daily a corn- and corn-byproduct-based diet and supplemented with 0 (control) or 10 g/d Smartamine M to provide 6 g/d metabolizable methionine. Pen weights were collected weekly to determine feed offerings the following week. Individual BW and tail-vein blood samples were collected on d 0, 14, and 45. Plasma haptoglobin was measured to assess inflammatory status. Incidences of morbidity (1.6% for control, 2.6% for SM) and mortality (0.5% for both control and SM) were low. Between d 0 and 45, no differences were observed for ADG or G:F ($P \geq 0.28$). An interaction between treatment and linear effect of day was detected for plasma haptoglobin ($P = 0.05$); over time haptoglobin increased more for control than for SM. In the second study, six ruminally cannulated Holstein steers (200 kg) were used in a 6 × 6 Latin square design with 10-d periods to evaluate the effects of modulated methyl group status on protein deposition and immune function. Factorial treatments included 3 methyl group modulators (MGM: control; 15 g/d guanidinoacetic acid [GAA]; or 16.8 g/d creatine) and 2 levels of choline (0 or 5 g/d choline ion); treatments were continuously infused abomasally. Providing GAA or creatine increases body creatine supply, but GAA will consume methyl groups to synthesize creatine, whereas supplemental creatine spares methyl groups otherwise used for its synthesis. Total fecal and urine collections occurred from d 7 to 9 to measure N balance and jugular blood was collected on d 10. No interactions between MGM and choline were observed. GAA increased N retention ($P = 0.04$), whereas creatine did not ($P = 0.56$). Plasma and urinary creatine were increased by GAA and creatine ($P \leq 0.01$), with

GAA leading to a larger increase. No effects of MGM on plasma haptoglobin ($P = 0.97$) or ex vivo neutrophil oxidative burst or phagocytosis ($P \geq 0.30$) were observed, but creatine did reduce plasma antioxidant capacity ($P \leq 0.01$). Choline did not affect N retention ($P = 0.69$) but increased plasma creatine ($P = 0.04$). Choline tended to reduce plasma haptoglobin ($P = 0.07$) but did not affect antioxidant capacity. Choline tended to reduce neutrophil phagocytosis in the presence of LPS ($P = 0.09$) but did not affect neutrophil phagocytosis in absence of LPS or neutrophil oxidative burst in the presence or absence of LPS ($P \geq 0.29$). These data demonstrate that GAA has potential to improve protein deposition in growing cattle fed corn-based diets. Also, choline may improve body creatine status and alter neutrophil function. Overall, methionine and choline appear to mitigate inflammation in growing cattle.

Table of Contents

List of Figures

List of Tables

Acknowledgements

As a native of Columbia, Missouri, the state of Kansas is often equated to the darkness of the elephant graveyard in Walt Disney's *The Lion King*. Throughout the last two and a half years of my graduate training at K-State, I have come to know a much fonder version of Kansas and the people it holds. Here, I will attempt to thank the numerous individuals that made the completion of this thesis possible.

I wouldn't be in Kansas if not for my major professor, Dr. Evan Titgemeyer. Evan, thank you for mentoring me throughout my master's program and for sharing your wit and brilliance with me. You continually show me what it means to do good science and how to think critically; your impact upon my life has been profound. I am forever grateful for the opportunity to work with you and am so thankful to continue my journey in higher education under your advisement.

To my committee members, Drs. Dale Blasi and Barry Bradford, thank you for your guidance, unwavering support, and practical contributions to my research. Dale, I appreciate your kindness and generosity so much; I know your door is always open. Thank you for always keeping me on my toes and never giving me the easy way out. Barry, thank you for your input on both my studies and for helping me not only understand, but also appreciate, the world of inflammation and immune function. Also, many thanks to Dr. Matt Miesner for being instrumental in the monitoring of my steers' health and for being an incredibly patient teacher.

I would likely still be in the lab today if it weren't for the following people's assistance. Haixia Liu, thank you for your assistance in analyzing samples for my first study and for answering every question I had with a smile. Additionally, Reshma Antony and Cheryl Armendariz were instrumental to sample analysis for my second study, thank you both for everything. Theresa Rathbun, thank you for your patience and flexibility when I had samples to

analyze in Weber. Hope Aufdemberge, thank you for all your work on my second trial and for helping make time in the lab more enjoyable. Finally, Dr. Laman Mamedova, thank you for every minute you spent with me in the lab or your office. I am by no means a molecular biologist, but through your training I feel less like an accident waiting to happen while running sensitive assays. You are a true friend and I appreciate you.

To my incredible lab group, Kim Pearl and Hannah Speer, thank you for your training in the art of conducting metabolism studies. Few people could make fecal and urine sampling and washing metabolism crates fun, but you both did. Kim, thank you for always bringing a smiling face and kind heart to the table, even when times were tough. I know you're never more than a message away when I need something, that and your friendship mean so much. Hannah, thank you for being a tremendous colleague and an even better friend. I know if I ever have a question about methyl group metabolism or life that I can come to you. I miss our late-night discussions about science (and not science) over Bang energy drinks in the graduate office so much. Additionally, thank you to the undergraduate research assistants for their assistance with my metabolism study. Shelby, Brigid, and D. J., your duties were far from glamorous, but without you my life would've been much more challenging.

Next, I would also like to thank Bill Hollenbeck, Ross Wahl, and the rest of the K-State Beef Stocker Unit crew. Bill, thank you for challenging me to conduct practical, high-quality research; I have learned so much from you about cattle. Ross, I appreciate your patience and willingness to help whenever I needed it, especially in training me in the art of tail bleeding. Marissa Johnson, thank you for all your help during my receiving trial. Thank you all for accepting me into the Stocker Unit family, I am incredibly grateful for the camaraderie.

Thank you to the fellow graduate students in the Ruminant Nutrition Graduate Office; whether it was 7:00 a.m. or p.m., there was rarely a dull moment. Adrian (and Danelle), Luis, Eduarda, Vanessa, and Steve Bear—you are all incredible friends and colleagues. Kelsey Batson and Brennan Hammers, I'm so glad I had the opportunity to really get to know you both here in Kansas. You are great neighbors and even better friends; I'm going to miss you both dearly when you move. A special shout out to Zach Duncan for helping with my research when you didn't have to, putting up with me as a roommate, and constantly telling me to, "Go write your thesis!" You make grad school so much more fun and are like the brother I never had.

My home is in Missouri, as is the home of many people in my life that made my journey to Kansas for graduate school possible. Kaitlin Epperson, you are my closest and most cherished friend and I miss our time in Missouri together so much. Although graduate school has taken us to different states, I have never questioned your unwavering faith in me and support of my goals. Without your consistent encouragement to reach those goals, it is unlikely that I would be where I am today. Although life has thrown us immense curve balls, your friendship has always been as true as Missouri is home, thank you for that. Perhaps most importantly, I want to thank my family. Mom and Dad, you have pushed me to seek out and achieve every goal I've ever set and loved and supported me every step of the way. Thank you for instilling in me an appreciation for hard work and a genuine love for agriculture. Grandma Dorothy, thank you for participating in our endless game of phone tag and for always pushing me forward, even if it means I don't live a mile down the road anymore. To my siblings, Lily and Sarah, we haven't always been the best of friends but I'm glad we've grown closer with age. Lily, I am especially thankful that you always keep up with me; even though I act annoyed with you sometimes, I truly appreciate your support. I love all of you so much, thank you.

Dedication

To my late grandpa, James Earl Grant, who would have been so proud.

Thank you for teaching me some of the most important life lessons:

"Don't take any wooden nickels!"

"Don't stick beans up your nose!"

"Keep your powder dry!"

I love and miss you so much.

Chapter 1 - Review of Literature

Introduction

Optimal protein nutrition in cattle is important for ensuring correct nutrient delivery to the animal while managing diet costs. Technically, animals do not have a true requirement for protein itself, but rather requirements for amino acids, the individual building blocks that construct proteins. Optimizing the dietary amino acid profile for monogastric animals is much easier than for ruminants. This is because ruminants rely on amino acids of microbial and dietary origin to support growth and other metabolic processes involving amino acids. Additionally, the requirements for these amino acids in cattle varies depending on stage of production and desired level of performance. Growing cattle have a greater amino acid requirement than do mature cattle because skeletal muscle growth demands are much greater in growing animals. Direct provision of growth-limiting amino acids (or products they produce when metabolized in the body) to growing cattle may improve animal performance and growth efficiency. Methionine is considered the first-limiting amino acid for growth in cattle when microbial protein is the primary source of metabolizable protein (Richardson and Hatfield, 1978). Beyond its importance for protein synthesis, when converted to *S*-adenosylmethionine (SAM), methionine participates in over a hundred methylation reactions in the body (Lobley, 1992). Two of the most quantitatively important methylation reactions are choline synthesis and the conversion of guanidinoacetic acid (GAA) to creatine. Once synthesized, choline is actively involved in several metabolic processes, including serving as a potential methyl donor to resynthesize methionine. Beyond their use as methyl donors, methionine and choline have potential to modulate immune function in cattle. The review herein will outline the pathways and interrelationships between

methionine, choline, and creatine metabolism and the effects of their supplementation on performance, methyl group metabolism, and immune function in cattle.

Methionine metabolism

Methionine is an essential sulfur-containing amino acid that participates in numerous essential bodily processes, which makes determining its requirement and efficiency of use difficult. Namely, it is involved in protein synthesis, acts as the initiating amino acid in translation during protein synthesis, participates in polyamine synthesis, and acts as a widely utilized methyl group source in the body.

Methionine can be activated to form *S*-adenosylmethionine (SAM), which serves as the primary methyl group donor in hundreds of transmethylation reactions in the body (Lobley, 1992). Once SAM donates its methyl group to an acceptor, it becomes *S*-adenosylhomocysteine (SAH), which is then hydrolyzed to yield homocysteine. Once formed, homocysteine can either be remethylated to resynthesize methionine or move through the transsulfuration pathway to produce cysteine. Transmethylation, remethylation, and transsulfuration are the three predominant reactions in methionine metabolism beyond protein synthesis.

Synthesis of *S*-adenosylmethionine occurs when methionine adenosyltransferase (MAT) transfers an adenosyl group from ATP to methionine. Three protein variants of MAT (MAT I, MAT II, and MAT III) exist throughout the body. Both MAT I and III are present in the liver, whereas MAT II predominates in all other tissues (Finkelstein, 2006). Tight enzymatic regulation of methionine metabolism allows methionine to be conserved when supply is limited and cleared when supply is in excess. The three MAT isoforms differ in their regulation; MAT I and II activities decrease with increasing methionine supply and are feedback inhibited by SAM,

whereas MAT III is activated by both dietary methionine and SAM production (Finkelstein, 1998). In situations of surplus methionine, MAT III allows for continued hepatic production of SAM because of its low affinity and high capacity for methionine (Finkelstein, 2006). Once activated, SAM can either be decarboxylated for polyamine synthesis (less than 10% of total SAM) or, more significantly, serve as a methyl donor in hundreds of methyltransferase reactions (Finkelstein, 1998). Namely, these reactions include synthesis of creatine, phosphatidylcholine, neurotransmitters, as well as methylation of histones, DNA, and RNA (Walker, 1979). Although these reactions are catalyzed by numerous enzymes, all are feedback inhibited by SAH, the product of SAM-dependent transmethylation reactions (Finkelstein, 1998).

Because accumulation of SAH hinders its further production, it must be removed from the system to allow for transmethylation reactions to proceed (Finkelstein, 1998). First, *S*-adenosylhomocysteine hydrolase (SAHH) catalyzes a reversible reaction converting SAH into homocysteine and adenosine (Brosnan and Brosnan, 2006). The equilibrium of this reaction favors SAH production from homocysteine and adenosine, so constant homocysteine removal is necessary to prevent SAH accumulation (Finkelstein, 1998). Once formed, homocysteine can either be remethylated to form methionine or move through the transsulfuration pathway.

Synthesis of methionine via remethylation proceeds through one of two reactions. Methionine synthase (MS), also referred to as methyltetrahydrofolate-homocysteine methyltransferase, utilizes a methyl group from 5-methyltetrahydrofolate (5-MTHF) to methylate homocysteine (Finkelstein, 1998) and depends on methylcobalamin (vitamin B_{12}) as a cofactor. In addition to producing methionine, this reaction is critical for regeneration of tetrahydrofolate (THF), which is a part of the folate cycle (Finkelstein, 1998). Once THF is regenerated, it reacts with serine by serine hydroxymethyltransferase, a vitamin B_6 dependent enzyme, to form 5,10-

methylenetetrahydrofolate (5,10-MTHF) and glycine (Brosnan and Brosnan, 2006). Then, methylenetetrahydrofolate reductase (MTHFR) can reduce the methylene bond of 5,10-MTHF to produce 5-MTHF for use in further homocysteine methylation (Finkelstein, 1998; Brosnan and Brosnan, 2006), which relies on vitamin B_2 derived flavin adenine dinucleotide (FAD) as a cofactor. The second remethylation reaction occurs when betaine-homocysteine methyltransferase (BHMT) transfers a methyl group from betaine to homocysteine to reform methionine. Betaine used for remethylation can be derived from either the diet or from choline oxidation by enzymatic action of choline oxidase. In addition to methionine, dimethyl glycine is produced from the BHMT reaction, which can donate its two methyl groups for the reformation of 5,10-MTHF, which can be converted to 5-MTHF and donate its methyl group to homocysteine to form methionine via MS (Finkelstein, 1998).

Activities of MS and BHMT have been well researched in monogastrics, with less work conducted in ruminants. Methionine synthase is active in all mammalian tissues (Finkelstein et al., 1971; Lambert et al., 2002), which is not surprising given its importance in THF regeneration in addition to its role in methionine resynthesis. Conversely, BHMT is only present in select tissues throughout the body. In monogastrics, BHMT is most active in the liver, but also present in the pancreatic and renal tissues (Finkelstein et al., 1971; Brosnan et al., 2009). In sheep, BHMT is present in the liver, pancreas, and kidney, with pancreatic activity being five-fold that of liver (Xue and Snoswell, 1985). In contrast, in growing cattle Lambert et al. (2002) found comparable BHMT activity in hepatic and pancreatic tissues, with minor activity also present in splenic tissue. Both MS and BHMT are activated when methionine supply is limited and inhibited when methionine is in excess (Finkelstein, 1998). Additionally, BHMT and MS have a high affinity for homocysteine, suggesting that remethylation is preferential when dietary

methionine is limiting (Finkelstein, 1998). Homocysteine that is not remethylated must be consumed via the transsulfuration pathway to maintain the equilibrium of SAH hydrolysis toward homocysteine production.

The transsulfuration pathway begins when cystathionine β-synthase (CBS), a vitamin B_6 (pyridoxal phosphate) dependent enzyme, combines homocysteine with serine in an irreversible reaction to form cystathionine (Finkelstein, 1998). Then, in another pyridoxal phosphate dependent reaction, cystathionine is hydrolyzed by cystathionase (cystathionine γ-lyase) to form cysteine, α-ketobutyrate, and an ammonium ion (Brosnan and Brosnan, 2006). Once formed, cysteine can be utilized for protein synthesis or diverted toward synthesis of glutathione, taurine, or sulfate (Finkelstein, 2006). Both SAM and SAH are activators of CBS, which further supports that methionine metabolism is diverted toward transsulfuration when dietary methionine is high (Finkelstein, 1998). Because the transsulfuration pathway is irreversible, it represents methionine oxidation as opposed to methionine conservation via remethylation (Brosnan and Brosnan, 2006). Activity of CBS is present in the liver, kidney, pancreas, and intestine of mammals (Finkelstein et al., 1971; Brosnan and Brosnan, 2006), and additionally in ruminal tissue of growing cattle (Lambert et al., 2002).

Methionine present in excess of protein synthesis and methyl group requirements can also be disposed in conjunction with activity from the enzyme glycine *N*-methyltransferase (GNMT). Because MAT III is activated by high dietary methionine, methionine will continue to produce SAM in the liver when its supply is greater than metabolic needs for methyl donation. Then, GNMT, which is activated by SAM, can transfer a methyl group from SAM to glycine to form sarcosine, also known as methyl-glycine (Finkelstein, 1998). Sarcosine can then donate its methyl group to form 5,10-MTHF, which is reduced to 5-MTHF and can in turn be diverted

toward methionine resynthesis via MS (Finkelstein, 1998). The GNMT pathway is particularly useful because it consumes excess methyl groups while producing a benign product.

Choline metabolism

Choline is an essential nutrient that is present in some feedstuffs or can be produced through *de novo* synthesis in the liver. Choline is extensively degraded in the rumen, so very little dietary choline is available for absorption in the small intestine (Sharma and Erdman, 1988). As a result, choline metabolism in ruminants appears to be somewhat different than in monogastric animals.

Choline is synthesized in the body during phospholipid modification, where phosphatidylethanolamine *N*-methyltransferase (PEMT) transfers three methyl groups from SAM to phosphatidylethanolamine, which produces phosphatidylcholine (Vance, 2014). Once synthesized, phosphatidylcholine can be utilized in cell membranes or incorporated into very light density lipoproteins (VLDL) for liver lipid export. When biological needs for phosphatidylcholine are met, choline ion can also be cleaved from phosphatidylcholine to provide free choline (Zeisel, 2006). Dietary choline can also be phosphorylated and in turn be used to synthesize phosphatidylcholine (Pelech and Vance, 1984). Choline of dietary or de novo origin can be oxidized to form betaine by the enzyme choline oxidase. Betaine acts as a methyl donor in the body and betaine-homocysteine methyltransferase (BHMT) utilizes a methyl group from betaine to methylate homocysteine to form methionine. The second and third methyl groups from betaine (as dimethylglycine or sarcosine) can be transferred to 5,10-MTHF, which is further converted to 5-MTHF to remethylate homocysteine to methionine.

Enzymatic activity of choline metabolism differs between species. Because dietary choline bioavailability is low in ruminants, choline synthesis via PEMT is a major consumer of methyl groups in ruminants (Lobley et al., 1996; Stead et al., 2006). Activity of PEMT in rat liver was increased when rats were fed a diet deficient in choline (Cui and Vance, 1996). This creates a scenario similar to ruminants where very little dietary choline is absorbed in the small intestine. As a result, ruminant choline availability is limited by methyl group availability for its synthesis and is logically regulated differently than in monogastrics under normal conditions. Choline oxidation is an active pathway in rat liver, but remarkably less so in sheep liver (Xue and Snoswell, 1986). Activity of BHMT is also strikingly lower in sheep liver than rat liver (Xue and Snoswell, 1985a). In growing cattle, Lambert et al. (2002) detected similarly low BHMT activity in liver tissue. Low choline oxidase and BHMT activity suggests that much of choline synthesized in the body may not be used to support homocysteine remethylation in ruminants.

Creatine metabolism

Creatine is a molecule that functions to maintain energy homeostasis in muscle tissue throughout the body. It serves as a shuttle of high-energy phosphate bonds when phosphorylated by creatine kinase, and subsequently it can be used to replenish ATP supplies in tissues with high energy demand, such as skeletal and cardiac muscle (Wyss and Kaddurah-Daouk, 2000). Growing animals have a particularly high creatine requirement to support tissue development and protein deposition needs (Walker, 1979). Creatine synthesis begins when arginine and glycine react to form guanidinoacetic acid (GAA).

Guanidinoacetic acid (GAA) acts as the precursor to creatine. The enzyme L-arginine:glycine amidinotransferase (AGAT) catalyzes the reaction where the amidino group

from arginine is transferred to the amino group of glycine, producing GAA and L-ornithine (Brosnan and Brosnan, 2007). From there, guanidinoacetate *N*-methyltransferase (GAMT) transfers a methyl group from SAM to GAA, yielding creatine and SAH (Wyss and Kaddurah-Daouk, 2000). In mammals, AGAT activity is most active in the kidneys and pancreas (Wyss and Kaddurah-Daouk, 2000; Brosnan et al., 2009). Activity of AGAT is feedback inhibited by creatine production, which prevents GAA production from exceeding the endogenous need for creatine (Stead et al., 2001). Conversely, GAMT activity is greatest in mammalian liver and pancreas and low in the kidney (Brosnan et al., 2009). Feedback inhibition of GAMT by creatine has not been observed, but its other product, SAH, may inhibit further methylation of GAA to creatine (Clarke and Banfield, 2001). A majority of creatine synthesized in mammals originates from GAA produced in the kidney and transported to the liver to be methylated to form creatine, which can be exported to extrahepatic tissues in the blood (Wyss and Kaddurah-Daouk, 2000).

Once transported to extrahepatic tissues, creatine can be utilized to replenish ATP when energy demand is high. Creatine kinase catalyzes the reversible reaction which transfers a phosphate group from ATP to creatine, forming phosphocreatine and ADP (Brosnan and Brosnan, 2007). When ATP stores are depleted, creatine kinase can transfer the phosphate group from phosphocreatine to ADP, yielding ATP and creatine (Wyss and Kaddurah-Daouk, 2000), thus allowing for further energetic use of ATP. This shuttle system is particularly useful in tissues with high and fluctuating energy demand, because phosphocreatine can be stored in tissues and is readily available when needed (Brosnan and Brosnan, 2007). Although creatine may undergo multiple phosphorylation/dephosphorylation cycles by creatine kinase, it is not indefinitely recycled; creatine is subject to irreversible conversion to creatinine.

The spontaneous conversion of creatine and phosphocreatine to creatinine is nonenzymatic and represents permanent loss of creatine from the body (Brosnan and Brosnan, 2007). Once formed, creatinine is quantitatively lost by urinary excretion at a rate of approximately 1.7% of total body creatine per day (Wyss and Kaddurah-Daouk, 2000). The constant loss of creatine as creatinine represents a need for constant replenishment of body creatine stores, either through the diet or de novo synthesis (Brosnan and Brosnan, 2007). Because creatine is only synthesized by vertebrates, animals that consume a vegetarian diet rely exclusively on de novo creatine synthesis to meet their demand (Brosnan and Brosnan, 2007). This includes ruminants, which often consume an entirely plant-based diet and therefore little to no dietary creatine. Because hepatic methylation of GAA to creatine by GAMT is not inhibited by creatine, exogenous GAA provision has the potential to increase endogenous creatine production. This process also irrevocably consumes methyl groups from SAM, which has potential to create a methyl group deficiency if methionine is not available in excess. This has been demonstrated in studies supplementing GAA in excess to rats (Stead et al., 2001; Setoue et al., 2008).

Guanidinoacetic acid supplementation to growing cattle

Recent work has evaluated the effects of GAA supplementation to growing cattle. Delivery of GAA in these studies was via either abomasal (Ardalan et al., 2016; Ardalan et al., 2020; Speer, 2019) or ruminal (Speer, 2019) infusion. To date, research has evaluated the effects of GAA supplementation in conjunction with methionine, which serves as a methyl donor (Ardalan et al., 2016, 2020; Speer, 2019). In Holstein heifers fed corn-based diets, Ardalan et al. (2020) evaluated the effects of abomasal infusions of 0, 10, 20, 30, or 40 g/d GAA combined

with either 0 or 12 g/d of methionine on creatine and methyl group status. Plasma and creatine concentrations responded quadratically to increasing GAA supplementation, with all levels increasing creatine concentrations up to 30 g/d; however, at 40 g/d GAA plasma creatine concentration returned to levels similar to when no GAA was provided in the absence of methionine. Urinary creatine concentration responded similarly, increasing up to 30 g/d GAA, but decreased when 40 g/d GAA was infused in the absence of methionine. But, when 40 g/d GAA was provided with 12 g/d methionine, a decrease in urinary GAA was not observed. Plasma homocysteine concentrations increased when 30 or 40 g/d GAA was supplemented in the absence of methionine; whereas, when 12 g/d methionine was supplemented plasma homocysteine did not respond. This could be a result of supplemental methionine correcting a methyl group deficiency induced by high levels of GAA supplementation. These data demonstrate GAA's ability to obligatorily consume methyl groups for creatine synthesis, even when supplemented at levels greater than endogenous GAA synthesis.

In a methionine deficient model, Ardalan et al. (2016) supplemented growing steers with GAA at levels of 0, 7.5, or 15 g/d with either 0 or 6 g/d of methionine. Nitrogen retention was utilized as a measure of protein deposition and creatine status. Nitrogen retention was not affected by GAA supplementation alone; however, there was a tendency for an interaction between GAA × methionine, where GAA linearly improved N retention in the presence of 6 g/d methionine. Plasma creatine increased linearly with GAA supplementation but was lower in the presence of supplemental methionine. This effect could be a result of increased tissue growth with methionine supplementation, and therefore greater creatine uptake in muscle tissue.

Speer (2019) evaluated the effects of GAA supplementation (0, 7.5, or 15 g/d) in the presence or absence of 5 g/d methionine to growing steers consuming corn-based diets. Nitrogen

retention was numerically decreased by GAA supplementation, which is in contrast to Ardalan et al. (2016) who used intentionally methionine deficient steers. Nitrogen retention was increased when methionine was provided, suggesting that, although unintentional, methionine was limiting in the basal diet. Supplemental GAA linearly increased plasma creatine concentrations and tended to linearly increase urinary creatine output. This agrees with previous work conducted in heifers fed corn-based diets (Ardalan et al., 2020) and growing steers in a methionine deficient model when methionine was not present (Ardalan et al., 2016). Methionine methyl group flux tended to increase with GAA supplementation, but only in the presence of supplemental methionine. Because GAA supplementation failed to demonstrate an increase in net methyl flux in the absence of methionine, it is likely that other transmethylation reactions (e.g., phosphatidylcholine synthesis) were decreased (Speer, 2019). Additionally, Speer (2019) evaluated the relative bioavailability of GAA between abomasal and ruminal infusion. Plasma and urinary creatine concentrations were used to estimate ruminal GAA escape using the slope-ratio methodology, and they observed that ruminal GAA infusion was roughly half as effective as abomasal GAA infusion at increasing plasma and urinary creatine. This suggests that if supplemented to ruminants commercially (i.e., through the feed), GAA would require either ruminal protection or to be fed in twice the desired dose. In total, data from Ardalan et al. (2016; 2020) and Speer (2019) show that GAA supplementation is effective at generating a methyl group deficiency model for research in cattle.

Effects of methionine and choline on performance of growing cattle

Methionine effects on performance

Methionine is often the first limiting amino acid for growth in cattle (Richardson and Hatfield, 1978). This is a result of the low methionine content of microbial protein synthesized in the rumen (Ørskov, 1982). In addition to its quantitatively most important role in protein synthesis, methionine serves an essential role in transsulfuration and numerous transmethylation reactions in the body. The additional metabolic reactions methionine is required for may explain why it is used less efficiently for growth than other amino acids.

Because free AA are readily degraded or utilized by ruminal microbes, supplemental AA (e.g., methionine, lysine) must be supplied in a protected form to prevent ruminal degradation. Four primary forms of rumen protected methionine in current use are 1) encapsulation by a pH-sensitive coating, 2) fat encapsulation, 3) ethyl-cellulose coating, and 4) Met derivatives and analogs. One of the most widely studied and utilized commercially available product is Smartamine M (Adisseo USA Inc., Alpharetta, GA). A pH sensitive co-polymer coating encapsulates methionine, which is resistant to degradation in the ruminal environment (pH = 5 to 7), but is solubilized in lower pH environments, such as the abomasum (pH = 2 to 3), which allows for direct absorption in the small intestine (Schwab, 1995).

Studies investigating the impact of ruminally protected methionine on growth and finishing performance in cattle have shown variable effects. Deetz et al. (1985) evaluated a 3 × 3 factorial of including 3 levels of ruminally protected methionine (RPMet; 0, 0.08, and 0.16% of diet DM) and 3 levels of ruminally protected lysine (RPLys; 0, 0.06, and 0.12% of diet DM) in steers fed corn-based diets. No treatment effects on average daily gain (ADG) were observed, but RPMet supplementation improved feed efficiency at both 0.08% and 0.16% inclusion levels in

combination with 0 or 0.06% RPLys. In contrast, Wright and Loerch (1988) observed no differences in performance when RPMet was supplemented at increasing levels to steers consuming a diet based on high-moisture corn. Moreover, plasma Met concentrations increased with increasing levels of RPMet provision, suggesting that Met may not have been limiting (Wright and Loerch, 1988). In growing calves with ad libitum access to bermudagrass hay, RPMet was supplemented at increasing levels (0, 1, 2, 3, 4, and 5 g/d available Met) and showed linear improvements in ADG and feed efficiency with increasing RPMet provision (Kunkle and Hopkins, 1999). This is not surprising because in cattle consuming low-quality forages, a majority of post-ruminal AA are of microbial origin, which is deficient in methionine (Ørskov, 1982), so increased provision of Met to the small intestine would be expected to improve performance. Löest et al. (2001) supplemented 10 g/d methionine hydroxy analog (MHA) to steers limit-fed a soybean hull-based diet at either 1.5% or 2.5% of body weight daily; performance was not impacted by MHA supplementation. Because Met was expected to be limiting in this scenario, a failure of MHA to improve performance suggests that either 1) MHA was extensively degraded in the rumen, 2) another AA was first- or co-limiting for growth, or 3) no AA were limiting. Across two trials, Tripp et al. (1998) failed to detect a difference in growth performance in cattle supplemented with 0 or 6 g/d RPMet when fed a corn silage-based diet and detected a tendency for increased serum Met concentrations, suggesting that Met was not first limiting. Klemesrud et al. (2000a) supplemented increasing levels of RPMet to growing steers fed a diet based on sorghum silage and corn cobs containing supplemental meat and bone meal (which has high ruminal bypass but is Met deficient) to establish a methionine requirement by breakpoint analysis. Maximal performance was observed at 3 g/d supplemental metabolizable Met and plasma Met concentrations increased at this level of supplementation, suggesting that

the Met requirement was achieved. Hosford et al. (2015) evaluated the effects of supplemental RPMet and RPLys in steers fed zilpaterol hydrochloride (ZH; a β-adrenergic agonist) in the last phase of finishing. Steers treated with RPMet and RPMet+Lys gained faster than the negative (not supplemented with AA or ZH) and positive (given ZH but not AA) control cattle. This suggests that cattle fed a β-agonist at the end of finishing may require additional post-ruminal Met supply to optimize performance. In growing Holstein heifers (6 to 9 months old) fed corn-based diets, Li et al. (2019) evaluated the effects of partial deletion of lysine, methionine, and threonine on growth performance and N retention to determine their limiting AA sequence and ideal ratios of supplementation; a theoretical ideal AA profile was used as a positive control for comparison (100 Lys:33 Met:72 Thr). From months 8 to 9, heifers fed the Lys and Met partially deleted diets had depressed ADG and feed efficiency compared to the control and Thr groups. Additionally, Lys and Met deletion decreased N retention compared to controls, and Thr was intermediate to Met and controls. From this, they determined Lys was first limiting, followed by Met and then Thr with an ideal ratio determined to be 100 Lys:31.95 Met:56.84 Thr (Li et al., 2019). These mixed responses to RPMet supplementation are likely due to differences in 1) whether or not Met was the diet's first-limiting AA, 2) if the product utilized was adequately protected from ruminal degradation, or 3) if the product was available in the small intestine (providing adequate ruminal bypass occurred).

Numerous studies have evaluated the effects of ruminally protected methionine and lysine (RPMet+Lys) supplementation on growth performance in cattle. In steers supplemented with RPMet+Lys at increasing levels while consuming a corn-based diet, Deetz et al. (1985) demonstrated increased ADG and a tendency for improved feed efficiency at the lowest level of AA supplementation over control. Oke et al. (1986) demonstrated similar responses in growing

steers fed a corn-silage based diet; however, ADG and feed efficiency only showed improvement at their highest level of supplemental RPMet+Lys. The same steers were transitioned to high-moisture corn-based finishing diet with increasing RPMet+Lys levels (steers were maintained on same respective treatments for growing and finishing periods), but no differences were observed in performance except a tendency for improved ADG at the highest level of supplemental AA provision. In contrast, Wright and Loerch (1988) detected no differences in growing, finishing, or carcass performance of steers supplemented with increasing levels of RPMet+Lys when fed corn-based diets with urea as the primary protein source. In growing steers fed 0.5 kg/d barley with *ad libitum* access to grass silage, RPMet+Lys supplementation (2.6 g/d Met and 8.2 g/d Lys) increased ADG by 16.3% and feed efficiency by 13.6% (Veira et al., 1991). Additionally, plasma concentrations of Met and Lys increased when RPMet+Lys was supplemented, suggesting that the requirement for both AA was met. Hussein and Berger (1995) fed corn-based diets to Holstein steers supplemented with four levels of RPMet+Lys, but observed no differences in ADG suggesting that the basal diets were not limiting in Met or Lys. In a similar trial, Holstein steers fed corn-based diets were supplemented with four levels of RPMet+Lys (Healy et al., 1995) and feed efficiency in this trial tended to degrade quadratically in response to increasing RPMet+Lys supplementation; however, no effect on ADG was observed, so it is unlikely the basal diet was deficient either AA. Klemesrud et al. (2000b) conducted a finishing trial on fed corn-based diets and supplemented with 8 increasing levels of RPMet+Lys or a RPMet control. In the first phase of the trial, maximal performance was observed at 3 g/d supplemental Lys (which included 0.9 g/d supplemental Met); however, RPMet+Lys did not improve performance in the later phase of the trial. Supplemental RPMet alone did not improve performance at any stage of the trial suggesting it was not first limiting. In the aforementioned

trials, it is likely that any performance increases by RPMet+Lys were a result of correcting a lysine deficiency, but the Met and Lys effects cannot be separated from these data.

Choline effects on performance

Limited work has been conducted on the effects of supplemental choline on performance in beef cattle. Because choline is extensively degraded in the rumen (Sharma and Erdman, 1988), it must be provided in a ruminally protected form to ensure its intact passage from the rumen and availability for absorption in the small intestine. Fat-encapsulation is the most common form of protection used in ruminally protected choline (RPC) products.

In finishing beef heifers consuming corn-based diets, Bindel et al. (2000) supplemented RPC at four levels of available choline ion (0, 5, 10, and 15 g/d) throughout the finishing period. Choline supplemented heifers had improved ADG and feed efficiency compared to controls (8.6% and 7.6% greater when 5 g/d available choline was provided, respectively), with no advantage to additional RPC above the lowest level of supplementation. Bryant et al. (1999) supplemented RPC at four levels (0, 5, 10, and 20 g/d available choline) to finishing steers fed a 90% concentrate diet and observed that 5 g/d choline improved ADG 11.6% and feed efficiency 6.8% compared to control animals. No advantage to supplementing RPC above the lowest level was observed. In finishing steers consuming a diet based on steam-flaked corn, Sexson et al. (2010) evaluated the effects of short-term RPC supplementation in the final 29 days of the finishing period; steers received either 0 or 5 g/d available choline. No differences were observed in ADG or feed efficiency between steers supplemented with or without choline. It appears that when choline is supplemented for the entire finishing period, improvements in gain and feed efficiency can be expected. Bryant et al. (1999) suggested that supplemental RPC may improve

performance by modifying lipid metabolism; however, the true mechanism of choline's action in finishing cattle is unknown. It does appear that the effects of RPC on performance may require greater than short-term (i.e., 29 days as utilized by Sexson et al., 2010) supplementation. No work has been published on effects of RPC on performance in cattle in the receiving and growing phases.

Effects of methionine and choline on methionine metabolism in cattle

Methionine effects on methionine metabolism

In addition to methionine's effects on protein synthesis in cattle, it serves an equally important role is as a methyl donor in the body. As mentioned previously, methionine serves as a precursor to cysteine via homocysteine moving through the transsulfuration pathway. Cysteine is not considered an essential AA as long as methionine supply is adequate to support its synthesis (Brosnan and Brosnan, 2006). Work in rats has shown that cysteine supplementation can spare methionine's use for its synthesis, which will divert more methionine toward remethylation and protein synthesis (Finkelstein et al., 1988). Work in swine (Chung and Baker, 1992) and poultry (Graber and Baker, 1971) have shown that up to 50% of total sulfur-containing AA supply can be provided by cysteine. This same effect, however, has not been effectively demonstrated in ruminants. Campbell et al. (1997) was unable to demonstrate a methionine sparing effect of cysteine in growing steers used in a model where cysteine was predicted to be more limiting than methionine. In this study, methionine supplementation improved nitrogen retention (i.e., protein deposition) but cysteine did not, which may be attributed to low availability of methyl groups for homocysteine remethylation. In another study, Löest et al. (2002) was unable to demonstrate a methionine sparing effect of cysteine in the presence of choline and betaine as methyl donors.

Additionally, methionine methyl group flux (i.e., irreversible loss rate of methionine) was not affected by methionine, choline, or betaine, which Löest et al. (2002) attributed to a likely lack of change in homocysteine remethylation. Lambert et al. (2004) hypothesized that the cattle of Löest et al. (2002) may have been deficient in one or more vitamin cofactors related to methionine metabolism (i.e., folate, vitamin B_{12}, or pyridoxine), which may have limited methionine remethylation. Lambert et al. (2004) supplemented growing steers with a mixture of these three vitamins in a methionine-deficient model and observed increased protein deposition when vitamins were provided. Specific effects of vitamin supplementation on methyl group metabolism (i.e., homocysteine remethylation and transsulfuration) were not measured. Additional work has demonstrated that liver CBS activity is not regulated by methionine supply in cattle (Lambert et al, 2002), which may explain cysteine's inability to spare methionine under cysteine-deficient conditions. Relative to observed concentrations in rats, Lambert et al. (2002) observed high MS activity compared to CBS. In sheep, Xue and Snoswell (1985) demonstrated that MS activity was more important for homocysteine remethylation than BHMT. These data suggest that MS may serve as the quantitatively most important control point of homocysteine remethylation.

Effects of methionine supplementation on methionine metabolism have been studied less in lactating dairy cows than in growing cattle. Girard et al. (2005) evaluated the effects of supplemental folate on lactating Holstein cows in the presence or absence of supplemental ruminally protected methionine on methionine and folate metabolism. Cows supplemented with folate had reduced serum homocysteine concentrations. Serum cysteine concentrations decreased with folate supplementation when methionine was supplemented but were not affected when methionine was not fed (methionine × quadratic folate interaction). These data may suggest

greater homocysteine remethylation to methionine and lower transsulfuration of homocysteine when folate was supplemented, especially when in conjunction with methionine. In a companion study, Girard and Matte (2005) demonstrated that cows fed supplemental methionine and folate responded to vitamin B_{12} injections with increased lactational performance, which could be attributed to improved methionine utilization (i.e., remethylation of homocysteine to methionine via MS, which utilizes vitamin B_{12} as a cofactor).

In cows supplemented with RPMet, Osorio et al. (2014) observed a decrease in milk choline and a tendency for decreased hepatic phosphatidylcholine concentrations. This may demonstrate an increase of phosphatidylcholine utilization for VLDL assembly or export, which would indicate improved lipid metabolism. Methionine supplemented cows also demonstrated greater hepatic glutathione concentrations, which is a product of cysteine metabolism and a marker for increased antioxidant activity. Zhou et al. (2017) observed increased plasma cystathionine, homocysteine, taurine, and total sulfur compounds in response to methionine supplementation to transition dairy cows, all of which are indicative of increased flux of homocysteine through the transsulfuration pathway (Guttormsen et al., 2004). In addition to methionine directly altering methyl group metabolism, there may be potential for supplementation with other methyl donors (e.g., choline or betaine) to alter methionine metabolism.

Choline effects on methionine metabolism

Synthesis of choline consumes methyl groups by conversion of phosphatidylethanolamine to phosphatidylcholine; however, choline also has potential to serve as a methyl donor in the body if oxidized to form betaine, which can remethylate homocysteine

to form methionine. Supplementation of choline has been investigated to both spare methyl group consumption (i.e., spare phosphatidylcholine synthesis) and provide methyl groups for remethylation of homocysteine by BHMT. Löest et al. (2002) evaluated choline and betaine as methyl donors to methionine in growing cattle and observed no differences in methionine flux (i.e., irreversible loss rate of methionine, including transmethylation) with choline or betaine supplementation. This suggests that choline and betaine were unable to successfully alter homocysteine remethylation in their cattle. As mentioned previously in this review, Lambert et al. (2004) suspected this could be a result of a vitamin cofactor deficiency (i.e., folate, B_{12}, or pyridoxine). Choline supplementation also did not alter protein deposition in the cattle of Löest et al. (2002), which contrasts with work conducted in finishing cattle where feeding RPC improved performance (Bindel et al., 2000; Bryant et al., 1999). It is possible that positive responses in finishing cattle were a result of altered lipid metabolism via phosphatidylcholine synthesis from choline rather than improving remethylation of homocysteine.

In transition dairy cows supplemented with or without choline, Zhou et al. (2017) observed no differences in circulating cystathionine, homocysteine, taurine, or total sulfur compounds in response to choline supplementation. This may suggest that choline did not shift homocysteine away from transsulfuration and toward remethylation to methionine, which might be due to low choline oxidase activity in ruminants (Xue and Snoswell, 1986). In nutrient restricted cows supplemented with increasing amounts of choline, Coleman et al. (2019b) observed linear increases in activity of BHMT and a tendency for increased MS activity with increasing choline dose. In the same study, Coleman et al. (2019a) observed decreases in CBS activity with increased choline supply, which may suggest that choline was diverting homocysteine to remethylation to methionine rather than toward transsulfuration. Together, these

data suggest that choline supplementation may have increased methionine resynthesis. Further work evaluating the quantitative impact that choline supplementation may have on methionine metabolism may strengthen evidence for its use as a methyl donor in cattle.

Effects of methionine and choline on antioxidant capacity and immune function in cattle

Methionine effects on antioxidant capacity and immune function

The effects of methionine on immune function have been studied primarily in transition dairy cattle using commercially available ruminally protected products. Improved health in response to RPMet has been observed in transition dairy cows (Ardalan et al., 2010). Osorio et al. (2013) observed that cows supplemented with RPMet tended to have increased neutrophil phagocytosis, providing evidence that methionine may improve immune function in the transition period. In a companion study, Osorio et al. (2014) observed, in response to RPMet supplementation, lower ceruloplasmin and serum albumin A (SAA) concentrations, which are acute phase proteins that serve as biomarkers of inflammation. Greater concentrations of glutathione were observed in methionine supplemented cows, suggesting a shift toward greater antioxidant capacity. Similarly, Zhou et al. (2017) observed increased hepatic concentrations of antioxidants taurine and glutathione in methionine supplemented cows, further supporting improved antioxidant status when methionine is supplemented. Vailati-Riboni et al. (2017) observed increased oxidative burst in monocytes and neutrophils and increased phagocytosis by neutrophils in response to methionine. Zhou et al. (2018) observed a similar response, with methionine supplemented cows having increased oxidative burst in monocytes and neutrophils. In response to an *in vitro* whole blood lipopolysaccharide (LPS) challenge, Vailati-Riboni et al.

(2017) observed lower IL-1β concentrations in plasma harvested from cows supplemented with methionine, which may demonstrate anti-inflammatory effects of improved methyl group availability. This agrees with Zhou et al. (2016a), where anti-inflammatory responses were observed including reduced IL-1β and haptoglobin concentrations and improved phagocytic and oxidative capacity of neutrophils. Reduced circulating haptoglobin and increased albumin concentrations were observed by Batistel et al. (2018) when cows were supplemented with methionine. Methionine supplementation also increased neutrophil phagocytosis and oxidative burst, reduced reactive oxygen metabolites, and increased concentrations of several antioxidants including glutathione, tocopherol, and β-carotene, and also improved ferric-reducing antioxidant power, all suggesting improvements in antioxidant status (Batistel et al., 2018). In bovine polymorphonuclear leukocytes (PMN; primarily neutrophils), Abdelmegeid et al. (2017) demonstrated downregulation of several genes associated with inflammatory signaling when methionine was provided, signifying a reduction in immune activation in these cells. Bovine PMN have no activity of PEMT (i.e., no ability to synthesize choline via conversion of phosphatidylethanolamine to phosphatidylcholine), so effects of methionine on immune function related genes were not associated with increased choline synthesis (Abdelmegeid et al., 2017). In addition to methionine's direct impact on immune function, there is potential for similar effects through choline supplementation to cattle.

Choline effects on antioxidant capacity and immune function

Supplementation of choline to transition dairy cows has been well documented for its effects on lipid metabolism and immune function. Although choline may be synthesized using methyl groups from methionine, it appears beneficial to provide choline directly in a ruminally

protected form as opposed to or in addition to supplemental methionine. Ardalan et al. (2010) observed improved health in cows supplemented with RPC alone or in addition to RPMet in transition cows. Vailati-Riboni et al. (2017) observed increased phagocytosis and oxidative burst by monocytes from cows supplemented with choline. In contrast, another study observed no differences in oxidative burst in monocytes when choline was supplemented (Zhou et al., 2018). Choline did, however, decrease expression of numerous genes associated with inflammatory and oxidative stress-related pathways (Zhou et al., 2018). Zhou et al. (2016a) noted that methionine supplementation tended to produce more favorable responses than choline supplementation, but the amount of metabolizable choline delivered through the RPC product may have been a factor (i.e., greater amounts of choline may yield better responses). In in vitro assays utilizing bovine PMN, choline provision decreased expression of numerous genes associated with an inflammatory response (Abdelmegeid et al., 2017). As noted earlier, activity of BHMT is not present in PMN, and therefore, these responses to choline were not a result of increased remethylation of homocysteine to form methionine.

Garcia et al. (2018) studied the effects of choline supplementation on bovine immune cells to determine if the impact of choline on immune function is direct or indirect. Choline provision linearly decreased oxidative burst in neutrophils and increased lymphocyte proliferation, suggesting a modulation of immune cells. Choline supplementation also increased gene expression associated with several choline metabolites, suggesting that choline's effect on immune cells may involve choline directly or further downstream by one of its products (Garcia et al., 2018). I am unaware of any work related to choline's potential effects on immune function in growing cattle. Thus, it is unclear if the apparent effects of choline on the immune system in

transition dairy cattle can be translated to immune-challenged cattle (i.e., high-risk receiving cattle).

Conclusion

Methionine is an essential amino acid and is often the first-limiting amino acid for growth in cattle. In addition to its role in protein synthesis, methionine also serves as the most widely used methyl donor in the body when converted to SAM. Numerous methylation reactions rely on methyl groups from SAM, including synthesis of creatine and choline. Creatine is synthesized when SAM donates a methyl group to GAA and is important for maintaining energy homoeostasis in tissues. Supplementation of GAA to growing cattle can improve creatine status, may alter protein deposition, and, if supplemented in large enough quantities, can be used to modulate methyl group status for research purposes. Choline is an essential nutrient used in numerous metabolic processes including lipid transport and its role as a methyl donor for methionine resynthesis. Choline is synthesized when phosphatidylethanolamine receives three methyl groups from SAM to produce phosphatidylcholine. Although work has been conducted on the impact of methionine and choline supplementation on performance and methyl group metabolism in cattle, little to no data is available on these effects in receiving beef cattle. The effects of methionine and choline on immune function in transition dairy cows has been well documented, so there may be potential for these products to be useful for high-risk receiving cattle. Further research on the effects of methionine and choline supplementation on performance, methyl group metabolism, and immune function in growing beef cattle is necessary to determine the efficacy of these products in the receiving industry.

Literature Cited

Abdelmegeid, M. K., M. Vailati-Riboni, A. Alharthi, F. Batistel, and J. J. Loor. 2017. J. Dairy. Sci. 100:3155-3165. doi.org/10.3168/jds.2016-12025

Ardalan, M., E. D. Batista, and E. C. Titgemeyer. 2020. Effect of post-ruminal guanidinoacetic acid supplementation on creatine synthesis and plasma homocysteine concentrations in cattle. J. Anim. Sci. 98(3):1-9. doi:10.1093/jas/skaa072

Ardalan, M., K. Rezayazdi, and M. Dehghan-Banadaky. 2010. Effect of rumen-protected choline and methionine on physiological and metabolic disorders and reproductive indices of dairy cows. J. Anim. Physiol. Anim. Nutr, (Berl.) 94:e259–e265. doi: 10.1111/j.1439-0396.2009.00966.x

Ardalan, M., M. D. Miesner, C. D. Reinhardt, D. U. Thomson, C. K. Armendariz, and E. C. Titgemeyer. 2016. Guanidinoacetic acid as a precursor for creatine in steers. J. Anim. Sci. (E-Suppl. 5):753-754. (Abstr.). doi: 10.2527/jam2016-1577

Arshad, U., M. G. Zenobi, C. R. Staples, and J. E. P. Santos. 2020. Meta-analysis of the effects of supplemental rumen-protected choline during the transition period on performance and health of parous dairy cows. J. Dairy Sci. 103:282–300. doi: 10.3168/jds.2019-16842

Batistel, F., J. M. Arroyo, A. Bellingeri, B. Saremi, C. Parys, E. Trevisi, F. Cardoso, and J. J. Loor. 2017. Ethyl-cellulose rumen-protected methionine enhances performance during the periparturient period and early lactation in Holstein cows. J. Dairy Sci. 100:7455–7467. doi: 10.3168/jds.2017-12689

Batistel, F., J. M. Arroyo, C. I. M. Gares, E. Trevisi, C. Parys, M. A. Ballou, F. C. Cardoso, and J. J. Loor. 2018. Ethyl-cellulose rumen-protected methionine alleviates inflammation and oxidative stress and improves neutrophil function during the periparturient period and early lactation in Holstein dairy cows. J. Dairy Sci. 101:480-490. doi: 10.3168/jds.2017-13185

Bindel, D. J., J. S. Drouillard, E. C. Titgemeyer, R. H. Wessels, and C. A. Löest. 2000. Effects of ruminally protected choline and dietary fat on performance and blood metabolites of finishing heifers. J. Anim. Sci. 78:2497–2503. doi: 10.2527/2000.78102497x

Brosnan, J. T., and M. E. Brosnan. 2006. The sulfur-containing amino acids: an overview. J. Nutr. 136(6 Suppl.):1636S-1640S. doi: 10.1093/jn/136.6.1636S

Brosnan, J. T., and M. E. Brosnan. 2007. Creatine: endogenous metabolite, dietary, and therapeutic supplement. Annu. Rev. Nutr. 27:241-261. doi: 10.1146/annurev.nutr.27.061406.093621

Brosnan, J. T., E. P. Wijekoon, L. Warford-Woolgar, N. L. Trottier, M. E. Brosnan, J. A. Brunton, and R. F. Bertolo. 2009. Creatine synthesis is a major metabolic process in neonatal piglets and has important implications for amino acid metabolism and methyl balance. J. Nutr. 139:1292–1297. doi:10.3945/jn.109.105411

Bryant, T. C., J. D. Rivera, M. L. Galyean, G. C. Duff, D. M. Hallford, and T. H. Montgomery. 1999. Effects of dietary level of ruminally protected choline on performance and carcass characteristics of finishing beef steers and on growth and serum metabolites in lambs. J. Anim. Sci. 77:2893−2903. doi: 10.2527/1999.77112893x

Burris, W. R., J. A. Boling, N. W. Bradley, and A. W. Young. 1976. Abomasal lysine infusion in steers fed a urea supplemented diet. J. Anim. Sci. 42:699-705. doi: 10.2527/jas1976.423699x

Campbell, C. G., E. C. Titgemeyer, and G. St-Jean. 1997. Sulfur amino acid utilization by growing steers. J. Anim. Sci. 75:230–238. doi: 10.2527/1997.751230x

Chung, T. K. and D. H. Baker. 1992. Ideal amino acid pattern for 10-kilogram pigs. J. Anim. Sci. 70:3102-3111. doi: 10.2527/1992.70103102x

Clarke, S., and K. Banfield. 2001. S-adenosylmethionine-dependent methyltransferases. In: Carmel, R., Jacobsen, D. W. (Eds.), Homocysteine in health and disease. Cambridge University Press, Cambridge, England. p. 63-78.

Coleman, D. N., A. Alharthi, V. Lopreiato, E. Trevisi, M. Miura, Y. -X. Pan, and J. J. Loor. 2019b97. Choline supply during negative nutrient balance alters hepatic cystathionine β-synthase, intermediates of the methionine cycle and transsulfuration pathway, and liver function in Holstein cows. J. Dairy Sci. 102:8319-8331. doi: 10.3168/jds.2019-16406

Coleman, D. N., M. Vailati-Riboni, A. A. Elolimy, F. C. Cardoso, S. L Rodrigues-Zas, M. Miura, Y. -Z. Pan, and J. J. Loor. 2019b. Hepatic betaine-homocysteine methyltransferase and methionine synthase activity and intermediates of the methionine cycle are altered by choline supply during negative energy balance in Holstein cows. J. Dairy Sci. 102:8305-8318. doi: 10.3168/jds.2018-16204

Cooke, R. F., and J. D. Arthington. 2013. Concentrations of haptoglobin in bovine plasma determined by ELISA or a colorimetric method based on peroxidase activity. J. Anim. Physiol. Anim. Nutr. (Berl). 97:531-536. doi: 10.1111/j.1439-0396.2012.01298.x

Cui, Z. and D. E. Vance. 1996. Expression of phosphatidylethanolamine N-methyltransferase-2 is markedly enhanced in long term choline-deficient rats. J. Biol. Chem. 271:2839-2843. doi: 10.1074/jbc.271.5.2839

Deetz, L. E., A. M. Papas, and C. H. Benton. 1985. Performance of finishing steers fed rumen-protected methionine and/or lysine. J. Anim. Sci. 61(Suppl. 1):486 (Abstr.).

Drackley, J. K. 1999. ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: The final frontier? J. Dairy Sci. 82:2259–2273. doi: 10.3168/jds.S0022-0302(99)75474-3

Finkelstein, J. D. 1998. The metabolism of homocysteine: pathways and regulation. Eur. J. Pediatr. 157(Suppl. 2):S40-S44. doi: 10.1007/pl00014300

Finkelstein, J. D. 2006. Inborn errors of sulfur-containing amino acid metabolism. J. Nutr. 136: 1750S–1754S. doi: 10.1093/jn/136.6.1750S

Finkelstein, J. D., W. E. Kyle, and B. J. Harris. 1971. Methionine metabolism in mammals. Regulation of homocysteine methyltransferases in rat tissue. Arch. Biochem. Biophys. 146(1):84-92. doi: 10.1016/s0003-9861(71)80044-9

Finkelstein, J. D., J. J. Martin, and B. J. Harris. 1988. Methionine metabolism in mammals. The methionine-sparing effect of cystine. J. Biol. Chem. 263(24):11750-11754.

Garcia, M., L. K. Mamedova, B. Barton, B. J. Bradford. 2018. Choline regulates the function of bovine immune cells and alters the mRNA abundance of enzymes and receptors involved in its metabolism in vitro. Frontiers in Immunology. 9:1-14. doi: 10.3389/fimmu.2018.02448

Girard, C. L., H. Lapierre, J. J. Matte, and G. E. Lobley. 2005. Effects of Dietary Supplements of Folic Acid and Rumen-Protected Methionine on Lactational Performance and Folate Metabolism of Dairy Cows. J. Dairy Sci. 88:660-670. doi: 10.3168/jds.S0022-0302(05)72730-2

Girard, C. L., J. J. Matte. 2005. Effects of Intramuscular Injections of Vitamin B12 on Lactation Performance of Dairy Cows Fed Dietary Supplements of Folic Acid and Rumen-Protected Methionine. J. Dairy Sci. 88:671-676. doi: 10.3168/jds.S0022-0302(05)72731-4

Graber, G. and D. H. Baker. 1971. Sulfur Amino Acid Nutrition of the Growing Chick: Quantitative Aspects Concerning the Efficacy of Dietary Methionine, Cysteine and Cystine. J. Anim. Sci. 33:1005-1011. doi: 10.2527/jas1971.3351005x

Guttormsen, A. B., E. Solheim, H. Refsum. 2004. Variation in plasma cystathionine and its relation to changes in plasma concentrations of homocysteine and methionine in healthy subjects during a 24-h observation period. Am. J. Clin. Nutr. 79:76-79. doi: 10.1093/ajcn/79.1.76

Healy, B. J., R. T. Brandt, and E. C. Titgemeyer. 1995. Effect of rumen escape amino acids and multiple TBA implants on feedlot performance of lightweight Holstein steer calves. Cattlemen's Day. Kansas State Univ. pp. 31-33.

Hersom, M. J., M. Vázquez-Añón, K. P. Ladyman, M. S. Kerley, and J. D. Arthington. 2009. Effect of methionine source and level on performance of growing beef calves consuming forage-based diets. Prof. Anim. Sci. 25:465-474. doi: 10.15232/S1080-7446(15)30745-2

Hill, G. M., J. A. Boling, and N. W. Bradley. 1980. Postruminal lysine and methionine infusion in steers fed a urea-supplemented diet adequate in sulfur. J. Dairy Sci. 63:1242-1247. doi: 10.3168/jds.S0022-0302(80)83075-X

Hosford, A. D., J. E. Hergenreder, J. K. Kim, J. O. Baggerman, F. R. B. Ribeiro, M. J. Anderson, K. S. Spivey, W. Round, B. J. Johnson. 2015. Effects of supplemental lysine and methionine with zilpaterol hydrochloride on feedlot performance, carcass merit, and skeletal muscle fiber characteristics in finishing feedlot cattle. J. Anim. Sci. 93:4532-4544. doi: 10.2527/jas.2015-9047

Hussein, H. S. and L. L. Berger. 1995. Feedlot performance and carcass characteristics of Holstein steers as affected by source of dietary protein and level of ruminally protected lysine and methionine. J. Anim. Sci. 73:3503-3509. doi: 10.2527/1995.73123503x

Klemesrud, M. J., T. J. Klopfenstein, and A. J. Lewis. 2000a. Metabolizable methionine and lysine requirements of growing cattle. J. Anim. Sci. 78:199-206. doi: 10.2527/2000.781199x

Klemesrud, M. J., T. J. Klopfenstein, R. A. Stock, A. J. Lewis, and D. W. Herold. 2000b. Effect of dietary concentration of metabolizable lysine on finishing cattle performance. J. Anim. Sci. 78:1060-1066. doi: 10.2527/2000.7841060x

Kunkle, W. E., and D. I. Hopkins. 1999. Is methionine the first limiting amino acid for growing cattle fed forages? In: 10th FL. Rum. Nutr. Syp., Gainesville, FL. p. 19-29.

Lambert, B. D., E. C. Titgemeyer, C. A. Löest, and D. E. Johnson. 2004. Effect of glycine and vitamin supplementation on sulphur amino acid utilization by growing cattle. J. Anim. Physiol. Anim. Nutr. (Berl.) 88:288-300. doi: 10.1111/j.1439-0396.2004.00484.x

Lambert, B. D., E. C. Titgemeyer, G. L. Stokka, B. M. DeBey, and C. A. Löest. 2002. Methionine supply to growing steers affects hepatic activities of methionine synthase and betaine-homocysteine methyltransferase, but not cystathionine synthase. J. Nutr. 132:2004-2009. doi: 10.1093/jn/132.7.2004

Li, Y., Y. Bi, Q. Diao, M. Piao, B. Wang, F. Kong, F. Hu, M. Tang, Y. Sun, and Y. Tu. 2019. The limiting sequence and appropriate amino acid ratio of lysine, methionine, and threonine for seven- to nine-month-old Holstein heifers fed corn–soybean m-based diet. Animals. 9:750-762. doi: 10.3390/ani9100750

Lobley, G. E. 1992. Control of the metabolic fate of amino acids in ruminants: a review. J. Anim. Sci. 70:3264–3275. doi: 10.2527/1992.70103264x.

Lobley, G. E., A. Connell, and D. Revell. 1996. The importance of transmethylation reactions to methionine metabolism in sheep: effects of supplementation with creatine and choline. British J. Nutr. 75(1): 47-56. doi: 10.1079/BJN19960109

Löest, C. A., E. C. Titgemeyer, J. S. Drouillard, D. A. Blasi, and D. J. Bindel. 2001. Soybean hulls as a primary ingredient in forage-free diets for limit-fed growing cattle. J. Anim. Sci. 79:766-774. doi: 10.2527/2001.793766x

Löest, C. A., E. C. Titgemeyer, G. St-Jean, D. C. Van Metre, and J. S. Smith. 2002. Methionine as a methyl group donor in growing cattle. J. Anim. Sci. 80:2197-2206. doi: 10.1093/ansci/80.8.2197.

Marsh, W. H., B. Fingerhut, and H. Miller. 1965. Automated and manual direct methods for the determination of blood urea. J. Clin. Chem. 11:624-627. doi: 10.1093/clinchem/11.6.624

McBreairty, L. E., J. L. Robinson, K. R. Furlong, J. A. Brunton, and R. F. Bertolo. 2015. Guanidioacetate is more effective than creatine at enhancing tissue creatine stores while consequently limiting methionine availability in Yucatan miniature pigs. PLoS ONE. 10(6):e0131563. doi: 10.1371/journal.pone.0131563.

Oke, B. O., S. C. Loerch, and L. E. Deetz. 1986. Effects of rumen-protected methionine and lysine on ruminant performance and nutrient metabolism. J. Anim. Sci. 62:1101-1112. doi: 10.2527/jas1986.6241101x

Ørskov, E. R. 1982. Protein Nutrition in Ruminants. Acad. Press, London, England.

Osorio, J. S., E. Trevisi, P. Ji, J. K. Drackley, D. Luchini, G. Bertoni, and J. J. Loor. 2014. Biomarkers of inflammation, metabolism, and oxidative stress in blood, liver, and milk reveal a better immunometabolic status in peripartal cows supplemented with Smartamine M or MetaSmart. J. Dairy Sci. 97:7437–7450. doi: 10.3168/jds.2013-7679

Osorio, J. S., P. Ji, J. K. Drackley, D. Luchini, and J. J. Loor. 2013. Supplemental Smartamine M or MetaSmart during the transition period benefits postpartal cow performance and blood neutrophil function. J. Dairy Sci. 96:6248–6263. doi: 10.3168/jds.2012-5790

Pelech, S. L. and D. E. Vance. 1984. Regulation of phosphatidylcholine biosynthesis. Biochimica et biophysica acta 779(2):217-251. doi: 10.1016/0304-4157(84)90010-8

Re, R., N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology & Medicine. 26:1231-1237. doi: 10.1016/S0891-5849(98)00315-3

Richardson, C. R., and E. E. Hatfield. 1978. The limiting amino acids in growing cattle. J. Anim. Sci. 46:740-745. doi: 10.2527/jas1978.463740x

Schwab, C. G. 1995. Protected proteins and amino acids for ruminants. In: R. J. Wallace and A. Chesson, editor, Biotechnology in animal feeds and animal feeding. V.C.H. Press. Weinheim, Germany. p. 115-141.

Setoue, M., S. Ohuchi, T. Morita, and K. Sugiyama. 2008. Hyperhomocysteinemia induced by guanidinoacetic acid is effectively suppressed by choline and betaine in rats. Biosci. Biotechnol. Biochem. 72(7):1696-1703. doi: 10.1271/bbb.70791

Sexson, J. L., J. J. Wagner, T. E. Engle, J. W. Spears. 2010. Effects of water quality and dietary potassium on performance and carcass characteristics of yearling steers. J. Anim. Sci. 88:296-305. doi: 10.2527/jas.2009-1899

Sharma, B. K., and R. A. Erdman. 1989. In vitro degradation of choline from selected feedstuffs and choline supplements. J. Dairy Sci. 72:2772–2776

Shingfield, K. J., and N. W. Offer. 1999. Simultaneous determination of purine metabolites, creatinine and pseudouridine in ruminant urine by reversed-phase high-performance liquid chromatography. J. Chrom. B. 723:81-94. doi: 10.1016/S0378-4347(98)00549-0.

Speer, H. S. 2019. Efficacy of guanidinoacetic acid supplementation to growing cattle and relative bioavailability of guanidinoacetic acid delivered ruminally or abomasally [Master's thesis]. Kansas State University, Manhattan, KS.

Stead, L. M., K. P. Au, R. L. Jacobs, M. E. Brosnan, and J. T. Brosnan. 2001. Methylation demand and homocysteine metabolism: effects of dietary provision of creatine and guanidinoacetate. Am. J. Physiol. Endocrinol. Metab. 281(5):E1095-E1100. doi: 10.1152/ajpendo.2001.281.5.E1095

Stead, L. M., J. T. Brosnan, M. E. Brosnan, D. E. Vance, and R. L. Jacobs. 2006. Is it time to reevaluate methyl balance in humans? Am. J. Clin. Nutr. 83(1):5-10. doi: 10.1093/ajcn/83.1.5

Titgemeyer, E. C., N. R. Merchen, L. L. Berger, and L. E. Deetz. 1988. Estimation of lysine and methionine requirements of growing steers fed corn silage-based or corn-based diets. J. Dairy Sci. 71:421. doi: 10.3168/jds.S0022-0302(88)79572-7

Tossenberger, J., M. Rademacher, K. Németh, V. Halas, and A. Lemme. 2016. Digestibility and metabolism of dietary guanidino acetic acid fed to broilers. Poult. Sci. 95:2058-2067. doi: 10.3382/ps/pew083

Tripp, M. W., T. A. Hoagland, G. E. Dahl, A. S. Kimrey, and S. A. Zinn. 1998. J. Anim. Sci. 76:1197-1203. doi: 10.2527/1998.7641197x

Vailati-Riboni, M., J. S. Osorio, E. Trevisi, D. Luchini, and J. J. Loor. 2017. Supplemental Smartamine M in higher energy diets during the prepartal period improves hepatic biomarkers of health and oxidative status in Holstein cows. J. Anim. Sci. Biotechnol. 8:17. doi: 10.1186/s40104-017-0147-7

Vance, D. E. 2014. Phospholipid methylation in mammals: from biochemistry to physiological function. Biochimica et Biophysica Acta. 1838:1477–1487. doi: 10.1016/j.bbamem.2013.10.018

Veira, D. M., J. R. Seoane, and J. G. Proulx. 1991. Utilization of grass silage by growing cattle: effect of a supplement containing ruminally protected amino acids. J. Anim. Sci. 69:4703-4709. doi: 10.2527/1991.69124703x

Walker, J. B. 1979. Creatine: biosynthesis, regulation, and function. Adv. Enzymol. Relat. Areas Mol. Biol. 50:177-242. doi: 10.1002/9780470122952.ch4

Wright, M. D., and S. C. Loerch. 1988. Effects of rumen-protect amino acids on ruminant nitrogen balance, plasma amino acid concentrations and performance. J. Anim. Sci. 66:2014-2027. doi: 10.2527/jas1988.6682014x

Wyss, M., and R. Kaddurah-Daouk. 2000. Creatine and creatinine metabolism. Physiol. Rev. 80:1107–1213. doi:10.1152/physrev.2000.80.3.1107

Xue, G. -P., and A. M. Snoswell. 1985. Comparative studies on the methionine synthesis in sheep and rat tissues. Comp. Biochem. Physiol. 80B:489-494. doi: 10.1016/0305-0491(85)90278-0.

Xue, G. -P., and A. M. Snoswell. 1986. Developmental changes in the activities of enzymes related to methyl group metabolism in sheep tissues. Comp. Biochem. Physiol. 83B:115-120. doi: 10.1016/0305-0491(86)90340-8.

Zeisel, S. H. 2006. Choline: critical role during fetal development and dietary requirements in adults. Annual review of nutrition. 26:229-250. doi: doi.org/10.1146/annurev.nutr.26.061505.111156

Zhou, Z., O. Bulgari, M. Vailati-Riboni, E. Trevisi, M. A. Ballou, F. C. Cardoso, D. N. Luchini, and J. J. Loor. 2016a. Rumen-protected methionine compared with rumen-protected choline improves immunometabolic status in dairy cows during the peripartal period. J. Dairy Sci. 99:8956–8969. doi: 10.3168/jds.2016-10986

Zhou, Z., T. A. Garrow, X. Dong, D. N. Luchini, J. J. Loor. 2017. Hepatic Activity and Transcription of Betaine-Homocysteine Methyltransferase, Methionine Synthase, and Cystathionine Synthase in Periparturient Dairy Cows Are Altered to Different Extents by Supply of Methionine and Choline. J. Dairy Sci. 147:11-19. doi: 10.3945/jn.116.240234

Zhou, Z., M. Vailati-Riboni, E. Trevisi, J. K. Drackley, D. N. Luchini, and J. J. Loor. 2016b. Better postpartal performance in dairy cows supplemented with rumen-protected methionine compared with choline during the peripartal period. J. Dairy Sci. 99:8716–8732. doi: 10.3168/jds.2015-10525

Zhou, Z., F. Ferdous, P. Montagner, D. N. Luchini, M. N. Corrêa, and J. J. Loor. 2018. Methionine and choline supply during the peripartal period alter polymorphonuclear leukocyte immune response and immunometabolic gene expression in Holstein cows. J. Dairy Sci. 101:10374-10382. doi.org/10.3168/jds.2018-14972

Chapter 2 - Effect of Smartamine M on performance and acute phase protein response in receiving beef heifers

M. S. Grant, H. F. Speer, W. R. Hollenbeck, R. N. Wahl, N. D. Luchini, D. A. Blasi, and E. C. Titgemeyer

Abstract

Methionine supplementation can improve immune function in transition dairy cattle. Our objective was to determine if supplemental methionine could improve health and performance of newly received growing cattle. Crossbred heifers (n = 384; 222 ± 4.9 kg initial BW; southeastern U.S. origin) were received in 4 truckloads (blocks) over 9 d. Heifers were weighed at arrival. The following day (d 0) cattle were vaccinated for viral and clostridial diseases, received 2.5 mg tulathromycin/kg BW, and were stratified within block by arrival BW to 1 of 8 pens containing 12 heifers each. Cattle were limit-fed at 2.2% of BW daily (DM basis) a diet containing 40% Sweet Bran, 34.5% dry-rolled corn, 10% corn silage, 7.5% supplement, 4% alfalfa hay, and 4% prairie hay. Within blocks, pens were assigned to 1 of 2 treatments: 0 (control) or 10 g/d Smartamine M (SM) to provide 6 g/d metabolizable methionine. Pen weights were collected weekly to determine feed offered the following week. Individual BW and tail-vein blood samples were collected on d 0, 14, and 45. Plasma haptoglobin was measured to assess acute phase protein response. Incidences of morbidity (1.6% for control, 2.6% for SM) and mortality (0.5% for both control and SM) were low. Between d 0 and 45, no differences were observed for ADG (1.24 vs. 1.27 kg/d; control vs. SM, $P = 0.55$) or G:F (0.107 vs. 0.110, $P = 0.28$), although DMI was 1.3% greater ($P < 0.01$) for control than SM due to differences in diet DM. An interaction between treatment and linear effect of day was detected for plasma haptoglobin ($P < 0.05$); over time, haptoglobin increased more for control (2.15, 2.28, and 2.95 mg/mL at 0, 14, and 45 d) than for SM (2.35, 2.37, and 2.58 mg/mL). Supplemental methionine may alleviate acute phase protein responses in stressed receiving cattle.

Introduction

Methionine is an essential amino acid (AA) that is often first limiting for growth in cattle (Richardson and Hatfield, 1978). This is a result of the low methionine content of microbial protein synthesized in the rumen (Ørskov, 1982). In addition to its quantitatively most important role in protein synthesis, when methionine is converted to *S*-adenosylmethionine (SAM), it serves as the most widely used methyl group donor in the body (Finkelstein, 1990). As SAM, methionine participates in over a hundred essential methylation reactions throughout the body (Lobley, 1992), including synthesis of creatine and phosphatidylcholine as well as methylation of histones, DNA, and RNA (Walker, 1979). Because AA are rapidly degraded by ruminal microbes, ruminally protected AA (RPAA) must be fed to ensure that they are available for absorption in the small intestine. One of the most widely studied and utilized forms of ruminally protected methionine (RPMet) is Smartamine M (Adisseo USA Inc., Alpharetta, GA), which utilizes a pH sensitive co-polymer coating to resist ruminal degradation but allow solubilization in the abomasum and absorption in the small intestine.

In the transition period, dairy cattle face numerous physiological stressors due to reduced dry matter intake (DMI) near calving and increased nutrient partitioning toward fetal growth and the onset of lactation (Drackley, 1999). Supplementation of RPMet to transition dairy cattle has been shown to improve performance, health, and immune function (Ardalan et al., 2010; Osorio et al., 2014; Zhou et al., 2016a, b). Newly received beef cattle face stress associated with marketing and transport to the feedlot including commingling, pathogen exposure, and low DMI. As a result, high-risk receiving cattle often become ill shortly after feedlot arrival. Because these stressors may be physiologically similar to those of dairy cattle in the peripartal period, methionine may have potential as an immunomodulator in receiving cattle. Our objective was to

evaluate the effect of supplemental methionine on performance, health, and acute-phase protein response in high-risk receiving beef cattle.

Materials and Methods

All procedures involving the use of animals were approved by the Kansas State University Institutional Animal Care and Use Committee.

Animals and Experimental Diets

A total of 384 crossbred heifers (222 ± 4.9 kg initial BW) of southeastern U.S. origin were purchased from auction markets in Tennessee, transported and commingled at an order buyer's facility in Dixon, TN, then transported 1,086 km to the Kansas State University Beef Stocker unit where they were received over 9 d from October 4 to October 13, 2018. Cattle were blocked by truckload (4) and stratified by individual arrival BW within a block to pens containing 12 animals each. Within block, pens were assigned randomly to 1 of 2 treatments creating 16 pens/treatment, with a total of 32 pens. All pens (9.1 × 15.2 m) were soil surfaced and had a concrete fenceline bunk (9.1 m) and 3.6-m apron. Experimental diets were offered at 2.2% BW daily (DM basis) and were formulated to contain 1.32 Mcal NEg/kg DM. Experimental diets (Table 2.1) were offered at 2.2% of BW daily (DM basis) and contained either 0 (control) or 0.1725% Smartamine M (SM). This was targeted to provide approximately 10 g/d Smartamine M or 6 g/d supplemental metabolizable methionine initially, increasing slightly as cattle grew and feed offerings increased. During the first 14 d of the experiment (i.e., during step up) SM heifers received on average 7.5 g/d Smartamine M (i.e., 4.5 g/d supplemental metabolizable methionine). For the entire 45 d trial, SM heifers received on average 9 g/d Smartamine M (i.e., 5.4 g/d metabolizable methionine). Smartamine M was mixed with dry-rolled corn before being added to the diet. The mixture was prepared in a paddle mixer; corn and Smartamine M were combined and mixed for 60 seconds to ensure an even distribution of

Smartamine M without damaging the pH sensitive coating on the product. Two bins were utilized to store corn, one with control corn and the other containing the corn-Smartamine M mixture to be used for control and SM diets, respectively.

Upon arrival, heifers were individually weighed and received an individual identification ear tag. An ear notch was collected from each animal and immediately analyzed for persistent infection with Bovine Viral Diarrhea Virus using a rapid visual ELISA assay (IDEXX BVDV PI X2 Test, IDEXX Laboratories, Inc., Westbrook, ME). Animals not displaying illness were assigned randomly to pens containing 12 heifers and offered 0.5% BW (DM basis) prairie hay and *ad libitum* access to water overnight.

The following morning (d 0), heifers were individually weighed, assigned an ear tag for pen number, and were vaccinated for respiratory and clostridial disease. For respiratory pathogens, Pyramid 5 + Presponse SQ (Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO), a modified-live vaccine against infectious bovine rhinotracheitis virus (IBR), bovine viral diarrhea virus types I and II (BVDV I and II), parainfluenza$_3$ virus (PI$_3$), and bovine respiratory syncytial virus (BRSV), was administered. For clostridial pathogens, Vision 7 Somnus with Spur (Merck Animal Health, Madison, NJ) was used. Animals were treated on d 0 for subclinical respiratory disease with 2.5 mg/kg tulathromycin (Draxxin; Zoetis, Parisippany, NJ). Heifers were also treated on d 0 for internal parasites with 10% fenbendazole (Safe-Guard; Merck Animal Health, Madison, NJ) and external parasites with pour on ivermectin (Bimectin, Bimeda US, Oakbrook Terrace, IL). On d 14, all heifers were revaccinated for respiratory pathogens with Vista 5 SQ (Merck Animal Health, Kenilworth, NJ), a modified live vaccine for IBR, BVDV I and II, PI$_3$, and BRSV.

Individual BW were measured on d 0, at revaccination (d 14), and at the conclusion of the study (d 45). Pen weights were measured weekly (d 14, 21, 28, 35, and 45) using a pen scale (Rice Lake Weighing Systems, Rice Lake, WI). Weekly pen weights were used to calculate feed offered for the following week. Animals were fed once daily at 0700 h using a Roto-Mix feed wagon (Model #414-14B; Roto-Mix, Dodge City, KS). When transitioning from the SM diet to control, the feed wagon was emptied first by the auger and then manually where all reachable remaining feed was removed and discarded. Approximately 100 kg corn silage was added to the wagon as a flushing agent, mixed for 60 seconds, and removed via wagon auger and manual cleaning to reduce possibility of treatment carryover. Cattle were transitioned to the treatment diets by offering 1% BW (DM basis) on d 0 and increasing feed offered by 0.2% BW each day if the previous day's feed was totally consumed until the pen reached a DMI of 2.2% BW. During step-up, refusals were left in the bunk; if greater than approximately 5% refusals were present, feed call was decreased accordingly to avoid excessive refusals the following day. By d 14 all pens were fully stepped up to feed delivered at 2.2% BW (DM basis) and consistently ate all feed offered prior to the next day's feeding. Amount of feed delivered to each pen was recorded daily at feeding.

Heifers were observed twice daily for clinical signs of illness including depression, anorexia, gauntness, and ocular or nasal discharge. Animals showing signs of morbidity were walked to treatment facilities where rectal temperature was measured and clinical illness score was determined. Clinical illness scores were defined as: 1: normal and healthy, 2: slightly ill, with mild depression or gauntness, 3: moderately ill, with severe depression/labored breathing/ocular or nasal discharge or 4: severely ill, near death with little response to human approach. Animals with a rectal temperature ≥40 °C and a clinical illness score ≥2 were treated.

Upon first morbidity, animals were treated with fluorfenicol and flunixin meglumine (300 mg/mL and 16.5 mg/mL respectively; Resflor, Merck Animal Health, Madison, NJ). If symptoms did not improve within 72 h of first treatment, second morbidity was declared and animals were treated with enrofloxacin (100 mg/mL; Baytril 100, Bayer Animal Health, Shawnee Mission, KS). Upon third morbidity, heifers were treated with oxytetracycline (200 mg/mL; Bio-Mycin 200, Boehringer Ingelheim Vetmedica, Inc., St. Joeseph, MO). At third treatment, animals were considered chronic and removed from the trial.

Sample Collection and Analysis

Feed ingredient and total mixed ration samples were collected weekly and frozen at -20 °C until analysis. Previously frozen feed and ingredient samples were composited on a biweekly basis, dried in a forced-air oven at 55 °C for 48 h, and ground through a 1-mm screen using a Thomas Wiley Mill (Thomas Scientific, Swedesboro, NJ). All samples were analyzed in duplicate. Ground samples were dried for 24 h at 105 °C in a forced-air oven for total dry matter (DM) determination and then heated to 450 °C in a muffle furnace for 18 h to measure organic matter (OM) content. Samples were analyzed for neutral detergent fiber (NDF) using alpha amylase and sequentially analyzed for acid detergent fiber (ADF) using an ANKOM Fiber Analyzer (Model 200, ANKOM Technology, Macedon, NY). Starch content was measured by the method of Herrera-Saldana and Huber (1989) with glucose measured using a commercial colorimetric assay (Autokit Glucose, FUJIFILM Wako Diagnostics U.S.A. Corp., Mountain View, CA). Nitrogen content of samples was analyzed using a LECO TruMac N Analyzer (LECO Corporation, Saint Joseph, MI). Measured N content was multiplied by 6.25 to determine crude protein (CP) content of the samples.

Prior to feeding on d 0, 14, and 45 a coccygeal vein blood sample was collected from each animal into a 10-mL evacuated tube (BD Vacutainer; Beckton, Dickinson, and Company, Franklin Lakes, NJ) containing sodium heparin. Following collection, tubes were immediately inverted several times and stored on ice until centrifuged at 1,200 × g at 4 °C for 20 min. Plasma was harvested and stored in 2-mL microcentrifuge tubes at -20 °C until analysis for haptoglobin concentration to assess inflammation and acute phase protein response.

Plasma haptoglobin concentrations were measured by colorimetric assay based on peroxidase activity following the methods of Cooke and Arthington (2013). Briefly, 10 µL of plasma or serially diluted plasma standard was added to 7.5 mL *o*-dianisidine solution (0.6 g/L *o*-dianisidine, 0.5 g/L EDTA, and 13.8 g/L sodium phosphate monobasic in distilled water) in a borosilicate tube (16 × 100 mm). Immediately, 25 µL hemoglobin solution (0.3 g/L bovine hemoglobin dissolved in distilled water) was added to each tube. After sample and hemoglobin addition to all tubes, samples were covered with parafilm and incubated at 37 °C for 45 min in a VWR gravity convection incubator (VWR International, Radnor, PA). Immediately following incubation, 100 µL of a freshly prepared 156 m*M* hydrogen peroxide solution was added to each tube. Tubes were then incubated at room temperature for 1 h. After incubation, 200 µL from each tube was pipetted in quadruplicate into a 96-well flat-bottom microplate and read at 450 nm using a microplate reader (BioTek PowerWave XS, BioTek Instruments, Inc., Winooski, VT). A test run was conducted, and the plasma sample with the highest concentration was subsequently used as the standard. The selected standard plasma sample was serially diluted with water, analyzed identically to samples during each run, and used to calculate haptoglobin concentrations for all samples for this study.

Calculations

Average pen BW was calculated for d 0 based on individual weights collected at processing. All subsequent average pen BW (d 14, 21, 28, 35, and 45) were based on pen scale weights. Day 0, 14, and 45 BW were used to calculate ADG and G:F. Throughout the trial, only 2 pens experienced death loss; all calculations for performance data were made with data from dead heifers removed. For these pens, adjustments were made by adjusting weekly pen weights to the proportion the dead individual contributed to d 14 pen weights applied to subsequent pen weights prior to death to effectively remove that animal's weight contribution until mortality (e.g. for a d-14 individual weight of 250 kg, d 14 total pen weight 3000 kg, (3000-250) / 3000 = 0.917, all subsequent pen weights prior to the animal's death would be multiplied by a factor of 0.917). The same factor was used to adjust DMI for the affected pens.

Statistical Analysis

Performance data was analyzed as a randomized block design using the mixed procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC) with a model including the fixed effect of treatment and the random effect of block.

A large proportion of plasma samples collected for haptoglobin measurement contained hemolysis to some degree, which interfered with the haptoglobin assay. Thus, samples containing any visible hemolysis were not analyzed for haptoglobin concentration. As a result, only 695 individual observations of plasma haptoglobin concentration were available across the 3 blood sampling days. To generate pen means for plasma haptoglobin concentration for each of the 3 sampling days, individual observations were analyzed using the mixed procedure of SAS with a model including fixed effects of pen, day, and pen × day as well as the random effect of

heifer(pen). The 96 pen means were then analyzed using the mixed procedure of SAS with fixed effects in the model including treatment, day, and treatment × day as fixed effects and block as a random effect. Day was considered a repeated measure with spatial power as the covariance structure. Treatment by day interactions were evaluated using contrasts for assessing treatment × time interactions, with linear and quadratic effects constructed for unequal spacing of sampling days. Significance was declared at $P \leq 0.05$ and tendencies at $0.05 \leq P \leq 0.10$.

Results

Animal Performance

Performance data are presented in Table 2.2. Dry matter intake (DMI) was greater d 0 to 14 and d 0 to 45 for control than for SM ($P < 0.008$) but did not differ between treatments over d 14 to 45 ($P = 0.54$). This was the result of differences in dietary DM among treatments as analyzed from weekly feed samples. Because cattle were fed based on predicted ingredient and dietary DM, these differences were not measured until after the trial's conclusion. No differences in BW ($P > 0.65$), ADG ($P > 0.52$), or G:F ($P > 0.28$) were observed at any time point throughout the trial.

Health and Acute Phase Protein Response

Very low incidence of morbidity and mortality were observed for this trial (Table 2.3); morbidity and mortality data were not analyzed statistically. A total of 8 animals were treated once (2.08%) for respiratory illness, 5 from the SM treatment (2.60%) and 3 from the control treatment (1.56%). Two previously treated animals died during the trial, one from each treatment, resulting in mortality rates of 0.52% for each treatment. One of the deceased animals

was considered chronically infected with respiratory illness and removed from the trial prior to death. The other died within 24 h of second treatment for respiratory illness.

For plasma haptoglobin, an interaction between dietary treatment and linear effect of day was observed ($P = 0.05$; Figure 2.1). Over the duration of the trial, plasma haptoglobin concentrations for the SM treatment group remained relatively stable. Control cattle had numerically lower d-0 haptoglobin than did cattle receiving SM, but control cattle then demonstrated increases over the duration of the trial. It appears that supplemental methionine mitigated this increase in plasma haptoglobin over time.

Discussion

Animal Performance

In our study, heifers were limit fed at a targeted 2.2% of BW daily (DM basis). In the early phase of the trial (i.e., d 0 to 14) DMI was greater for control than SM supplemented heifers; however, because this was not observed from d 14 to 45 and differences in performance were not observed, the effect of differing diet DM is assumed to be negligible. As mentioned previously, analyzed dietary DM was numerically greater for the control diet. Because diet composition was nearly identical between treatment groups, this is attributed to sampling error rather than true differences in dietary DM between treatments.

Work investigating the effects of RPMet supplementation on growth and finishing performance in beef cattle has shown variable effects. In 280-kg steers fed corn- and corn silage-based diets, Deetz et al. (1985) observed no differences in ADG but lower DMI in steers fed 2 levels of RPMet compared to unsupplemented controls. Feed efficiency was greater for RPMet-supplemented steers. In contrast, Wright and Loerch (1988) observed no differences in DMI,

ADG, or feed efficiency when RPMet was supplemented to finishing steers fed high-moisture corn-based diets. Limited response to methionine supplementation in the cattle of our trial, Deetz et al. (1985), or Wright and Loerch (1988) may be explained by either a lack of methionine deficiency, or possibly another AA either more limiting or co-limiting performance increases. Titgemeyer et al. (1988) concluded that cattle fed corn-based diets are most likely first-limited by lysine rather than methionine. Therefore, in our cattle fed a corn- and corn-byproduct-based diet, it is not particularly surprising that we did not observe improvements in performance with RPMet supplementation.

Improved lactational performance (i.e., increased milk yield, milk fat and protein yield) in post-partum dairy cows supplemented with RPMet in the peripartum period has been reported (Osorio et al., 2013; Zhou et al., 2016b; Batistel et al., 2017). This may in part be a result of a corrected methionine deficiency; however, postpartum DMI was also greater in RPMet supplemented cows in these studies. Improved DMI following calving may explain increased lactational performance in the cows of Osorio et al. (2013), Zhou et al. (2016b), and Batistel et al. (2017). Our heifers were limit-fed, so theoretical improvements in ADG that could have been attributed to improved voluntary DMI above our restricted intakes could not be measured.

Health and Acute Phase Protein Response

Despite being subjected to numerous stressors prior to arrival (e.g., marketing, commingling, transport, pathogen exposure, feed restriction, etc.), our cattle had unexpectedly low incidence of respiratory illness. It appears that our heifers' immune function was not significantly challenged and, as a result, any effect of RPMet supplementation on general health could not be detected. One explanation for our low morbidity incidence is the use of

metaphylaxis at initial processing, which would be expected to limit expression of any respiratory illness existing at arrival. In a similar study conducted at this research facility with metaphylaxis, Spore et al. (2019) observed an average 12% incidence of first-treatment respiratory disease in heifers of similar size and origin. Step et al. (2008) observed 31.9% incidence of respiratory illness in commingled auction-market cattle that did not receive antibiotic treatment upon arrival, markedly greater than that of Spore et al. (2019) or our cattle. Because our cattle were relatively healthy throughout the trial's duration, we are unable to draw conclusions about the ability of RPMet to affect clinical illness in receiving cattle.

Our heifers maintained relatively stable concentrations of plasma haptoglobin between d 0 and 14 regardless of treatment; however, by d 45 control heifers had greater haptoglobin concentrations than SM heifers. Haptoglobin is a positive acute phase protein produced by the liver during an inflammatory response (Ackerman, 2017); for this reason, it serves as useful a marker of general inflammatory status in the body. Our results appear to indicate that the increase in haptoglobin (i.e., inflammation) observed in control cattle by d 45 was mitigated by RPMet supplementation in the SM cattle. It is possible that plasma haptoglobin concentrations may have spiked and returned to baseline sometime between d 0 and 14 (i.e., likely due to transportation and/or initial processing stress); however, it is not certain that treatment would have had adequate time to quantitatively affect acute phase protein response within the first week of the experiment. To our knowledge, no previous work evaluating the effects of RPMet supplementation on receiving cattle health and inflammation has been conducted. In transition dairy cows, variable haptoglobin responses to RPMet supplementation have been observed; in some cases, RPMet decreased plasma haptoglobin (Batistel et al., 2018; Zhou et al., 2016a) whereas in others RPMet had no effect on haptoglobin (Osorio et al., 2014; Vailati-Riboni et al.,

2017). In our cattle, because RPMet supplementation mitigated inflammation without altering performance, it is likely that this response was due to methionine's role in metabolism rather than as a limiting AA.

Conclusion

Our data suggest that methionine was not likely first limiting for growth performance in our heifers. Because our cattle were predominantly healthy, we were unable to characterize any effects of methionine supplementation on clinical illness. In contrast, our observation that methionine supplementation reduced haptoglobin late in the trial suggests that it mitigated inflammation at the physiological level. Further work evaluating the effects of methionine and other methyl group sources on health and inflammation in high-risk cattle would be helpful to determine if RPMet has utility as an immunomodulator for receiving cattle.

Literature Cited

Ackermann, M. R. 2017. Inflammation and healing. In: J. F. Zachary, editor, Pathologic Basis of Veterinary Disease (Sixth Edition). Elsevier. St. Louis, MO. pp. 73-131.

Ardalan, M., K. Rezayazdi, and M. Dehghan-Banadaky. 2010. Effect of rumen-protected choline and methionine on physiological and metabolic disorders and reproductive indices of dairy cows. J. Anim. Physiol. Anim. Nutr. 94:e259–e265. doi: 10.1111/j.1439-0396.2009.00966.x

Batistel, F., J. M. Arroyo, A. Bellingeri, B. Saremi, C. Parys, E. Trevisi, F. Cardoso, and J. J. Loor. 2017. Ethyl-cellulose rumen-protected methionine enhances performance during the periparturient period and early lactation in Holstein cows. J. Dairy Sci. 100:7455–7467. doi: 10.3168/jds.2017-12689

Batistel, F., J. M. Arroyo, C. I. M. Gares, E. Trevisi, C. Parys, M. A. Ballou, F. C. Cardoso, and J. J. Loor. 2018. Ethyl-cellulose rumen-protected methionine alleviates inflammation and oxidative stress and improves neutrophil function during the periparturient period and early lactation in Holstein dairy cows. J. Dairy Sci. 101:480-490. doi: 10.3168/jds.2017-13185

Deetz, L. E., A. M. Papas, and C. H. Benton. 1985. Performance of finishing steers fed rumen-protected methionine and/or lysine. J. Anim. Sci. 61(Suppl. 1):486 (Abstr.).

Drackley, J. K. 1999. ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: The final frontier? J. Dairy Sci. 82:2259–2273. doi: 10.3168/jds.S0022-0302(99)75474-3

Lobley, G. E. 1992. Control of the metabolic fate of amino acids in ruminants: a review. J. Anim. Sci. 70:3264–3275. doi: 10.2527/1992.70103264x.

Ørskov, E. R. 1982. Protein Nutrition in Ruminants. Acad. Press, London, England.

Osorio, J. S., E. Trevisi, P. Ji, J. K. Drackley, D. Luchini, G. Bertoni, and J. J. Loor. 2014. Biomarkers of inflammation, metabolism, and oxidative stress in blood, liver, and milk reveal a better immunometabolic status in peripartal cows supplemented with Smartamine M or MetaSmart. J. Dairy Sci. 97:7437–7450. doi: 10.3168/jds.2013-7679

Osorio, J. S., P. Ji, J. K. Drackley, D. Luchini, and J. J. Loor. 2013. Supplemental Smartamine M or MetaSmart during the transition period benefits postpartal cow performance and blood neutrophil function. J. Dairy Sci. 96:6248–6263. doi: 10.3168/jds.2012-5790

Richardson, C. R., and E. E. Hatfield. 1978. The limiting amino acids in growing cattle. J. Anim. Sci. 46:740-745. doi: 10.2527/jas1978.463740x

Spore, T. J., S. P. Montgomery, E. C. Titgemeyer, G. A. Hanzlicek, C. I. Vahl, T. G. Nagaraja, K. T. Cavalli, W. R. Hollenbeck, R. A. Wahl, and D. A. Blasi. 2019. Effects of a high-energy programmed feeding protocol on nutrient digestibility, health, and performance of newly received growing beef cattle. Appl. Anim. Sci. 35:397–407. doi:10.15232/aas.2019-01853

Step, D. L., C. R. Krehbiel, H. A. DePra, J. J. Cranston, R. W. Fulton, J. G. Kirkpatrick, D. R. Gill, M. E. Payton, M. A. Montelongo, and A. W. Confer. 2008. Effects of commingling beef calves from different sources and weaning protocols during a forty-two-day receiving period on performance and bovine respiratory disease. J. Anim. Sci. 86:3146–3158. doi: 10.2527/jas.2008-0883

Titgemeyer, E. C., N. R. Merchen, L. L. Berger, and L. E. Deetz. 1988. Estimation of lysine and methionine requirements of growing steers fed corn silage-based or corn-based diets. J. Dairy Sci. 71:421-434. doi: 10.3168/jds.S0022-0302(88)79572-7

Vailati-Riboni, M., J. S. Osorio, E. Trevisi, D. Luchini, and J. J. Loor. 2017. Supplemental Smartamine M in higher energy diets during the prepartal period improves hepatic biomarkers of health and oxidative status in Holstein cows. J. Anim. Sci. Biotechnol. 8:17. doi: 10.1186/s40104-017-0147-7

Walker, J. B. 1979. Creatine: biosynthesis, regulation, and function. Adv. Enzymol. Relat. Areas Mol. Biol. 50:177-242. doi: 10.1002/9780470122952.ch4

Wright, M. D., and S. C. Loerch. 1988. Effects of rumen-protect amino acids on ruminant nitrogen balance, plasma amino acid concentrations and performance. J. Anim. Sci. 66:2014-2027. doi: 10.2527/jas1988.6682014x

Zhou, Z., O. Bulgari, M. Vailati-Riboni, E. Trevisi, M. A. Ballou, F. C. Cardoso, D. N. Luchini, and J. J. Loor. 2016a. Rumen-protected methionine compared with rumen-protected choline improves immunometabolic status in dairy cows during the peripartal period. J. Dairy Sci. 99:8956–8969. doi: 10.3168/jds.2016-10986

Zhou, Z., M. Vailati-Riboni, E. Trevisi, J. K. Drackley, D. N. Luchini, and J. J. Loor. 2016b. Better postpartal performance in dairy cows supplemented with rumen-protected methionine compared with choline during the peripartal period. J. Dairy Sci. 99:8716–8732. doi: 10.3168/jds.2015-10525

Table 2.1. Composition of diet fed to receiving heifers

	Treatment	
Item	Control	Smartamine M
Ingredient, % of DM		
Corn, dry rolled	34.5	-
SM corn, dry rolled[1]	-	34.5
Wet corn gluten feed[2]	40.0	40.0
Corn silage	10.0	10.0
Alfalfa hay	4.0	4.0
Prairie hay, chopped	4.0	4.0
Supplement[3]	7.5	7.5
Nutrient composition, % of DM		
DM, % as is	62.8	62.6
OM	93.5	93.2
NDF	22.1	23.7
ADF	8.7	9.6
Starch	53.8	54.3
CP	13.2	13.3

[1]Dry-rolled corn and Smartamine M were combined and mixed for 60 seconds in a paddle mixer according to Smartamine M User Guide instructions. The final mixture was designed to contain 99.5% dry-rolled corn and 0.5% Smartamine M. The SM diet contained 0.1725% Smartamine M.

[2]Sweet Bran, Cargill Animal Nutrition, Blair, NE.

[3]Supplement pellets formulated to contain (DM basis) 10.6% CP, 8.7% Ca, 0.62% P, 4.6% NaCl, 0.70% K, 0.20% Mg, 5.1% fat, and 330 mg/kg monensin (Rumensin; Elanco, Greenfield, IN). Supplement ingredients were (as % of DM) 70.7% wheat middlings, 23.4% $CaCO_3$, 5.0% NaCl, 0.35% soybean oil, 0.18% Rumensin 90, 0.11% $ZnSO_4$, 0.08% $MnSO_4$ (32%), 0.06% vitamin E (500,000 IU/kg), 0.05% $CuSO_4$, 0.01% Se (0.99%), 0.007% EDDI (50 grain), 0.004% vitamin A (650,000 IU/g).

Table 2.2. Effect of Smartamine M on performance in beef receiving heifers

	Treatment			
Item	Control	SM	SEM	*P*-value
No. of pens	16	16		
No. of animals	191	191		
BW, kg				
d 0	222	222	4.9	0.70
d 14	242	242	2.9	0.87
d 21	251	251	2.5	0.88
d 28	259	259	2.4	0.66
d 35	267	268	2.9	0.87
d 45	278	279	3.0	0.65
ADG, kg/d				
d 0 to 14	1.44	1.44	0.167	0.95
d 14 to 45	1.15	1.19	0.049	0.52
d 0 to 45	1.24	1.27	0.064	0.55
DMI, kg/d				
d 0 to 14	4.43	4.34	0.044	0.008
d 14 to 45	5.65	5.63	0.051	0.54
d 0 to 45	5.28	5.21	0.045	0.001
G:F, kg/kg				
d 0 to 14	0.148	0.152	0.0187	0.70
d 14 to 45	0.093	0.096	0.0034	0.45
d 0 to 45	0.107	0.110	0.0064	0.28

Table 2.3. The effects of Smartamine M on morbidity and mortality in beef heifers

	Treatment	
Item	Control	SM
Morbidity, %		
Treated once	1.56	2.60
Treated twice	0.52	0.52
Treated thrice [1]	0.00	0.52[2]
Mortality, %	0.52	0.52[2]

[1]Heifers requiring 3 treatments were considered chronic and removed from the experiment

[2]These two values both resulted from a single chronic heifer that died following removal from the experiment

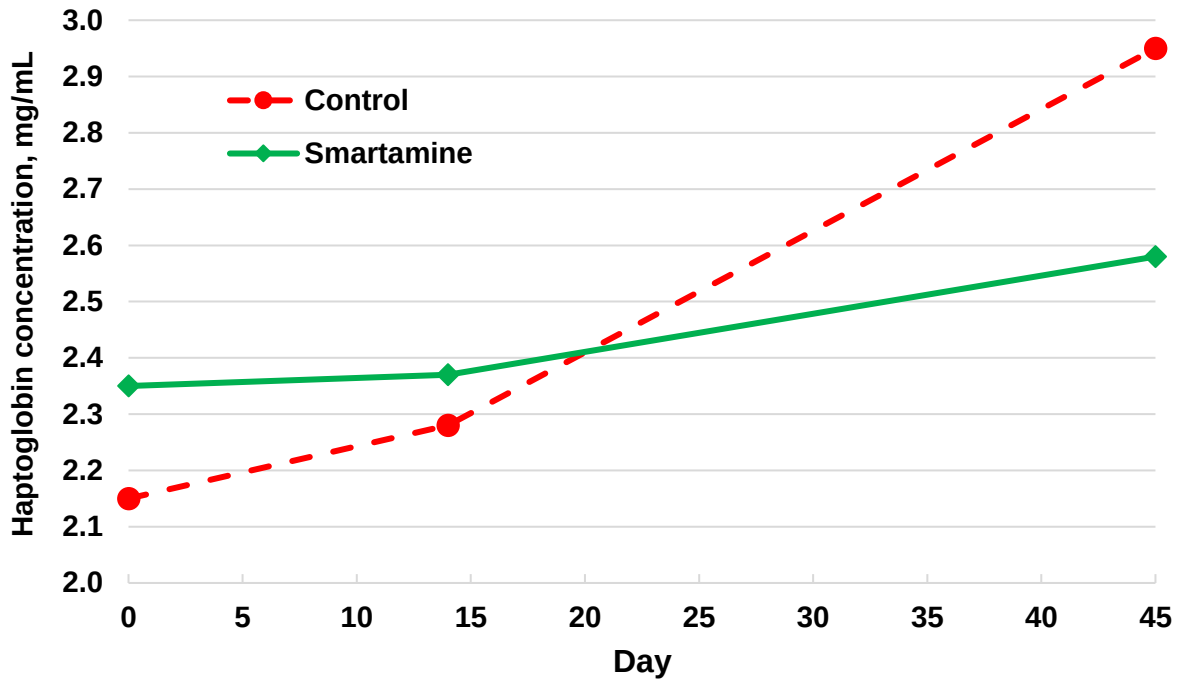

Figure 2.1. Effect of Smartamine M on plasma haptoglobin concentrations in receiving beef heifers. Treatment × linear day interaction, *P* = 0.05, SEM = 0.22.

Chapter 3 - Effects of supplemental guanidinoacetic acid, creatine, and choline on protein deposition and immune function in growing steers

M. S. Grant[1], H. D. Aufdemberge[1], L. K. Mamedova[1], M. D. Miesner[2], B. J. Bradford[1], and E. C. Titgemeyer[1]

[1]Department of Animal Sciences and Industry, Kansas State University, Manhattan, KS, 66506

[2]Department of Clinical Sciences, Kansas State University, Manhattan, KS, 66506

Abstract

Methionine and choline can improve performance and immune function in transition dairy cows. Our objective was to determine how modulation of methyl group status would affect protein deposition and immune function in growing cattle. Six ruminally cannulated Holstein steers (200 kg) were used in a 6×6 Latin square design with 10-d periods. Factorial treatments, which were continuously infused abomasally, included 3 methyl group modulators (MGM: control; 15 g/d guanidinoacetic acid [GAA]; or 16.8 g/d creatine) and 2 levels of choline (0 or 5 g/d choline ion; provided as 6.7 g/d choline chloride). Providing GAA or creatine increases body creatine supply; however, GAA consumes methyl groups to synthesize creatine, whereas supplemental creatine directly spares methyl groups that would otherwise be used for its synthesis. Steers received 4 kg/d of a corn-based diet. Total collection of urine and feces occurred from d 7 to 9 to measure N retention. Jugular blood was collected on d 10. No interactions between MGM and choline were observed. GAA increased N retention ($P = 0.04$), whereas creatine did not ($P = 0.56$). Urinary creatine excretion was increased by GAA and creatine supplementation ($P < 0.0001$). Urinary GAA output was greater for GAA supplemented steers than creatine and control ($P < 0.0001$). Plasma creatine concentration was increased by GAA and creatine supplementation ($P \leq 0.01$), with GAA leading to a larger increase. Plasma GAA concentration increased with GAA provision ($P < 0.0001$) but tended to be decreased by creatine ($P = 0.09$). Plasma urea-N and urinary urea-N output were not affected by MGM ($P \geq 0.25$). No effects of MGM on plasma haptoglobin ($P = 0.97$) or ex vivo neutrophil oxidative burst or phagocytosis ($P \geq 0.30$) were observed, but creatine did reduce plasma Trolox-equivalent antioxidant capacity ($P \leq 0.01$). Choline did not affect N retention ($P = 0.69$) or urinary GAA or creatine excretions ($P \geq 0.14$). Choline increased plasma creatine ($P = 0.04$), but

not plasma GAA. Choline did not affect plasma urea-N or urinary urea-N output ($P \geq 0.68$). Choline tended to reduce plasma haptoglobin ($P = 0.07$) but did not affect plasma Trolox-equivalent antioxidant capacity. Choline tended to reduce ex vivo neutrophil phagocytosis in the presence of LPS ($P = 0.09$) but did not affect ex vivo neutrophil phagocytosis in the absence of LPS or ex vivo neutrophil oxidative burst in the presence of absence of LPS ($P \geq 0.29$). These data demonstrate that GAA has potential to improve protein deposition in growing cattle fed corn-based diets. Additionally, choline provision may allow for greater creatine synthesis in the body and may have potential to improve inflammatory status and alter neutrophil function independent of methyl group status.

Introduction

Choline is an essential nutrient that is present in some feedstuffs, and it can be synthesized in the liver. In ruminants, very little dietary choline is available for absorption in the small intestine due to extensive degradation by ruminal microbes (Sharma and Erdman, 1988). For this reason, ruminants rely almost solely on *de novo* choline synthesis, which is produced when phosphatidylethanolamine *N*-methyltransferase (PEMT) transfers three methyl groups from *S*-adenosylmethionine (SAM; derived from methionine) to phosphatidylethanolamine to produce phosphatidylcholine (Vance, 2014). Phosphatidylcholine can be utilized in cell membranes, incorporated into very light density lipoproteins (VLDL), or have the choline ion cleaved to provide free choline (Zeisel, 2006). Although choline synthesis consumes methyl groups, it also has potential to serve as a methyl donor in the body. Free choline can be oxidized to form betaine, a methyl donor that can remethylate homocysteine to form methionine by action of betaine-homocysteine methyltransferase (BHMT). Choline supplementation can improve performance in finishing beef cattle (Bindel et al., 2000; Bryant et al., 1999) and transition dairy cows (Arshad et al., 2019). Effects of choline on health and immune function in transition dairy cattle have also been studied (Ardalan et al., 2010; Zhou et al., 2016, 2018; Vailati-Riboni et al., 2017).

Guanidinoacetic acid (GAA) is the sole precursor of creatine, which acts as a shuttle for high-energy phosphate bonds to maintain energy homeostasis in muscle tissue (Brosnan et al., 2009; Wyss and Kaddurah-Daouk, 2000). Creatine is formed when guanidinoacetate *N*-methyltransferase (GAMT) transfers a methyl group from SAM to GAA. Once synthesized, creatine does not appear to feedback to inhibit GAMT activity (Clarke and Banfield, 2001). As a result, exogenous GAA supplementation has the potential to increase endogenous creatine

production; however, this process also consumes methyl groups from SAM, which may produce a methyl group deficiency if methionine supply is limited (Stead et al., 2001; Setoue et al., 2008).

Recent work in our lab has investigated GAA supplementation to growing cattle (Ardalan et al., 2016; Speer, 2019). In cattle of Ardalan et al. (2016), supplemental GAA tended to improve protein deposition, measured by N retention, when methionine was provided to meet requirements but not when methionine as deficient. In contrast, Speer (2019) observed no improvements in protein deposition when GAA was supplemented to steers fed corn-based diets. Supplementation of GAA or creatine in conjunction with methyl sources other than methionine (i.e., choline) has not been investigated. Our objective was to evaluate the effects of choline, GAA, and creatine on performance and immune function in growing cattle fed corn-based diets.

Materials and Methods

All procedures involving the use of animals were approved by the Kansas State University Institutional Animal Care and Use Committee.

Animals and Treatments

Six ruminally cannulated Holstein steers (average initial body weight 146 ± 5.9 kg) were used in a 6 × 6 Latin square design with 2 × 3 factorial arrangement of treatments, with treatment sequence balanced for carryover effects. All treatments were infused abomasally and included 3 levels of methyl group status by providing saline solution (control), 15 g/d GAA (which consumes methyl groups), or 16.8 g/d creatine (which spares methyl groups; equimolar with the GAA treatment) with either 0 or 5 g/d of choline.

Steers were housed in a temperature-controlled room (20°C) with *ad libitum* access to water and were fed a corn-based diet (3.5 kg/d DM) in 2 equal portions at 12-h intervals (0700 and 1900 h). Three weeks prior to the study, calves were adapted to the diet while housed in tie-stalls. The diet (Table 3.1) was formulated to be identical to that of Speer (2019) and was designed to be comparable to a diet fed in a production-type setting. Three days prior to initiation of treatment infusions, calves were placed in individual metabolism crates to allow total collection of urine and feces for the duration of the study. Flexible Tygon tubing infusion lines (2.38 mm i.d.; Fisher Scientific, Pittsburgh, PA) were placed in the abomasum through the ruminal cannula prior to the study. Infusion lines were held in the abomasum by a rubber flange (10 cm in diameter) placed near the end of the line. A peristaltic pump (Ismatec ISM444A-230V BVP Standard; Cole-Parmer Instrument Company, Vernon Hills, IL) was used to infuse treatments continuously at a rate of 2.77 mL/min, with total infusion amounts of 4 kg/d per steer.

Periods were 10 d in length, and included 6 d of adaptation to treatments, 3 d for total collection of urine and feces, and 1 d for blood sampling.

The GAA treatment solution was prepared by dissolving 91 g GAA in 17,450 g water containing 218.4 g 9.25% (wt/wt) HCl. After the GAA had dissolved, approximately 436 g of 5% (wt/wt) NaOH was added to raise the final solution pH between 6 and 7. Finally, water was added to bring the total solution weight to 18,200 g, resulting in a 5 g/kg GAA solution. To prepare the creatine solution, 116 g creatine monohydrate (102 g creatine) was dissolved in 17,500 g water. Once the creatine was dissolved in solution, 32 g NaCl was added and brought to a total weight of 18,200 g with water to create a 5.6 g/kg creatine solution. For the control base infusion, 32 g NaCl was dissolved in 17,500 g water. Once dissolved, the solution was brought up to a final weight of 18,200 g, creating a 1.75 g/kg saline solution. The NaCl was added to the creatine and saline solutions to equalize treatments for electrolytes, specifically balanced for Na content across treatments. The choline treatment solution was prepared by dissolving 67 g choline chloride (50 g choline) into a 2 L solution with deionized H_2O, creating a 2.5% (wt/vol) choline solution. The GAA, creatine, and choline solutions were stored at 4°C until use. Infusion bottles (providing enough material for 12 h of infusion) were prepared by adding 1.5 kg of the corresponding methyl group treatment solution (i.e., GAA, creatine, or saline) to each bottle (3 kg/d per steer). For steers receiving 5 g/d choline, 100 mL choline solution was added to each bottle (200 mL/d per steer). Bottles were then made to final daily weights of 4 kg with water. Infusion bottles were changed at 12-h intervals (0700 and 1900 h).

Sample Collection

Prior to diet mixing, feed ingredient samples were collected and stored frozen at -20°C until analysis. Representative diet samples were collected on d 6 to 8 of each period and frozen at -20°C. Within period, feed samples were composited, subsampled, and frozen at -20°C until analysis. If present, orts were collected and weighed at 1900 h daily from d 6 to 8 of each period and frozen at -20°C. Orts were composited by steer within period, subsampled, and frozen at -20°C until analysis. Total urine and fecal outputs were measured for d 7 to 9 of each period; collections were weighed daily for each steer. Urine was collected in buckets containing 900 mL of 10% (wt/wt) H_2SO_4 to prevent ammonia losses; 4 L H_2O was also added to each bucket to limit precipitation from the urine. Urine buckets were mixed prior to sampling. Feces were collected into metal pans lined with plastic bags and mixed thoroughly prior to sampling. Representative samples of urine and feces were collected daily and frozen at -20°C. Within period, samples of urine and feces were composited by steer in proportion of the total outputs and frozen at -20°C until analysis.

On d 10 of each period, blood was collected via jugular venipuncture into vacutainer tubes (Becton, Dickinson, and Company; Franklin Lakes, NJ) containing sodium heparin (50 mL blood) or potassium EDTA (10 mL blood) at 0800 h (1 h after morning feeding). Tubes were immediately placed on ice and 20 mL of blood containing sodium heparin was centrifuged at 2,000 × *g* at 4°C for 20 min, plasma harvested, and stored at -20°C until analysis. Frozen plasma was used for analysis of GAA, creatine, creatinine, urea, glucose, haptoglobin, and Trolox-equivalent antioxidant capacity. Blood containing EDTA (10 mL) was analyzed for complete blood count (CBC) with differential at the Kansas State University Veterinary Diagnostic Lab.

At the same time that blood was collected for analyses described above, the remaining 30 mL of heparinized whole blood was placed on ice until use for neutrophil isolation and analysis for phagocytosis and oxidative burst.

Laboratory Analyses

Subsamples of diet and orts were dried at 55°C in a forced-air oven for 48 h, allowed to air-equilibrate for 24 h, weighed, and ground through a 1-mm screen using a Thomas Wiley Mill (Thomas Scientific, Swedesboro, NJ). Partially dried feed and orts and wet fecal samples were dried at 105°C for 24 h in a forced-air oven for DM analysis and then ashed at 450°C for 8 h in a muffle oven to determine OM content. All feed, orts, wet fecal, and urine samples were analyzed for N content with a LECO TruMac N Analyzer (LECO Corporation, Saint Joseph, MI). For feed samples, CP was calculated by multiplying N × 6.25. Feed was analyzed for NDF (with α-amylase and sodium sulfite) and ADF sequentially using an ANKOM Fiber Analyzer (ANKOM Technology, Macedon, NY).

Plasma and urine samples were analyzed for urea concentration by the method of Marsh et al. (1965). Plasma glucose concentrations were measured using an assay based on glucose oxidase producing H_2O_2, which reacts with 4-aminoantipyrine to produce a chromophore (Autokit Glucose; Wako Life Sciences, Inc., Mountain View, CA). Plasma concentrations of haptoglobin were measured as described by Cooke and Arthington (2013) as a biomarker for inflammation status; samples were analyzed in quintuplet and the median value was utilized. Plasma antioxidant potential (AOP) was measured by Trolox equivalent antioxidant capacity using the methods of Re et al. (1999). Briefly, plasma antioxidant potential was measured by comparison to a standardized reduction of Trolox (synthetic vitamin E analog; Sigma-Aldrich,

St. Louis, MO) in a generated radical solution of 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS; Sigma-Aldrich, St. Louis, MO). It is difficult to accurately quantify individual antioxidants (e.g., glutathione, superoxide dismutase, thiol groups, vitamins, etc.), but plasma AOP provides an estimate of the total antioxidant activity in the blood.

Plasma and urinary GAA, creatine, and creatinine concentrations were measured by procedures described by Shingfield and Offer (1999) with modifications described by Ardalan et al. (2020). Analyses were performed on an Agilent 1100 series liquid chromatograph (Agilent Technologies; Santa Clara, CA), Acutect 500 UV/VIS detector (Thermo Fisher Scientific Inc., Waltham, MA), and SpectraSYSTEM AS 1000 autosampler equipped with a 5-µL loop for sample injection (Thermo Fisher Scientific Inc., Waltham, MA). A single channel data system with PeakSimple chromatography software (Model 333; SRI Instruments, Menlo Park, CA) was used for data acquisition. Compounds were separated on a reversed-phase 5-µm Discovery BIO Wide Pore C18 column (250 × 4.6 mm i.d., Supelco 568223-U; Sigma-Aldrich, St. Louis, MO) with a 5-µm Discovery BIO Wide Pore C18 guard column (20 cm × 4.6 mm i.d.; Sigma-Aldrich, St. Louis, MO), and the eluted compounds were measured by absorbance at 200 nm.

Plasma was prepared by adding 10% (wt/vol) sulfosalicylic acid (SSA) 1:1 to deproteinize samples, then vortexed and placed on ice for 30 minutes. Samples were then centrifuged at 13,000 × *g* for 10 min at room temperature (22°C), and the supernatant was filtered through a 0.22-µm membrane syringe filter (Thermo Fisher Scientific Inc., Waltham, MA) into an HPLC vial for injection. Urine samples were diluted 10:1 with a diluent that was prepared by dissolving 0.09 g of monobasic ammonium phosphate and 0.101 g of sodium 1-heptane sulfonic acid in 100 mL of double-deionized H_2O (pH adjusted to 2.2 with 50% phosphoric acid). Samples were then vortexed and filtered through a 0.22-µm membrane syringe

filter (Thermo Fisher Scientific Inc., Waltham, MA) into an HPLC vial for injection. The aqueous mobile phase contained 0.09% ammonium monophosphate (wt/vol), 0.101% (wt/vol) sodium 1-heptane sulfonic acid, 70 µL triethylamine (vol/vol) and 3.5% methanol (vol/vol). The final pH of the mobile phase was adjusted to 2.85 with 50% phosphoric acid, and it was filtered through a 0.22-µm membrane filter (Thermo Fisher Scientific Inc., Waltham, MA) and degassed prior to use. The column temperature was maintained at room temperature (22°C). The elution was performed with a flow rate of 0.5 mL/min for 14 min, then increased to 1.2 mL/min for 12 min. Column was then washed with 100% methanol for 10 min at a flow rate of 1.2 mL/min, after which the column was re-equilibrated with the initial eluent before the injection of another sample. Total analysis time per sample was 55 min.

For phagocytosis and oxidative burst measures, polymorphonuclear cells (PMN; primarily neutrophils) were isolated from heparinized jugular blood similarly to Garcia et al. (2018). Briefly, 30 mL heparinized whole blood was diluted with equal parts PBS (approximately 4°C) followed by differential centrifugation with Ficoll-paque PLUS (GE Healthcare; Pittsburg, PA) at 1,700 × *g* at 4°C for 30 minutes. Following centrifugation, the upper layer containing plasma, mononuclear cells, and Ficoll was removed using a vacuum pump, leaving red blood cells and PMN behind. This phase was subjected to red blood cell lysis with ACK lysing buffer (Gibco by Life Technologies; Thermo Fisher Scientific, Waltham, MA) and cells were washed with Hanks' balanced salt solution (HBSS; Gibco by Life Technologies; Thermo Fisher Scientific, Waltham, MA) until only PMN remained. Cell viability of PMN was measured using trypan blue exclusion and averaged 96%.

Once isolated, PMN were primed with or without lipopolysaccharide (LPS; 1 µg/mL final concentration, *E. coli* 055:B5; Sigma-Aldrich Co.; St. Louis, MO) and incubated at 37°C for

30 minutes. Dihydrorhodamine (DHR; final concentration 100 µM) was added and cells incubated an additional 10 minutes. Finally, *E. coli* covalently labeled with Texas Red (Molecular Probes Inc.; Eugene, OR) was added at a ratio of 20:1 (bacteria:neutrophil) and incubated 40 min. Following incubation, PMN activity was stopped by cold shock and cells were washed with PBS and pelleted three times. Cells were then reconstituted with 200 µL PBS for fluorescence measurement. Activity of PMN was measured using a capillary flow cytometer (Guava EasyCyte Mini Flow Cytometry System; Millipore Sigma, Billerica, MA). Five thousand cells were counted for each sample and PMN were gated based on side scatter- and forward scatter-area. Cells incubated without bacteria but with DHR served as negative controls to correct for nonspecific fluorescence. The percentage of neutrophils containing red fluorescence represents phagocytosis of Texas Red labeled *E. coli*. The percentage of neutrophils with green fluorescence represents oxidative burst.

Calculations

Nitrogen retention was calculated as the difference between total N intake (feed and infused N) and total N output (fecal and urinary N). Dietary dry matter and organic matter digestibilities were calculated based on feed alone as a portion of intake (i.e., intake via abomasal infusion was not included).

Statistical Analysis

Data were analyzed using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). Our model included period and treatment (choline, MGM, and choline × MGM) as fixed effects and steer as a random effect. The LSMEANS statement was used to calculate treatment means.

Individual treatment means within MGM factor were separated by pairwise t-test when the f-test was significant or tended to be significant. Significance was declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$. Data for two steers was removed from periods 1 and 2 as a result of excessive feed refusals.

Results and Discussion

Nutrient Intake and Digestibility

No effects of choline or MGM were observed for DM or OM intake ($P \geq 0.40$; Table 3.2) or DM or OM apparent digestibility ($P \geq 0.34$); moreover, no interactions between choline and MGM were observed for DM or OM intake or apparent digestibility ($P \geq 0.45$). Apparent digestibilities of DM and OM were 78.8% and 79.7%, respectively. Speer (2019) fed a similar diet to growing steers and observed lower apparent DM and OM digestibilities of 73.9% and 74.5%, respectively. Cattle of Speer (2019) had greater DM intake than our cattle (5.3 kg/d vs. 3.5 kg/d) but consumed less as a percent of BW (2.1% vs. 2.4%, respectively); however, it isn't clear why our digestibilities were somewhat greater.

Nitrogen Retention

No interactions between choline and MGM were observed for any N intake, N excretion, or N retention measures ($P \geq 0.45$; Table 3.2). Total N intakes were greater for choline, GAA, and creatine supplemented steers compared to unsupplemented controls ($P \leq 0.001$), which was expected due to the treatment structure.

Effects of choline

Neither urinary N excretion nor urinary urea-N were affected by choline ($P > 0.44$). Additionally, urinary urea-N as a proportion of total urinary N was not affected by choline ($P = 0.50$; data not shown). Fecal N output was not affected by choline ($P = 0.66$). No effect of choline on retained N was observed ($P = 0.69$). The lack of a N retention response to choline is consistent with the work of Löest et al. (2002), where abomasal infusion of 8 g/d choline to methionine-deficient steers failed to improve N retention. These data are in contrast to work in finishing cattle (Bryant et al., 1999; Bindel et al., 2000), where supplemental choline provided in a ruminally protected form improved average daily gain and feed efficiency. The positive response to choline in finishing cattle may have been a result of altered lipid metabolism via phosphatidylcholine synthesis from choline or other mechanisms unrelated to alleviation of a methionine deficiency.

Effects of methyl group modulators

No effects of MGM on urinary N or urinary urea-N excretion were observed ($P \geq 0.69$). Urinary urea-N excretion as a percentage of total N excretion was also not affected by MGM ($P = 0.61$; data not shown). There was an effect of MGM on fecal N excretion ($P < 0.001$), with creatine supplemented steers excreting the most fecal N, followed by GAA, then control. There was a tendency for MGM to affect N retention ($P = 0.10$); GAA increased N retention compared to the control ($P = 0.04$), whereas creatine did not ($P = 0.56$). Previous work in our lab demonstrated that 256-kg growing steers fed the same basal diet as our steers were limited by methionine supply, as demonstrated by an increase in N retention when 5 g/d methionine was abomasally infused (Speer, 2019). Thus, we expected the steers in our model to be methionine deficient. Corn-based diets are often first-limiting in lysine (Burris et al., 1976; Hill et al., 1980;

Titgemeyer et al., 1985), so it may be that the dietary soybean meal provided enough lysine to meet the steers' requirement. Speer (2019) observed no significant effect of increasing GAA supplementation (0, 7.5, or 15 g/d infused abomasally) on N retention in the presence or absence of 5 g/d supplemental methionine; however, a numerical trend for decreased N retention with increasing GAA infusion was observed. This is in contrast with our data where 15 g/d GAA improved N retention compared to control. In growing steers starkly deficient in methionine, Ardalan et al. (2016) observed a tendency for an interaction between GAA and methionine, with N retention improving linearly when 6 g/d methionine was provided but decreasing linearly with methionine absent. It isn't abundantly clear why our cattle responded to GAA supplementation but those of Speer (2019) did not when fed the same diet; however, some differences between the two groups of cattle were present. Cattle of Speer (2019) had a greater initial BW (256 kg vs. 146 kg) and greater DM intake (5.3 kg/d vs. 3.5 kg/d), but less intake as a percent of BW (2.1% vs 2.4%) compared to our cattle. Total protein deposition was greater for control cattle of Speer (2019) compared to our control cattle (38.5 g/d vs. 27.2 g/d, respectively); however, Speer's cattle had lower protein deposition when scaled to BW than ours (0.15 g retained N/kg BW vs. 0.19 g retained N/kg BW, respectively). Steers of Speer (2019) that received 5 g/d supplemental Met but no GAA also had greater protein deposition than our control cattle (46.2 g/d vs. 27.2 g/d, respectively), but still had similar protein deposition when scaled to BW (0.18 g retained N/ kg BW vs 0.19 g retained N/ kg BW, respectively). Growing animals have a particularly high creatine requirement to support tissue development and protein deposition needs (Walker, 1979). It is possible that because our cattle were younger and smaller than those of Speer, (2019), they may have had a greater requirement for creatine and were therefore more able to respond to GAA supplementation for creatine synthesis. It is unclear why creatine did not improve retained

N, because direct supplementation of 16.8 g/d creatine should provide equal molar amounts of creatine as 15 g/d GAA without increasing methyl group demand. One possibility is that creatine may not have been effectively absorbed in the small intestine, which would prevent it from acting in the body. This could explain why fecal N excretion was greatest in creatine supplemented steers compared to control and GAA (i.e., creatine was likely the source of the greater fecal N excretion).

Plasma and Urinary Metabolites

No interactions between choline and MGM treatments were observed for any plasma or urinary metabolites measured ($P \geq 0.51$; Table 3.3).

Effects of choline

Supplemental choline increased plasma creatine concentrations ($P = 0.04$) but did not affect plasma GAA or creatinine ($P \geq 0.25$). No effects of choline supplementation on urinary excretion of GAA, creatine, or creatinine were observed ($P \geq 0.14$). Increased plasma creatine concentrations in response to choline provision suggests that choline provision may allow for greater body creatine synthesis. This could be because direct choline supplementation may limit the body's need to consume methyl groups for its synthesis via phosphatidylcholine, which could make more methyl groups available to be used to methylate GAA to form additional creatine. Although not statistically significant, there was also a numeric increase in urinary creatine excretion when choline was provided ($P = 0.14$), which could readily be explained as a consequence of the increase in plasma concentrations of creatine in response to choline supplementation.

No effects of choline on plasma urea N ($P = 0.68$; Table 3.4) or glucose ($P = 0.78$) concentrations were observed, suggesting that general energy and protein status were not affected by choline supplementation.

Effects of methyl group modulators

Supplemental GAA increased plasma GAA concentrations ($P < 0.0001$) whereas creatine tended to decrease plasma GAA compared to controls ($P = 0.09$). Urinary GAA excretion increased with GAA supplementation ($P < 0.0001$) but not creatine ($P = 0.72$). Increased plasma GAA concentrations, in response to GAA supplementation is consistent with results of Ardalan et al. (2016) in growing steers and Ardalan et al. (2020) in dairy heifers at similar levels of supplementation. Urinary GAA excretion averaged 0.18 g/d for control and creatine treatments and 0.38 g/d for GAA. Although GAA supplementation led to a roughly twofold increase in urinary GAA excretion compared to control and creatine, this 0.21 g/d increase only accounted for 1.4% of the 15 g/d GAA provided. Increased urinary excretion of GAA in response to GAA supplementation has also been observed in growing cattle (Speer, 2019; Ardalan et al., 2020), and tended to increase in methionine deficient growing steers (Ardalan et al., 2016). The tendency for decreased plasma GAA in response to creatine supplementation may suggest that feedback inhibition of L-arginine:glycine amidinotransferase (AGAT; which synthesizes GAA) by creatine occurred (Stead et al., 2001).

GAA and creatine provision both increased plasma creatine concentrations ($P < 0.0001$), with GAA producing a greater increase ($P = 0.01$). Supplemental creatine and GAA increased urinary creatine excretion ($P < 0.0001$); a greater increase was observed with GAA than creatine ($P < 0.001$). Creatine requirements are greatest for growing animals because of its essentiality for

development of lean tissue (Brosnan et al., 2009). The increase in plasma creatine concentration and urinary creatine output in response to GAA supplementation was expected and demonstrates that GAA was effectively methylated to form creatine. This agrees with previous work conducted in growing cattle fed both corn-based diets (Ardalan et al., 2020; Speer, 2019) and in a methionine deficiency model (Ardalan et al., 2016). Creatine supplementation also increased plasma creatine concentration and urinary creatine excretion, but less so than GAA. Urinary creatine excretion averaged 2.4 g/d for control, 4.9 g/d for creatine, and 7.0 g/d for GAA. The twofold increase in creatine excretion for creatine supplemented steers represents approximately 14.9% of the 16.8 g/d creatine infused. Additionally, the nearly threefold increase in creatine excretion for GAA supplemented steers is representative of approximately 27.4% of the 16.8 g/d creatine that would be expected to result from 15 g/d GAA infusion. Renal reabsorption of creatine followed a similar pattern and was greatest for control steers at 87.3%, followed by creatine supplemented steers at 77.2%, and lowest for GAA supplemented steers at 71.8% ($P < 0.0001$; data not shown). The greater increase in creatine excretion and lower renal reabsorption efficiency of creatine in GAA supplemented steers compared to creatine may be attributed to a possible lower absorption of creatine as mentioned previously. Nevertheless, it appears that as body creatine status improves (either from direct creatine provision or supplemental GAA), the efficiency that creatine is reabsorbed in the kidney decreases. It is possible that the body has some mechanistic control to decrease creatine retention as supply increases (i.e., possibly once the body's creatine requirement is met). Ostojic et al. (2016) observed numerically greater serum creatine in humans supplemented with GAA compared to creatine. Our plasma creatine results are in contrast to the work in Yucatan miniature pigs of McBreairty et al. (2015) who observed greater plasma creatine concentrations in creatine supplemented pigs than those supplemented

with GAA. In humans, GAA supplementation was more effective than supplemental creatine at increasing creatine concentrations in muscle and brain tissue (Ostojic et al., 2016). McBreairty et al. (2015) observed greater hepatic creatine concentrations in GAA supplemented pigs compared to creatine supplemented counterparts. This, in addition to work in cattle, demonstrates efficacy of GAA supplementation to improve body creatine supply, possibly more effectively than creatine alone.

No effects of MGM on plasma creatinine concentration or urinary creatinine excretion were observed ($P \geq 0.29$). Urinary creatinine excretion averaged 5.5 g/d across all treatments. Creatinine is spontaneously formed from creatine in an irreversible, nonenzymatic reaction and represents the permanent loss of body creatine (Brosnan and Brosnan, 2007). Creatinine is synthesized at a rate of approximately 1.7% of total body creatine per day and excreted in the urine (Wyss and Kaddurah-Daouk, 2000). Other work in growing steers has demonstrated no effect of GAA on plasma creatinine concentration (Ardalan et al., 2016; Speer, 2019). Speer (2019) observed no effect of GAA on urinary creatinine excretion, whereas Ardalan et al. (2016) observed a tendency for increased creatinine excretion with increasing GAA provision. In dairy heifers, Ardalan et al. (2020) saw a linear increase in plasma and urinary creatinine concentrations in response to GAA supplementation up to 40 g/d. Tossenberger et al. (2016) has suggested that this may be a result of excess creatine production, which may increase creatinine excretion, and could suggest that creatine provision and/or synthesis in our steers and those of Speer (2019) was not excessive enough to produce such a response.

No effects of MGM treatments on plasma urea N ($P = 0.25$) or glucose concentrations were observed ($P = 0.32$). This suggests that energy and protein status of our steers was not impacted by supplemental GAA or creatine.

Plasma Inflammation and Antioxidant Measures

No interactions were observed between choline and MGM treatments for plasma Trolox-equivalent AOP or haptoglobin ($P \geq 0.21$; 3.4).

Effects of choline

Choline supplementation did not affect plasma AOP ($P = 0.50$). Supplemental choline tended to reduce plasma haptoglobin concentration ($P = 0.07$). Although choline did not impact total antioxidant capacity in our steers, the tendency for choline to reduce plasma haptoglobin suggests a reduction in systemic inflammation. This is in contrast to the work of Zhou et al. (2016a) where no differences in haptoglobin concentration or other acute phase proteins were observed when transition dairy cows were supplemented with ruminally protected choline. Abdelmegeid et al. (2017) supplemented PMN isolated from neonatal Holstein calves with choline and observed downregulation of numerous genes associated with an inflammatory response. Because activity of betaine-homocysteine methyltransferase (which uses betaine to remethylate homocysteine to form methionine) is not present in PMN, it can be assumed that responses to choline were not a result of methionine resynthesis but instead some other pathway.

Effects of methyl group modulators

A main effect of MGM treatment on plasma AOP was observed ($P = 0.008$). Supplemental creatine decreased plasma AOP compared to both GAA supplemented and control steers ($P \leq 0.01$). No effect of MGM on plasma haptoglobin concentrations was observed ($P = 0.97$).

Because GAA consumes methyl groups when methylated to form creatine, we expected a possible increase in inflammation and/or oxidative stress associated with its supplementation as a result of some degree of reduced methyl group availability in the body. This is in part because

GAA obligatorily consumes methyl groups from SAM, which has the ability to directly ameliorate oxidative stress (Zhao et al., 2018; Cavallaro et al., 2010). Additionally, once SAM donates its methyl group it becomes *S*-adenosylhomocysteine (SAH) and then homocysteine, both of which can increase oxidative compounds (Tehlivets, 2011; Zhang et al., 2017). So, because the conversion of GAA to creatine consumes SAM methyl groups and produces SAH, it would be expected to have a negative impact on the body's oxidative status. When oxidative stress overwhelms the body's ability to mitigate it (i.e., via antioxidant enzymes and agents), the stability of cellular compounds such as proteins and lipid membranes can be damaged, leading to systemic inflammation (Freital et al., 2016). In contrast, direct creatine supplementation would be expected to decrease methyl demand (i.e., less GAA synthesis would be required to support creatine synthesis), which, may have positive impacts on inflammatory or oxidative stress. However, creatine-supplemented cattle had reduced plasma AOP compared to GAA-supplemented and control steers, which suggests lower antioxidant capacity. This is consistent with work of Percário et al (2012), where Brazilian handball athletes supplemented with creatine had lower AOP than placebo or unsupplemented control athletes. Creatine is often supplemented in humans to improve muscle strength as a result of greater ATP regeneration; however, it appears that it may also induce oxidative stress and decrease overall antioxidant status in humans and cattle. But, the lack of effect of GAA or creatine on plasma haptoglobin concentrations suggests that overall inflammatory status was likely unchanged by short term methyl status modulation. Speer (2019) also observed no impact of up to 15 g/d GAA on haptoglobin in the presence or absence of 5 g/d supplemental methionine in growing steers fed the same diet.

Blood cell profile and neutrophil functionality

Choline tended to decrease erythrocyte concentrations for control steers, but not those receiving creatine or GAA ($P = 0.09$; Table 3.5). No other significant interactions between choline and MGM treatment were observed for CBC parameters ($P \geq 0.11$) or PMN phagocytosis or oxidative burst ($P \geq 0.45$; Table 3.6).

Effects of choline

Supplemental choline tended to increase blood monocyte counts ($P = 0.06$). No other effects of choline on parameters in the CBC analysis were observed ($P \geq 0.15$). The tendency for greater concentration of monocytes in choline-supplemented steers could suggest an elevated immune response compared to controls (Ackermann, 2017). Nevertheless, blood monocyte concentrations of steers with or without choline provided (0.630 nL^{-1} and 0.496 nL^{-1}, respectively) were well within the normal reference range of 0.0 to 0.8 nL^{-1} for cattle. Vailati-Riboni et al. (2017) observed greater phagocytosis and oxidative burst in monocytes isolated from transition dairy cows supplemented with choline. In contrast, other work in dairy cows has shown no effect of choline supplementation on oxidative burst and phagocytosis in monocytes (Zhou et al., 2018). Because we measured monocyte abundance rather than activity, a direct comparison between these studies and ours on effects of supplemental choline on monocytes cannot be made.

Choline supplementation tended to decrease PMN phagocytosis in the presence of LPS ($P = 0.09$). No effects of choline on PMN phagocytosis without LPS or PMN oxidative burst with or without LPS were observed ($P \geq 0.29$). The tendency for reduced phagocytosis of PMN in the presence of LPS when choline was provided may suggest that it may in some way modulate the inflammatory response in the presence of an immune challenge. Using PMN

isolated from dairy cows in *in vitro* assays, Garcia et al. (2018) observed a linear decrease in oxidative burst as choline supplementation increased. Although it appears choline has potential to modulate immune cell function in cattle, the precise mode of action is not known. It is also unclear how the observed changes might affect immune and performance responses in the animal.

Effects of methyl group modulators

A tendency for a main effect of MGM on mean cell volume (MCV) of erythryoctes was observed ($P = 0.06$). Supplemental creatine and GAA increased MCV compared to control ($P \leq 0.05$), but creatine and GAA did not differ from one another ($P = 0.74$). No other effects of MGM on CBC parameters were observed ($P \geq 0.17$). Although creatine and GAA supplementation increased MCV, overall our cattle had an average MCV of 33.9 fL that was lower than the reference range (38 to 50 fL). The lack of effect of MGM on other parameters in the CBC analysis indicates that short term modulation of methyl group status may not greatly impact the profile of immune cells.

No main effects for MGM treatment on PMN oxidative burst or phagocytosis with or without LPS present were observed ($P \geq 0.30$). This further suggests that modulated methyl status in our steers may not have directly affected the immune cell profile or functionality. Additionally, the lack of an interaction between choline and GAA or creatine provision suggests that choline may act on PMN function independent of methyl group status.

Conclusion

Previous work in our lab has demonstrated mixed results when studying the effects of GAA on N retention in growing cattle maintained under different dietary conditions. In our

study, supplemental GAA improved N retention in growing steers fed a corn-based diet, but this effect was not observed when creatine was supplemented. Supplemental choline did not affect N retention in our cattle. GAA and creatine supplementation effectively improved body creatine supply, and choline may have potential to have a similar effect as evidenced by increased plasma creatine concentration. Choline tended to reduce inflammation, as evidenced by lower plasma haptoglobin concentrations, and may alter neutrophil phagocytosis in the presence of an *in vitro* LPS challenge but did not affect plasma antioxidant capacity. The lack of interaction between choline supplementation and GAA or creatine provision may suggest that choline acted independent of methyl group status. A more robust understanding of methyl group metabolism may allow nutritionists to supply these nutrients to optimize performance and immune function.

Literature Cited

Abdelmegeid, M. K., M. Vailati-Riboni, A. Alharthi, F. Batistel, and J. J. Loor. 2017. J. Dairy. Sci. 100:3155-3165. doi.org/10.3168/jds.2016-12025

Ackermann, M. R. 2017. Inflammation and healing: Acute phase proteins. In: J. F. Zachary, ed., Pathologic Basis of Veterinary Disease (Sixth Edition). Elsevier. St. Louis, MO. pp. 98-99.

Ardalan, M., E. D. Batista, and E. C. Titgemeyer. 2020. Effect of post-ruminal guanidinoacetic acid supplementation on creatine synthesis and plasma homocysteine concentrations in cattle. J. Anim. Sci. 98(3):1-9. doi:10.1093/jas/skaa072

Ardalan, M., K. Rezayazdi, and M. Dehghan-Banadaky. 2010. Effect of rumen-protected choline and methionine on physiological and metabolic disorders and reproductive indices of dairy cows. J. Anim. Physiol. Anim. Nutr, (Berl.) 94:e259–e265. doi: 10.1111/j.1439-0396.2009.00966.x

Ardalan, M., M. D. Miesner, C. D. Reinhardt, D. U. Thomson, C. K. Armendariz, and E. C. Titgemeyer. 2016. Guanidinoacetic acid as a precursor for creatine in steers. J. Anim. Sci. (E-Suppl. 5):753-754. (Abstr.) doi: 10.2527/jam2016-1577

Arshad, U., M. G. Zenobi, C. R. Staples, and J. E. P. Santos. 2020. Meta-analysis of the effects of supplemental rumen-protected choline during the transition period on performance and health of parous dairy cows. J. Dairy Sci. 103:282–300. doi: 10.3168/jds.2019-16842

Bindel, D. J., J. S. Drouillard, E. C. Titgemeyer, R. H. Wessels, and C. A. Löest. 2000. Effects of ruminally protected choline and dietary fat on performance and blood metabolites of finishing heifers. J. Anim. Sci. 78:2497–2503. doi: 10.2527/2000.78102497x

Brosnan, J. T., E. P. Wijekoon, L. Warford-Woolgar, N. L. Trottier, M. E. Brosnan, J. A. Brunton, and R. F. Bertolo. 2009. Creatine synthesis is a major metabolic process in neonatal piglets and has important implications for amino acid metabolism and methyl balance. *J. Nutr*. 139:1292–1297. doi:10.3945/jn.109.105411

Bryant, T. C., J. D. Rivera, M. L. Galyean, G. C. Duff, D. M. Hallford, and T. H. Montgomery. 1999. Effects of dietary level of ruminally protected choline on performance and carcass characteristics of finishing beef steers and on growth and serum metabolites in lambs. J. Anim. Sci. 77:2893−2903. doi: 10.2527/1999.77112893x

Burris, W. R., J. A. Boling, N. W. Bradley, and A. W. Young. 1976. Abomasal lysine infusion in steers fed a urea supplemented diet. J. Anim. Sci. 42:699-705. doi: 10.2527/jas1976.423699x

Cavallaro, R. A., A. Fuso, V. Nicolia, and S. Scarpa. 2010. S-adenosylmethionine prevents oxidative stress and modulates glutathione metabolism in TgCRND8 mice fed a B-vitamin deficient diet. J. Alzheimers Dis. 20:997-2002. doi: 10.3233/JAD-2010-091666

Clarke, S., and K. Banfield. 2001. *S*-adenosylmethionine-dependent methyltransferases. In: Carmel, R., Jacobsen, D. W. (Eds.), Homocysteine in health and disease. Cambridge University Press, Cambridge, England. p. 63-78.

Cooke, R. F., and J. D. Arthington. 2013. Concentrations of haptoglobin in bovine plasma determined by ELISA or a colorimetric method based on peroxidase activity. J. Anim. Physiol. Anim. Nutr. (Berl). 97:531-536. doi: 10.1111/j.1439-0396.2012.01298.x

Freitas, I., E. Boncompagni, E. Tarantola, C. Gruppi, V. Bertone, A. Ferrigno, G. Milanesi, R. Vaccarone, M. E. Tira, and M. Vairetti. 2016. Oxid. Med. Cell. Long. 2016:1-14. doi:10.1155/2016/9307064

Garcia, M., L. K. Mamedova, B. Barton, B. J. Bradford. 2018. Choline regulates the function of bovine immune cells and alters the mRNA abundance of enzymes and receptors involved in its metabolism in vitro. Front. Immunol. 9:1-14. doi: 10.3389/fimmu.2018.02448

Hill, G. M., J. A. Boling, and N. W. Bradley. 1980. Postruminal lysine and methionine infusion in steers fed a urea-supplemented diet adequate in sulfur. J. Dairy Sci. 63:1242-1247. doi: 10.3168/jds.S0022-0302(80)83075-X

Löest, C. A., E. C. Titgemeyer, G. St-Jean, D. C. Van Metre, and J. S. Smith. 2002. Methionine as a methyl group donor in growing cattle. J. Anim. Sci. 80:2197-2206. doi: 10.1093/ansci/80.8.2197

Marsh, W. H., B. Fingerhut, and H. Miller. 1965. Automated and manual direct methods for the determination of blood urea. J. Clin. Chem. 11:624-627.

McBreairty, L. E., J. L. Robinson, K. R. Furlong, J. A. Brunton, and R. F. Bertolo. 2015. Guanidioacetate is more effective than creatine at enhancing tissue creatine stores while consequently limiting methionine availability in Yucatan miniature pigs. PLoS ONE. 10(6):e0131563. doi: 10.1371/journal.pone.0131563

Ostojic, S. M., J. Ostojic, P. Drid, and M. Vranes. 2016. Guanidinoacetic acid versus creatine for improved brain and muscle creatine levels: a superiority pilot trial in healthy men. Appl. Physiol. Nutr. Metab. 41:1005-1007. doi: 10.1139/apnm-2016-0178

Percário, S., S. P. D. T. Domingues, L. F. M. Teixeira, J. L. F. Vieira, F. de Vasconcelos, D. M. Ciarrocchi, E. D. Almeida, and M. Conte. 2012. Effects of creatine supplementation on oxidative stress profile of athletes. J. Soc. Sports Nutr. 9:56. doi: 10.1186/1550-2783-9-56

Re, R., N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology & Medicine. 26:1231-1237. doi: 10.1016/S0891-5849(98)00315-3

Setoue, M., S. Ohuchi, T. Morita, and K. Sugiyama. 2008. Hyperhomocysteinemia induced by guanidinoacetic acid is effectively suppressed by choline and betaine in rats. Biosci. Biotechnol. Biochem. 72(7):1696-1703. doi: 10.1271/bbb.70791

Sharma, B. K., and R. A. Erdman. 1989. In vitro degradation of choline from selected feedstuffs and choline supplements. J. Dairy Sci. 72:2772–2776. doi: 10.3168/jds.S0022-0302(89)79421-2

Shingfield, K. J., and N. W. Offer. 1999. Simultaneous determination of purine metabolites, creatinine and pseudouridine in ruminant urine by reversed-phase high-performance liquid chromatography. J. Chrom. B. 723:81-94. doi: 10.1016/S0378-4347(98)00549-0

Speer, H. S. 2019. Efficacy of guanidinoacetic acid supplementation to growing cattle and relative bioavailability of guanidinoacetic acid delivered ruminally or abomasally [Master's thesis]. Kansas State University, Manhattan, KS.

Stead, L. M., K. P. Au, R. L. Jacobs, M. E. Brosnan, and J. T. Brosnan. 2001. Methylation demand and homocysteine metabolism: effects of dietary provision of creatine and guanidinoacetate. Am. J. Physiol. Endocrinol. Metab. 281(5):E1095-E1100. doi: 10.1152/ajpendo.2001.281.5.E1095

Tehlivets, O. 2011. Homocysteine as a Risk Factor for Atherosclerosis: Is Its Conversion to S-Adenosyl-L-Homocysteine the Key to Deregulated Lipid Metabolism?. J. Lipids. 2011:1-11. doi: 10.1155/2011/702853

Titgemeyer, E. C., N. R. Merchen, L. L. Berger, and L. E. Deetz. 1988. Estimation of lysine and methionine requirements of growing steers fed corn silage-based or corn-based diets. J. Dairy Sci. 71:421. doi: 10.3168/jds.S0022-0302(88)79572-7

Tossenberger, J., M. Rademacher, K. Németh, V. Halas, and A. Lemme. 2016. Digestibility and metabolism of dietary guanidino acetic acid fed to broilers. Poult. Sci. 95:2058-2067. doi: 10.3382/ps/pew083

Vailati-Riboni, M., J. S. Osorio, E. Trevisi, D. Luchini, and J. J. Loor. 2017. Supplemental Smartamine M in higher energy diets during the prepartal period improves hepatic biomarkers of health and oxidative status in Holstein cows. J. Anim. Sci. Biotechnol. 8:17. doi: 10.1186/s40104-017-0147-7

Vance, D. E. 2014. Phospholipid methylation in mammals: from biochemistry to physiological function. Biochimica et Biophysica Acta. 1838:1477–1487. doi: 10.1016/j.bbamem.2013.10.018

Wyss, M., and R. Kaddurah-Daouk. 2000. Creatine and creatinine metabolism. *Physiol. Rev*. 80:1107–1213. doi:10.1152/physrev.2000.80.3.1107

Zeisel, S. H. 2006. Choline: critical role during fetal development and dietary requirements in adults. Annual review of nutrition. 26:229-250. doi: 10.1146/annurev.nutr.26.061505.111156

Zhang, B., J. L. Dong, Y. L. Chen, Y. Liu, S. S. Huang, X. L. Zhong, Y. H. Cheng, and Z. G. Wang. 2017. Mol. Med. Rep. 16:597-602. doi:10.3892/mmr.2017.6633

Zhao, G., F. He, C. Wu, P. Li, N. Li, J. Deng, G. Zhu, W. Ren, and Y. Peng. 2018. Betaine in inflammation: mechanistic aspects and applications. Front. Immunol. 9:1070. doi: 10.3389/fimmu.2018.01070

Zhou, Z., O. Bulgari, M. Vailati-Riboni, E. Trevisi, M. A. Ballou, F. C. Cardoso, D. N. Luchini, and J. J. Loor. 2016. Rumen-protected methionine compared with rumen-protected choline improves immunometabolic status in dairy cows during the peripartal period. J. Dairy Sci. 99:8956–8969. doi: 10.3168/jds.2016-10986

Zhou, Z., F. Ferdous, P. Montagner, D. N. Luchini, M. N. Corrêa, and J. J. Loor. 2018. Methionine and choline supply during the peripartal period alter polymorphonuclear leukocyte immune response and immunometabolic gene expression in Holstein cows. J. Dairy Sci. 101:10374-10382. doi.org/10.3168/jds.2018-14972

Table 3.1. Composition of diet fed to steers

Item	% of dietary DM
Ingredient	
Corn, dry-rolled	75.6
Alfalfa hay, chopped	12.7
Soybean meal, solvent extracted	6.2
Cane molasses	4.2
Limestone	1.0
Trace mineral salt[1]	0.3
Vitamin and mineral premix[2]	0.1
Chemical composition	
DM	87.1
OM	94.5
CP	12.7
NDF[3]	15.4
ADF[4]	6.2

[1]Composition: 96.0% NaCl, 0.24% Mn, 0.24% Fe, 0.032% Cu, 0.032% Zn, 0.007% I, and 0.004% Co.
[2]Provided (per kg of diet DM): 2,100 IU vitamin A, 600 IU vitamin D, 25 IU vitamin E, and 0.16 mg Se.
[3]Analysis conducted with α-amylase and sodium sulfite; not corrected for ash.
[4]Sequential ADF analysis; not corrected for ash.

Table 3.2. Effects of choline on diet digestibility and nitrogen balance in steers supplemented with guanidinoacetic acid (GAA) and creatine

	Choline, g/d									
	0			5						
	Methyl Group Modulator (MGM)							*P*-value		
Item	Control	Creatine	GAA	Control	Creatine	GAA	SEM[1]	Choline	MGM	Choline × MGM
n	5	6	5	5	6	5				
Intake, kg/d[2]										
DM	3.49	3.47	3.49	3.48	3.49	3.49	0.009	0.40	0.48	0.45
OM	3.29	3.28	3.29	3.29	3.29	3.29	0.009	0.40	0.48	0.45
Apparent digestibility, %										
DM	80.0	77.9	77.2	79.5	78.5	79.9	2.54	0.36	0.40	0.45
OM	80.8	78.8	77.9	80.4	79.3	80.8	2.63	0.34	0.44	0.46
N, g/d										
Feed	73.2	72.8	73.2	73.2	73.2	73.2	0.18	0.40	0.48	0.45
Infused	0.0	5.3	5.4	0.7	6.0	6.0	0.06	<0.0001	<0.0001[4]	0.58
Intake[3]	73.2	78.1	78.6	73.9	79.2	79.3	0.24	<0.001	<0.0001[4]	0.48
Urinary	24.0	22.9	23.3	24.1	23.6	24.8	1.79	0.44	0.72	0.84
Urinary urea N	14.3	12.7	13.3	13.8	13.5	13.4	1.62	0.90	0.69	0.84
Fecal	21.9	27.5	25.6	22.6	27.4	23.8	1.52	0.66	<0.001[5]	0.52
Retained	27.2	27.8	29.8	27.2	28.2	30.7	2.33	0.69	0.10[6]	0.93

[1]Average SEM across all treatments.

[2]Excludes abomasal infusions

[3]Feed N + infused N

[4]Pairwise means separated within MGM as control < GAA = creatine; $P \leq 0.05$.

[5]Pairwise means separated within MGM as control < GAA < creatine; $P \leq 0.05$.

[6]Pairwise means separated within MGM as control = creatine < GAA; $P \leq 0.05$.

Table 3.3. Effects of choline on plasma concentrations and urinary excretion of guanidinoacetic acid, creatine, and creatinine in growing steers supplemented with GAA or creatine

	Choline, g/d									
	0			5						
	Methyl Group Modulator (MGM)							*P*-value		
Item	Control	Creatine	GAA	Control	Creatine	GAA	SEM[1]	Choline	MGM	Choline × MGM
n	5	6	5	5	6	5				
Plasma, mg/L										
GAA	0.26	0.18	0.51	0.29	0.17	0.64	0.058	0.25	<0.0001[2]	0.51
Creatine	9.9	12.4	14.0	10.8	13.5	15.5	0.95	0.04	<0.0001[3]	0.89
Creatinine	2.92	3.29	3.12	3.17	3.30	3.38	0.277	0.42	0.29	0.77
Urine, g/d										
GAA	0.19	0.18	0.36	0.17	0.17	0.40	0.041	0.92	<0.01[2]	0.63
Creatine	2.12	4.82	6.57	2.68	5.00	7.42	0.440	0.14	<0.01[3]	0.74
Creatinine	5.37	5.51	5.39	5.41	5.46	5.56	0.283	0.78	0.89	0.89

[1]Average SEM across all treatments

[2]Pairwise means separated within MGM as creatine = control < GAA; $P \leq 0.05$.

[3]Pairwise means separated within MGM as control < creatine < GAA; $P \leq 0.05$.

Table 3.4. Effects of choline on plasma glucose, urea, antioxidant capacity, and haptoglobin concentrations in growing steers supplemented with guanidinoacetic acid (GAA) and creatine

	Choline, g/d									
	0			5						
	Methyl Group Modulator (MGM)							*P*-value		
Item	Control	Creatine	GAA	Control	Creatine	GAA	SEM[1]	Choline	MGM	Choline × MGM
n	5	6	5	5	6	5				
Glucose, mM	4.51	4.68	4.35	4.53	4.61	4.50	0.16	0.78	0.32	0.76
Urea, mM	3.87	3.46	3.55	3.87	3.49	3.75	0.28	0.68	0.25	0.89
Trolox-equivalent AOP, μL^{-1}	0.094	0.085	0.112	0.107	0.072	0.099	0.008	0.50	0.008[2]	0.21
Haptoglobin, g/L	2.42	2.56	3.00	1.91	1.96	1.60	0.48	0.07	0.97	0.68

[1]Average SEM across all treatments

[2]Pairwise means separated within MGM as creatine < control = GAA; $P \leq 0.05$.

Table 3.5. Effects of choline on complete blood count (CBC) parameters in growing steers supplemented with guanidinoacetic acid (GAA) and creatine

	Choline, g/d									
	0			5						
	Methyl Group Modulator (MGM)							*P*-value		
Item	Control	Creatine	GAA	Control	Creatine	GAA	SEM[1]	Choline	MGM	Choline × MGM
n	5	6	5	5	6	5				
Leukocyte count, nL^{-1}	10.6	11.3	10.2	10.8	11.3	10.7	0.70	0.52	0.22	0.88
Erythrocyte conc, pL^{-1}	9.1	8.7	8.7	8.6	8.9	8.8	0.24	0.67	0.64	0.09
Hemoglobin, g/dL	11.7	11.1	11.1	11.0	11.3	11.3	0.40	0.63	0.73	0.11
Cellular hemoglobin, g/dL	11.4	10.8	11.0	10.9	11.3	11.3	0.45	0.57	0.83	0.11
Mean cell volume, fL	33.6	34.1	33.9	33.8	33.9	34.2	0.76	0.62	0.06	0.42
Mean cell hemoglobin, pg	12.7	12.9	12.8	12.6	12.7	12.8	0.22	0.40	0.21	0.62
Mean cell hemoglobin conc, g/dL	37.8	37.8	37.7	37.4	37.6	37.6	0.37	0.25	0.98	0.84
Cell hemoglobin conc mean, g/dL	36.8	36.8	36.7	36.9	36.7	36.8	0.23	0.66	0.58	0.89
RBC distribution width, %	20.9	20.6	21.2	20.9	21.0	20.6	0.36	0.77	0.82	0.19
Platelet, nL^{-1}	472	543	506	457	478	552	54.6	0.64	0.17	0.20
Segmented neutrophil conc, nL^{-1}	2.91	3.87	2.70	4.30	3.47	3.21	0.48	0.15	0.22	0.13
Lymphocyte conc., nL^{-1}	6.61	6.37	6.34	5.38	6.76	6.26	0.61	0.35	0.35	0.13
Monocyte conc., nL^{-1}	0.54	0.47	0.49	0.67	0.67	0.55	0.12	0.06	0.64	0.73
Eosinophil conc., nL^{-1}	0.47	0.50	0.54	0.32	0.33	0.60	0.13	0.37	0.31	0.62
Basophil conc., nL^{-1}	0.142	0.083	0.239	0.117	0.119	0.082	0.069	0.38	0.68	0.39
Hematocrit, % (calc)	30.7	29.3	29.5	29.3	30.1	29.9	1.28	0.88	0.78	0.15
Hematocrit, % (spun)	33.3	31.7	32.2	31.5	34.7	32.1	1.31	0.74	0.68	0.11
Plasma protein (refractom.), g/dL	7.44	7.55	7.46	7.50	7.43	7.61	0.22	0.67	0.75	0.32
Fibrinogen (heat ppt), mg/dL	591	567	536	567	503	603	56.4	0.88	0.68	0.49

[1]Average SEM across all treatments.

Table 3.6. Effects of choline on immune cell function in growing steers supplemented with guanidinoacetic acid (GAA) and creatine

	Choline, g/d									
	0			5						
	Methyl Group Modulator (MGM)							*P*-value		
Item	Control	Creatine	GAA	Control	Creatine	GAA	SEM[1]	Choline	MGM	Choline × MGM
n	5	6	5	5	6	5				
Oxidative burst, %										
-LPS	43.9	48.0	46.9	46.9	46.3	41.7	5.46	0.68	0.75	0.58
+LPS	47.1	54.4	50.8	48.8	46.9	46.9	5.76	0.42	0.85	0.64
Phagocytosis, %										
-LPS	83.3	81.3	84.0	84.1	79.8	79.6	2.22	0.29	0.30	0.45
+LPS	86.6	83.0	81.6	80.8	80.3	76.9	3.09	0.09	0.35	0.87

[1]Average SEM across all treatments